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The role of the DLX genes in olfactory development

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THE ROLE OF THE *DLX* GENES IN OLFACTORY DEVELOPMENT

by

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DISSERTATION

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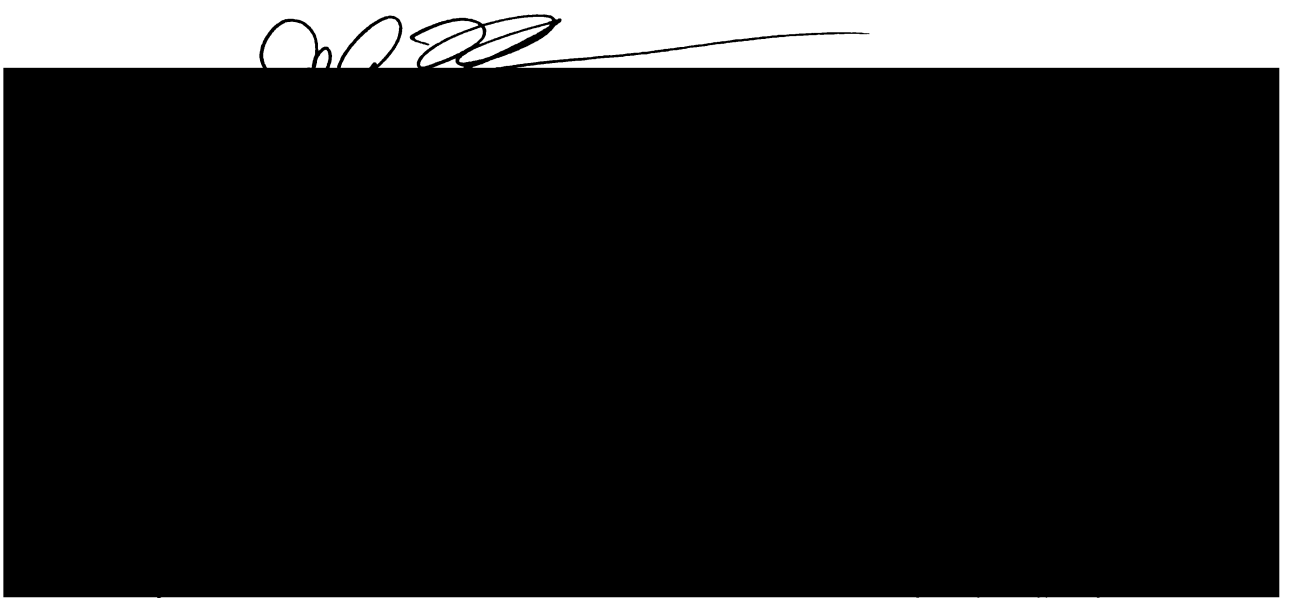

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ABSTRACT

The work presented in this thesis represents an analysis of the role of the *Dlx* genes in the development of the olfactory bulb (OB). The generation of the OB is a complex process, involving interactions from both within and outside of the central nervous system and migrations of neurons from distant sources. The *Dlx* genes are a family of homeodomain containing transcription factors expressed in both peripheral and central components of the olfactory system. Analysis of mouse mutations in the *Dlx* genes has revealed OB defects in all mutants studied to date. Therefore, the OB is an excellent model system in which to study the role of the *Dlx* genes during development. *Dlx5*$^{-/-}$ mutant mice have a hypoplastic olfactory epithelium in which a small amount of neuroepithelium differentiates. This neuroepithelium, however, does not contact the OB. The *Dlx5*$^{-/-}$ mutation also results in a small OB and a decrease in the expression of several local circuit neuron markers in the OB. This decreased expression is most likely a result of a requirement for DLX5 for differentiation of these local circuit neurons. In addition to this cell autonomous defect, there is a non-cell autonomous defect in the orientation of mitral cells and their processes, which results in an OB without clear morphological lamination. The mitral cell defect is most likely a result of the lack of input from the olfactory epithelium. The *Dlx1&2*$^{-/-}$ mutant mice have a near absence of OB local circuit neuron marker expression. This absence of marker expression is a result of a cell autonomous migration defect of the OB local circuit neuron precursors. The *Dlx1&2*$^{-/-}$ mutant has altered regulatory molecule expression, which may be the direct source of the migration defect. This work demonstrates the importance of the *Dlx* genes in OB neurogenesis.

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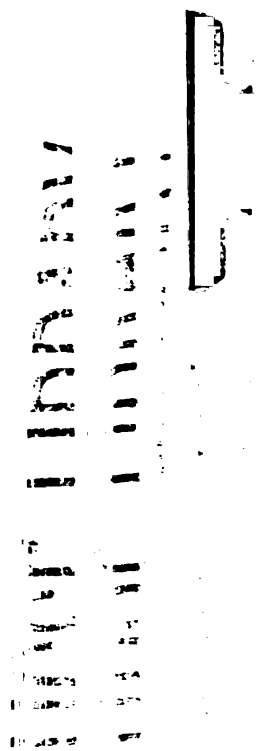
ABBREVIATIONS

AER	Apical Ectodermal Ridge
AOB	Accessory Olfactory Bulb
BA	Branchial Arches
CNC	Cranial Neural Crest
CNS	Central Nervous System
CP	Cribiform Plate
Cx	Cortex
E	Embryonic Day
EPL	External Plexiform Layer
FL	Fore Limb
GC	Granule Cell Layer
HyA	Hyoid Arch
IPL	Inner Plexiform Layer
l	Lateral
LFNP	Lateral Frontal Nasal Prominence
LGE	Lateral Ganglionic Eminence
LOT	Lateral Olfactory Tract
LV	Lateral Ventricle
m	Medial
MC	Mitral Cell Layer
MdA	Mandibular Branchial Arch



MFNP	Medial Frontal Nasal Prominence
MGE	Medial Ganglionic Eminence
MxA	Maxillary Branchial Arch
Mz	Mesenchyme
NS	Nasal Sinus
OB	Olfactory Bulb
OE	Olfactory Epithelium
ON	Olfactory Nerve
ONL	Olfactory Nerve Layer
OP	Olfactory Pit
OV	Otic Vesicle
PG	Periglomerular Layer
POB	Primordial Olfactory Bulb
RE	Respiratory Epithelium
RMS	Rostral Migratory Stream
Se	Septum
Str	Striatum
SVZ	Sub Ventricular Zone
v	Olfactory Ventricle
VNO	Vomeronasal Organ
VZ	Ventricular Zone

INTRODUCTION

Development of an organism requires an ordered series of processes beginning with induction and specification. After specification, genetic programs are deployed that regulate proliferation, migration and differentiation. The specific mechanisms that initiate and control these events are relatively unknown for many organisms, although through evolutionary analyses, it appears, not surprisingly, that the orthologues of many molecules play similar roles across species (Rossi et al., 1998; Smits et al., 1999; Ollmann et al., 2000; Shah et al., 2000; Beermann et al., 2001). The timing of expression and interactions between these molecules are crucial to the proper progression of an organism's development, and as such, disturbances to these programs frequently result in abnormalities such as birth defects, cancer and neurological disorders. In order to properly diagnose and treat these conditions, it is important to understand how normal development proceeds. Although much is unknown regarding normal developmental processes, inroads are being made through the analysis of molecules expressed in restricted patterns. One family of such molecules is the *Dlx* gene family. They are a family of homeodomain-containing transcription factors that are expressed in spatially and temporally restricted patterns in many areas of developing organisms (Stock et al., 1996; Panganiban et al., 1997; Quint et al., 2000; Zerucha and Ekker, 2000).

The Dlx Family

The *Dlx* family in *Mus musculus* (mouse) currently consists of six family members arranged in convergently transcribed pairs and distantly adjacent to neighboring *Hox* gene clusters (Fig. 1; the *Dlx* gene family will hereafter be referring to the mouse family members unless otherwise specified). The *Dlx* gene pairs are located nearest the

more posterior expressed genes of the *Hox* cluster (Rossi et al., 1994; Nakamura et al., 1996). *Dlx1* and *Dlx2* are a pair located near *HoxD* (Rossi et al., 1994); *Dlx3* and *Dlx4* (formally *Dlx7*) are the second pair and located near *HoxB* (Nakamura et al., 1996); *Dlx5* and *Dlx6* are the third pair and located near *HoxA* (Ruddle et al., 1994; Simeone et al., 1994). A *Dlx* gene pair located near the *HoxC* cluster has yet to be found, presumably due to a deletion, as it is theorized that the *Dlx* genes were formed by a tandem duplication, creating a linked pair, which was then followed by chromosomal duplications (Stock et al., 1996; Robinson-Rechavi et al., 2001). These duplications are thought to have occurred after the divergence of insects and vertebrates for the *Dlx* genes (Stock et al., 1996) and after the divergence of amphioxus and vertebrates for the *Hox* genes (Holland and Garcia-Fernandez, 1996).

In the insect *Drosophila melongaster*, there is the founding member of the *Dlx* gene family, *Distal-less* (*Dll*) (Cohen et al., 1989). The *Dlx* gene family members are *Dll* homologues, as are other members found in numerous species studied to date. In the nematode, *Caenorhabditis elegans*, there is one *Dll* homologue, *C28A5.4*, while in *Danio rerio* (zebrafish), there are eight known *Dlx* genes (Stock et al., 1996). This wide variation in the number of *Dlx* gene copies in different species allows for additional evolutionary categorization.

The duplication and evolution of the *Dlx* genes has resulted in the formation of clades, or groups of paralogues, which are more similar to one another, rather than the other members of the family. Although each member of the *Dlx* family is highly

Genomic *Dlx* Organization

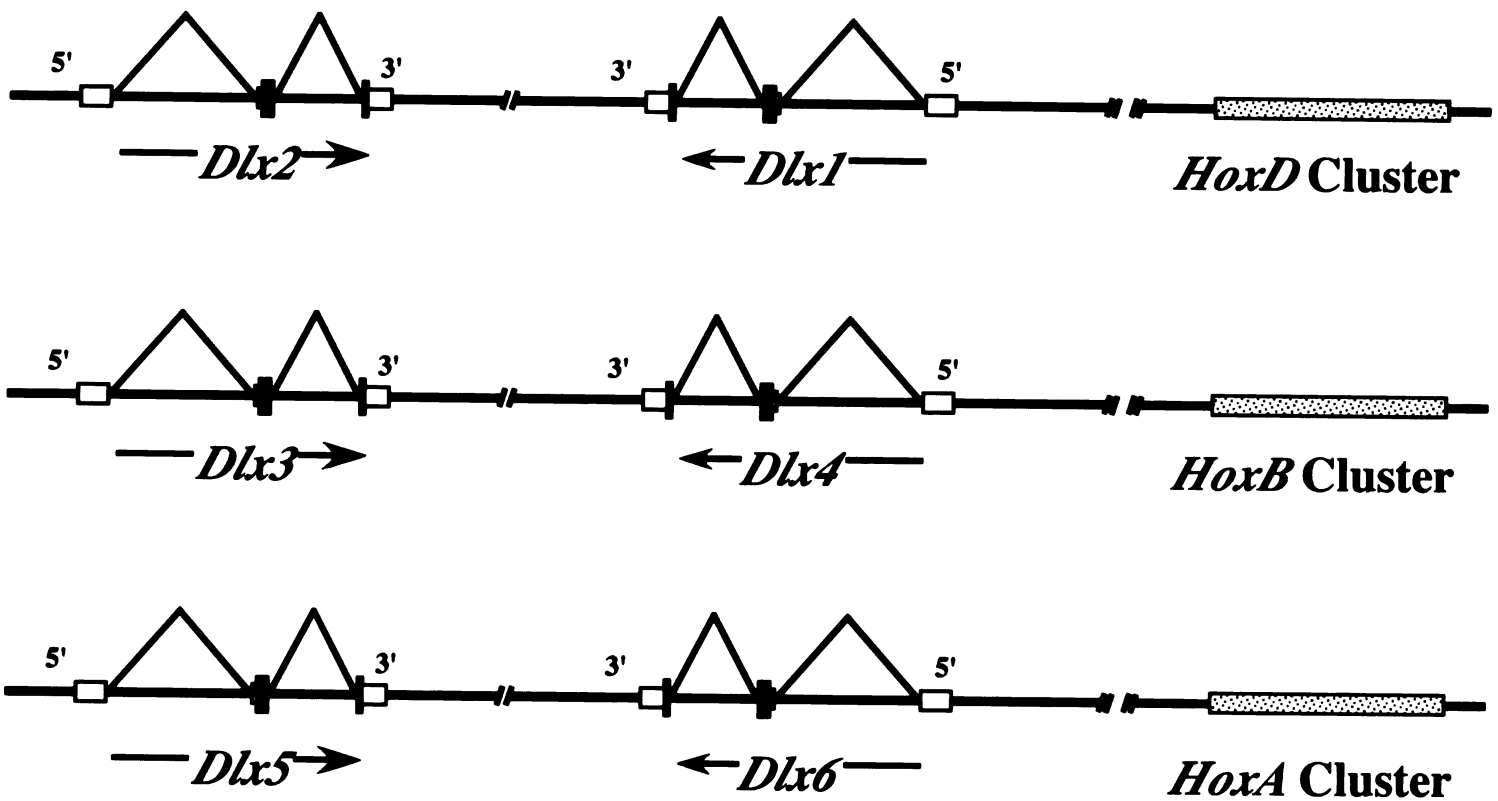


Figure 1: Schema of the genomic organization of the *Dlx* genes. The *Dlx* genes are organized as pairs of convergently transcribed genes located within approximately 15 Kb of one another. Each pair is on a different chromosome and is located near a *Hox* cluster. *Dlx1*, *Dlx4* and *Dlx6* are members of the first clade of *Dlx* family members, and *Dlx2*, *Dlx3* and *Dlx5* form the second. Each *Dlx* gene also has three exons, with the homeodomain (shown in black) split between exons 2 and 3. Additionally, *Dlx5* also has alternative splice variants (Liu et al., 1997) and *Dlx1* and *Dlx6* have anti-sense transcripts (not shown; (McGuinness et al., 1996; Liu et al., 1997).

conserved within the homeodomain, the regions outside of the homeodomain determine the clade to which each member belongs. *Dlx1*, *Dlx4* and *Dlx6* are one clade, while *Dlx2*, *Dlx3* and *Dlx5* form the second (Stock et al., 1996). However, functional similarity between members of a clade has yet to be established. Given the evolutionary context of the *Dlx* gene family, it is not parsimonious to suggest that the family members have similar expression patterns and regulatory controls.

Dlx Expression

The *Dlx* genes are primarily expressed in cranial neural crest derivatives, the surface ectoderm and the nervous system of the developing embryo. Cranial neural crest (CNC) cells migrate from the hindbrain to populate the branchial arches (BA). *Dlx5* is expressed in the anterior neural ridge (ANR) through embryonic day 9.0 (E9.0) (Acampora et al., 1999; Depew et al., 1999). These cells delaminate, becoming CNC cells and migrating away from the neural ridge. However, *Dlx* expression is downregulated before or slightly after beginning this migration. The CNC cells then populate the BA where the *Dlx* genes have a nested pattern along the proximodistal axis, with *Dlx1* and *Dlx2* expressed most proximally. *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* are expressed in the intermediate area of the BA, and in the most distal area, all six *Dlx* genes are expressed (Fig. 2). This nested pattern of expression is hypothesized to form a 'code' to specify tissue fate (Depew et al., 2002). This 'code', in concert with signals from the surface ectoderm, causes the BA to form the craniofacial skeleton and underlying connective tissue (Mina and Kollar, 1987; Lumsden, 1988; Hall, 1991; Webb et al., 1993).

Dlx Expression in the Branchial Arches and Frontonasal Prominences

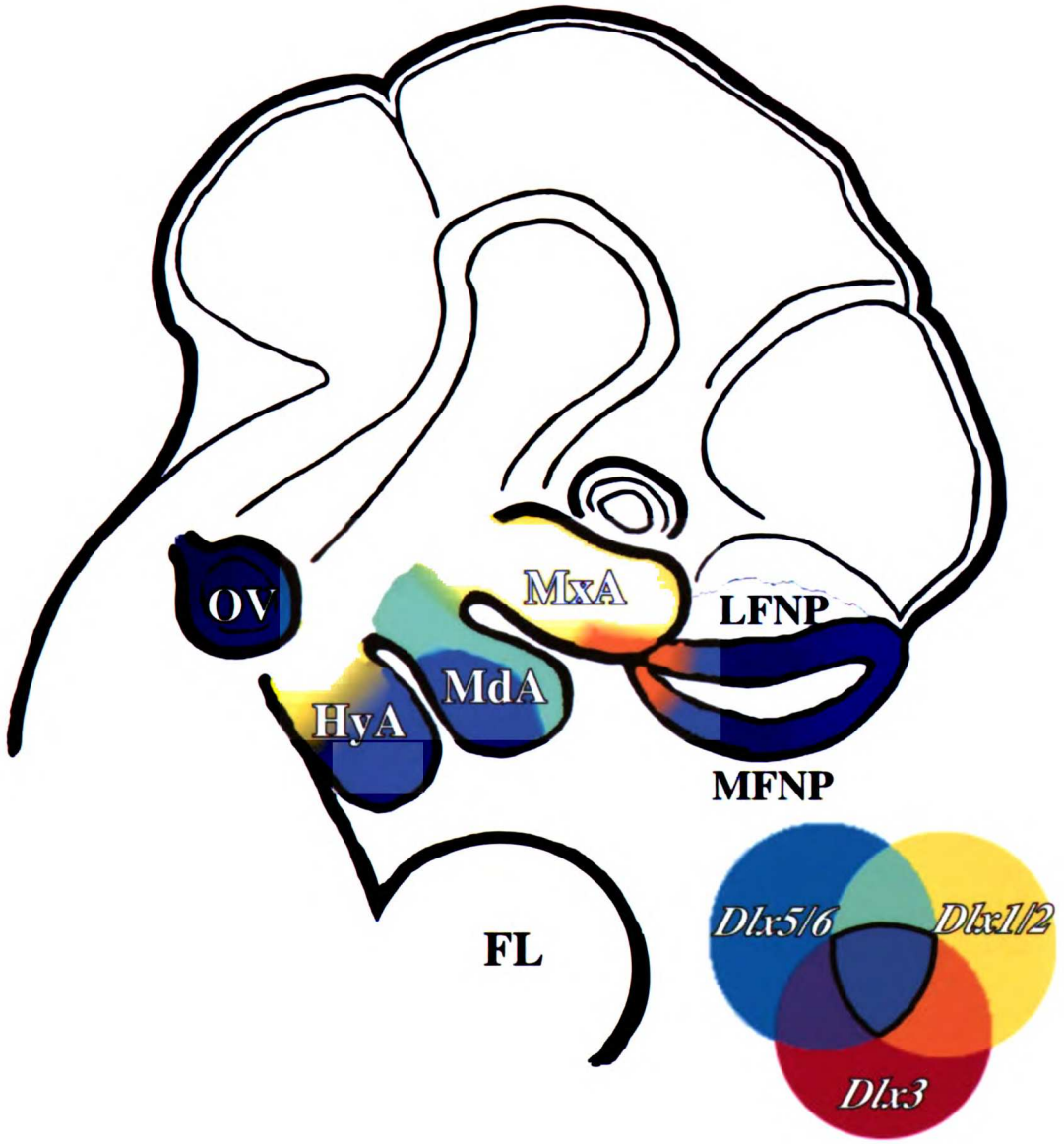


Figure 2: Schema of *Dlx* expression in the branchial arches and frontonasal prominences of an embryonic day 10.5 wild type embryo. The color wheel defines the overlapping expression patterns of the *Dlx* genes. *Dlx1* and *Dlx2* have similar expression patterns in the branchial arches, as do *Dlx5* and *Dlx6*, so they will be represented on the color wheel with only one color for each pair. *Dlx1&2* are expressed along most of the proximal distal axis of the maxillary (MxA), mandibular (MdA) and hyoid (HyA) arches; *Dlx3* and *Dlx5&6* expression is strong in the distal domains. This same arrangement holds true in the lateral and medial frontonasal prominences (LFNP and MFNP, respectively). There is a gradient of *Dlx* expression going from distal to proximal domains, with the most proximal domain having more compressed *Dlx* expression along its length. The otic vesicle (OV) has strong *Dlx5&6* expression distally, which reduces to a tight mix of all *Dlx* genes more proximal. Abbreviations: FL, forelimb; HyA, hyoid arch; LFNP, lateral frontonasal prominence; MdA, mandibular arch; MFNP, medial frontonasal prominence; MxA, maxillary arch; OV, otic vesicle.

In the surface ectoderm, the *Dlx* genes are expressed in multiple locations throughout embryonic development. Most of the family members have restricted expression in the surface ectoderm, the exception being *Dlx3*, which is expressed broadly (Morasso et al., 1996). *Dlx5* and *Dlx6* are initially expressed throughout the surface ectoderm of the BA from E8.0 to E8.75, and thereafter, their expression in the surface ectoderm is downregulated (Depew et al., 1999). Around E9.0, *Dlx2* and *Dlx3*, and to a lesser extent, *Dlx1* and *Dlx4*, are expressed along the midline of the mandibular BA and at the junction of the maxillary BA and the frontonasal prominences (Depew et al., 2002). Overall, the majority of *Dlx* expression in the surface ectoderm of the BA is in the distal portion, and whose 'code', unlike the ectomesenchyme beneath, has yet to be resolved.

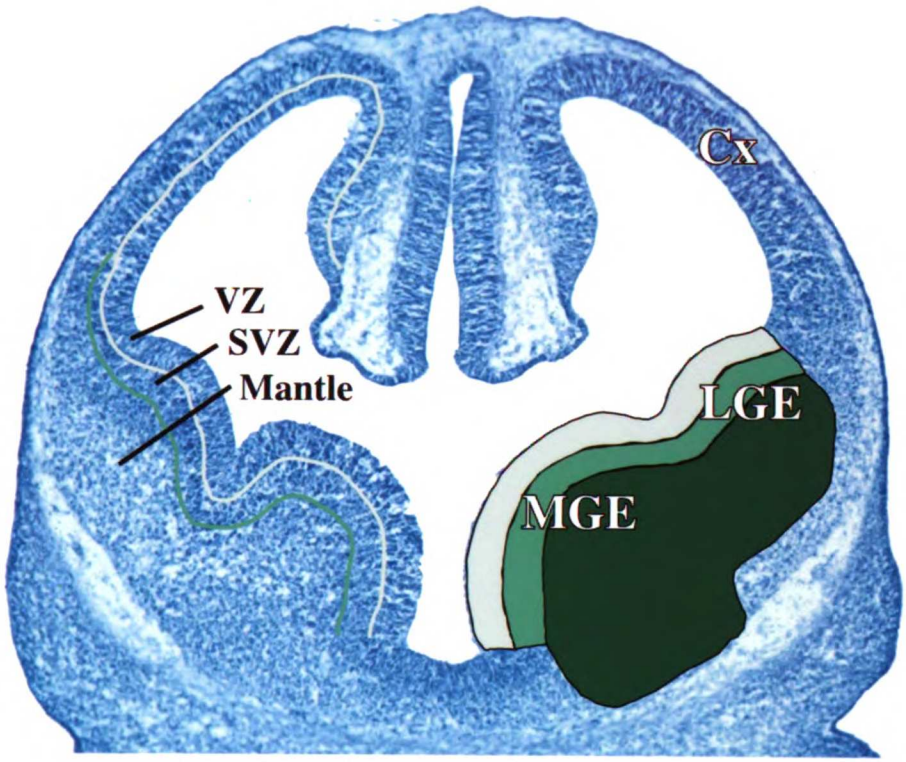
In other areas of the surface ectoderm, *Dlx3* (Akimenko et al., 1994; Pera and Kessel, 1999) and *Dlx5* (Yang et al., 1998) are expressed in the lateral edge of the neural plate. Later, they are both expressed in the olfactory placode and invaginating olfactory pit (Depew et al., 1999; Pera and Kessel, 1999). *Dlx3*, *Dlx5* and *Dlx6* are all expressed in the olfactory epithelium (Depew et al., 1999; Long and Rubenstein, unpublished observations). *Dlx1*, *Dlx2* and *Dlx5* are expressed in the apical ectodermal ridge of the developing limb bud (Dolle et al., 1992; Bulfone et al., 1993b; Ferrari et al., 1995). This expression is reminiscent of *Dll* expression in the developing fly limbs (Cohen et al., 1989; Cohen et al., 1991). In spite of the numerous locations of *Dlx* gene expression, their role in the surface ectoderm has not been fully elucidated.

In the central nervous system (CNS), *Dlx3* and *Dlx4* have not been detected. However, the other four genes have interesting spatial and temporal expression patterns. The *Dlx* genes are expressed in the same regions as glutamic acid decarboxylase (GAD),

an enzyme that synthesizes γ-amino butyric acid (GABA) from glutamic acid. These coincident expression patterns suggest the involvement of the *Dlx* genes in the development of GABAergic cells (Anderson et al., 1997a; Anderson et al., 1997b; Stuhmer et al., 2002a; Stuhmer et al., 2002b). Temporally, in the embryonic forebrain, there are two early domains of *Dlx* gene expression (Bulfone et al., 1993b; Bulfone et al., 1993a) and two later domains (Qiu et al., 1995; Anderson et al., 1997a; Anderson et al., 1997b; Bulfone et al., 1998). One early expression domain extends from the ventral thalamus to the suprachiasmatic nucleus. The second early domain includes the subcortical telencephalon, specifically the medial and lateral ganglionic eminences (MGE, LGE respectively). The first later expression domain is a result of migrating local circuit neurons from the basal ganglia, which populate the cortex (Porteus et al., 1994; Anderson et al., 1997a). The second later domain is in local circuit neurons of the olfactory bulb (OB; (Qiu et al., 1995; Anderson et al., 1997a; Anderson et al., 1997b; Bulfone et al., 1998).

In the basal ganglia, *Dlx1* and *Dlx2* are expressed in proliferating cells of the ventricular and subventricular zones (Fig. 3; VZ, SVZ respectively; (Bulfone et al., 1993b; Bulfone et al., 1993a; Porteus et al., 1994; Liu et al., 1997; Eisenstat et al., 1999)). *Dlx5* is expressed in more mature cells, the differentiating cells of the SVZ and with *Dlx6*, in the mantle zone (Liu et al., 1997; Eisenstat et al., 1999). These expression patterns suggest that the *Dlx* genes are expressed in progressively more differentiated cells of the basal ganglia, beginning with *Dlx2*, *Dlx1* and *Dlx5*, then *Dlx6* (Liu et al., 1997; Eisenstat et al., 1999; Zerucha et al., 2000). In addition, each *Dlx* gene may control

Wild Type *Dlx* Expression



Dlx1* and *Dlx2

Dlx1*, *Dlx2* and *Dlx5

Dlx5* and *Dlx6

Figure 3: Schema of *Dlx* expression in the developing basal ganglia in an E12.5 wild type embryo. The *Dlx* genes are expressed in an overlapping gradient of differentiation in the developing basal ganglia. *Dlx1* and *Dlx2* are expressed in the progenitor populations of the ventricular zone (VZ) and subventricular zone (SVZ). As the cells differentiate and move into the SVZ, they express *Dlx5*. Then, after exiting the cell cycle, the cells migrate out to the mantle, while they downregulate expression of *Dlx1* and *Dlx2*, continue to express *Dlx5* and upregulate expression of *Dlx6* as differentiation proceeds. Abbreviations: Cx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; SVZ, subventricular zone; VZ, ventricular zone.

different steps in this differentiation process. Furthermore, *Dlx* expression in the progenitor cell populations (e.g. *Dlx1* and *Dlx2*) may regulate the *Dlx* expression in the more differentiated cell populations (e.g. *Dlx5* and *Dlx6*).

Molecular Dlx Regulation

The similarities in *Dlx* gene expression suggest that each gene might be regulated by similar enhancers. These enhancers are most likely acted upon by regulatory molecules, but they may also be controlled by other members of the *Dlx* gene family. Several studies have suggested many developmental molecules which regulate the *Dlx* genes. One such molecule is sonic hedgehog (Shh), shown to be important for dorsoventral patterning in the CNS and limb bud by creating distinct areas of transcription factor expression (Briscoe et al., 2000; Ho and Scott, 2002; Marti and Bovolenta, 2002). It induces *Dlx* expression in the forebrain using a gain of function assay (Gaiano et al., 1999), suggesting it might be sufficient for *Dlx* expression. Analysis of $Shh^{-/-}$ mutant mice reveals reduced *Dlx2* expression, suggesting that it might be necessary for some, but not all aspects of *Dlx* expression (Ohkubo et al., 2002; Yun and Rubenstein, unpublished observations).

Other important molecules shown to regulate the *Dlx* genes are the bone morphogenic proteins (BMPs). The BMP family are dimeric ligands which act as osteogenic factors and have been shown to induce bone and cartilage growth in vivo (Reddi, 1998), two locations where *Dlx* gene expression is prominent. BMP2 induces *Dlx2* expression in chrondrocytes (Xu et al., 2001). BMP4 has been demonstrated to induce *Dlx1* and *Dlx2*, *Dlx3* and *Dlx5* in dental mesenchyme (Bei and Maas, 1998),

ectoderm (Feledy et al., 1999) and osteoblasts (Miyama et al., 1999), respectively. In addition, the fly homologue of the *Dlx* family, *Dll*, has also been shown to be regulated by a BMP homologue, Decapentaplegic (*Dpp*), not in its induction of expression, but rather in its maintenance (Goto and Hayashi, 1997).

A third family of molecules which are important for *Dlx* gene expression are the fibroblast growth factors (FGFs), which have been implicated in cell proliferation, migration and differentiation (Boilly et al., 2000). They are a large family of more than 20 members, many of which have overlapping expression domains with one another (Vasiliauskas and Stern, 2001). FGF8 has been shown to induce *Dlx1* and *Dlx2* in the mouse dentition (Bei and Maas, 1998) and chick BA (Shigetani et al., 2002). FGF2 alone or FGF8 in concert with Wnt8c can induce *Dlx5* expression in the chick limb (Ferrari et al., 1999) and inner ear (Ladher et al., 2000) respectively. However, loss of function analysis of $FGF8^{hyp}$ mutant mice continue to express the *Dlx* genes (Trumpp et al., 1999). This may be the result of compensation by other FGF family members, something which is also hypothesized for the *Dlx* genes (Qiu et al., 1995; Qiu et al., 1997).

There is also compelling evidence that cross regulation of the *Dlx* genes may be mechanism for induction or maintenance of their expression patterns. In the $Dlx1^{-/-}$ mutant mouse, there is a reduction in *Dlx2* expression in the BA and OB, while the converse is also true (Liu et al., 1997; Long and Rubenstein, unpublished observations). In the $Dlx1\&2^{-/-}$ mutant mouse, there is a loss of *Dlx5* and *Dlx6* expression in the OB and basal ganglia (Anderson et al., 1997b; Long and Rubenstein, unpublished observations). In zebrafish, it has been shown that Dlx3b can activate *dlx5a* expression (Zerucha et al., 1997) and through loss of function analysis, is required for *dlx5a* expression (Mendonsa

and Riley, 1999). Furthermore, in the fly, *Dll* is required for its own maintenance and refinement of expression (Lecuit and Cohen, 1997). All of these data support the idea that there may be cross-regulatory interactions of the *Dlx* gene family. This is hypothesized to be one of the mechanisms that cause the overlapping expression patterns of the *Dlx* genes (Zerucha et al., 1997).

Genomic Dlx Regulation

The overlapping expression patterns of the *Dlx* genes suggest potential regulation through shared enhancers. A 143 base pair (bp) element with 76% sequence identity was found upstream of both *Dlx2* and *Dlx5*, although this element was unable to drive expression of *lacZ* in tissue culture cells (Long and Rubenstein, unpublished observations). This finding suggests the presence of enhancers that are evolutionarily conserved to drive expression of the *Dlx* genes in similar zones, such as the SVZ, where *Dlx2* and *Dlx5* have overlapping expression patterns. Additional support for this idea was shown by comparison of the regions surrounding the *Dlx3* and *Dlx4* genes of the mouse and human, which were found to have similar enhancers (Sumiyama et al., 2002). The most compelling support for shared enhancers has been shown by regulatory elements from the intergenic region between zebrafish *dlx5a* and *dlx6a* which are sufficient to drive their expression (Zerucha et al., 2000). This sequence is highly conserved in the mouse, can drive expression of a *lacZ* transgene and is regulated by *Dlx1* and *Dlx2* (Zerucha et al., 2000; Stuhmer et al., 2002a; Stuhmer et al., 2002b). All of these data support the idea that the *Dlx* gene enhancers are not only evolutionarily conserved, but

are also shared in some cases to give rise to the overlapping *Dlx* expression patterns. These patterns also provide information about the function of the *Dlx* genes.

Dlx Function

Functional studies have been performed on the *Dlx* gene family in several species. However, most of the studies have been from the analysis of loss of function mutations. In the fly, *Dll* is required for the distal formation of the legs, antennae and mouthparts (Cohen et al., 1989). Two functions of *Dll* emerge from these loss of function studies. One function is the specification of distal leg pattern elements (Gorfinkiel et al., 1997; Wu and Cohen, 1999) and the other is specification of antennal identity (Cohen et al., 1989; Dong et al., 2000). Translating these findings across species to the mouse yields some interesting comparisons. The leg of the fly is analogous to the limb in the mouse and the antenna of the fly is an analogous structure to the ear and nose of the mouse. The phenotypes of some of the *Dlx* mutant mice in these structures are somewhat similar to the fly. However, a caveat being that there are multiple *Dlx* genes in the mouse, which may functionally compensate for one another in the absence of a given member.

In the single *Dlx* and compound *Dlx1&2*$^{-/-}$ mutant mice, no abnormalities have been found in the limb (Qiu et al., 1995; Qiu et al., 1997; Acampora et al., 1999; Depew et al., 1999). However, in compound *Dlx5&6*$^{-/-}$ mutant mice, the limbs have several distal malformations, including the loss or fusion of digits (Robledo et al., 2002). A comparison to the fly data reveals a strong similarity in the requirement for a *Dlx* gene(s) to specify distal element identity, most likely by affecting downstream target genes.

The known downstream targets of *Dll* in the developing leg are *bric a brac* (*bab*) (Gorfinkiel et al., 1997; Campbell and Tomlinson, 1998), *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). The last gene, *ser*, encodes a *Notch* ligand, and its homologue in the mouse is a tantalizing target gene due to an interesting finding by Yun et al. showing that the *Dlx* genes downregulate *Notch* signaling in the mouse nervous system (Yun and Rubenstein, unpublished observations). This evidence suggests that the *Dlx* gene family have evolutionarily conserved targets, in addition to the evolutionarily conserved function of specifying distal element identity.

The lack of phenotypes in the limb of single *Dlx* mutant mice is not true for the ear and nose of the mutant mice. These structures are profoundly affected in several loss of function studies in the mouse. In the $Dlx1^{-/-}$, $Dlx2^{-/-}$ and $Dlx1\&2^{-/-}$ mutant mice, two components of the inner ear are hypotrophic, the stapes and styloid process (Qiu et al., 1997). In the $Dlx5^{-/-}$ mutant mice, the stapes and styloid are intact, however, several other components of the inner ear are abnormal. The semicircular canals and pars canalicularis are malformed and hypoplastic, while the tegmen tympani is hypertrophic (Depew et al., 1999). The analysis of the $Dlx5\&6^{-/-}$ mutant mice in this region reveals the fusion and dysmorphic structure of both the inner ear capsule and middle ear cartilages (Robledo et al., 2002). Furthermore, the external ear cartilage is absent. The mutation results suggest that the *Dlx* genes are required for specifying element identity, a function similar to *Dll*.

Again, similar to the leg, several known genes are downstream of *Dll* in the developing antenna. In the fly, *spalt* (*sal*) (Dong et al., 2000) and *atonal* (*ato*) (Dong et

al., 2002) are expressed in the antenna, while the vertebrate counterparts, *SALL1* (Kohlhase et al., 1998; Buck et al., 2001) and *Math1/Zath1* (Bermingham et al., 1999) are expressed in the mouse ear. However, no studies have published any effect of the *Dlx* mutations on these downstream targets. This further suggests the evolutionary conservation of the *Dlx* genes targets in specifying element identity.

The noses of all the single *Dlx* mutant mice have been found to be normal with the notable exception of the $Dlx5^{-/-}$ mutant mice. These mice have hypoplasia of their nasal capsules, with the right side more severely affected than the left (Depew et al., 1999). Analysis of the compound mutants reveals no defects in the noses of the $Dlx1\&2^{-/-}$ mutant mice. However, the $Dlx5\&6^{-/-}$ mutant mice have severe clefting of the nasal cavity, a phenotype more dramatic than the $Dlx5^{-/-}$ single mutant mice (Robledo et al., 2002). Again, this provides further support for the involvement of the *Dlx* genes in specifying structural identity.

In all *Dlx* mutant mice studied to date, defects have been found in the craniofacial and skeletal structures of the head (Qiu et al., 1995; Qiu et al., 1997; Acampora et al., 1999; Depew et al., 1999; Robledo et al., 2002). The abnormalities are distinct for each mutant, and form the basis for the code previously described of the involvement of the *Dlx* genes in specifying the proximodistal axis of the BA (Depew et al., 2002). These craniofacial defects in the mouse may be analogous to the defects in the mouthparts found in the analysis of the fly (Panganiban, 2000). The phenotypes in the skull of the *Dlx* mutant mice are more difficult to relate to those of the fly, so a direct comparison is too complicated for our understanding at this time.

The CNS is another *Dlx* expression zone that displays a phenotype in the *Dlx* mutant mice. However, in the fly, *Dll* expression in the nervous system is not well understood. Therefore, it is difficult to draw parallels to the fly in the analysis of the mouse CNS phenotypes. In the single *Dlx* mutants, there has not been a readily detectable phenotype in the basal ganglia or hypothalamus. However, in the *Dlx1&2*$^{-/-}$ mutant mice, there is a dramatic effect on the development of the basal ganglia. They have a block in differentiation in the striatum where late born cells accumulate in the SVZ, or secondary proliferative population (Anderson et al., 1997b). Cells in this abnormal SVZ express markers of both the primary proliferative population, such as *Lhx2* and *Notch1*, and markers of differentiation, such as *GAD67* and microtubule associated protein 2 (MAP2) (Anderson et al., 1997b). In addition, cells in this abnormal SVZ also lose expression of other markers of differentiation, such as *Dlx5*, *Dlx6* and *Oct6* (Anderson et al., 1997b). This block in differentiation leads to a reduction of GABAergic projection neurons in the striatum. In addition, it also causes a reduction of GABAergic local circuit neurons in the cerebral cortex, hippocampus, olfactory cortex and olfactory bulb. These neurons would normally migrate to these locations via a tangential migration from the basal ganglia (Anderson et al., 1997a; Bulfone et al., 1998; Anderson et al., 1999; Pleasure et al., 2000; Anderson et al., 2001; Marin and Rubenstein, 2001). The *Dlx5&6*$^{-/-}$ mutant mice are currently under investigation for a CNS phenotype. The CNS phenotype of the *Dlx1&2*$^{-/-}$ compound mutant mice again supports the hypothesis that there is compensation by other members of the *Dlx* gene family in the single *Dlx* mutant mice.

Despite the lack of phenotypes in the basal ganglia in the *Dlx* single mutants, one CNS structure seems to be uniquely sensitive to mutations in the *Dlx* genes. That

structure is the OB, an evagination of the developing forebrain. All of the *Dlx* mutant mice generated have a phenotype in the OB. The *Dlx1*$^{-/-}$ mutant mice have a small reduction in the tyrosine hydroxylase (TH) population of local circuit neurons, the periglomerular cells (Long and Rubenstein, unpublished observations). The *Dlx2*$^{-/-}$ mutant mice have a more dramatic reduction in the same population (Qiu et al., 1995; Long and Rubenstein, unpublished observations). The *Dlx5*$^{-/-}$ mutant mice have a reduction of both TH and *GAD67* local circuit neurons in the periglomerular layer and granule cell layer (Long and Rubenstein, unpublished observations). The *Dlx1&2*$^{-/-}$ mutant mice have a virtual absence of all local circuit neuron markers (Bulfone et al., 1998; Long and Rubenstein, unpublished observations). The *Dlx5&6*$^{-/-}$ mutant mice have small OBs and are currently being examined in further detail. In summary, all *Dlx* mutant mice studied to date have abnormalities in the OB.

The OB provides a unique system in which to study the genetic programs of proliferation, migration and differentiation. A large majority of the OB local circuit neurons are thought to migrate from caudal locations via a tangential migration (Luskin and Boone, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1997). These cells proliferate as they migrate to the OB, and then differentiate once they reach their final destination (Garcia-Verdugo et al., 1998; Law et al., 1999; Peretto et al., 1999; Wozniak and Bruska, 1999; Mason et al., 2001; Fasolo et al., 2002). Due to its sensitivity to perturbations by the *Dlx* mutations, the OB can be a model to understand the role of the *Dlx* genes in the development of this structure. Our hypotheses are tested using *Dlx* single and compound mutant analyses. Furthermore, this information can then be applied to the understanding of the greater role of the *Dlx* genes in the development of the CNS and

possibly the treatment of human neurological disorders and skeletal malformations, such as schizophrenia and autism or Split Hand/Split Foot Malformation (SHSM) (McGuffin et al., 1995; Royston and Roberts, 1995; Weinberger, 1995; Crackower et al., 1996; Barondes et al., 1997; Rice and Barone, 2000; Gogos and Karayiorgou, 2001).

CHAPTER ONE

DLX5 Regulates Development of Peripheral and Central Components of the Olfactory System

ABSTRACT

Induction, neurogenesis and synaptogenesis of the olfactory bulb are thought to require interactions with the olfactory epithelium. The *Dlx* family of homeodomain containing genes is expressed in both the olfactory bulb and olfactory epithelium. In particular, *Dlx5* is expressed in the olfactory placode, olfactory epithelium and in local circuit neurons of the olfactory bulb. Here we have analyzed mice lacking DLX5 function. The $Dlx5^{-/-}$ mutation reduces the size of the olfactory epithelium. Although some olfactory neurons are formed, they fail to generate olfactory axons that innervate the olfactory bulb. Despite the lack of innervation, the olfactory bulb forms and neurogenesis of projection and local circuit neurons proceeds. However, the mutation has a cell-autonomous effect on the ability of neural progenitors to produce olfactory bulb local circuit neurons, with granule cells more severely affected than periglomerular cells. In addition, the mutation has a non-cell autonomous effect on the morphogenesis of mitral cells.

INTRODUCTION

The olfactory bulb (OB) is an evagination from the rostral telencephalon that receives primary olfactory axonal innervation from neurons in the olfactory epithelium (OE). The OB is a laminar structure (Fig. 9A; (Shepherd, 1998)). Its outer nerve layer (ONL) consists primarily of olfactory afferent axons and ensheathing glia. The axons from olfactory neurons expressing a specific olfactory receptor converge, and synapse upon, the dendrites of glutamatergic projection neurons (mitral and tufted cells) in structures called glomeruli. Interspersed between glomeruli are local circuit neurons (periglomerular cells), which send their dendrites into the glomeruli of the glomerular layer (PG). These neurons are both GABAergic (GABA is γ-amino butyric acid) and dopaminergic (about 85% of Tyrosine Hydroxylase$^+$ (TH) cells are also GABA$^+$ in the PG (Gall et al., 1987; Kosaka et al., 1995)). Deep to the PG is the external plexiform layer (EPL), which contains the horizontal processes of tufted and mitral cells, the cell bodies of tufted cells and the dendrites of a second type of GABAergic local circuit neuron (granule cells). The next layer, the mitral cell layer (MC), contains the cell bodies of the mitral cells. These neurons grow their axons into the fibrous internal plexiform layer (IPL). Below this fiber zone are the cell bodies of the granule cells, forming the granule cell layer (GC). Deep to the GC is the subventricular zone (SVZ), a reservoir of progenitor cells which produces new granule and periglomerular neurons in the adult brain (Hinds, 1968a, b; Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Goldman and Luskin, 1998). At the center of the OB are ependymal cells, the remnants of the neuroepithelial lining of the ventricle.

Development of the OB begins with its induction and evagination from the rostral telencephalon. There is evidence that signaling from the olfactory placode contributes to patterning the telencephalic anlage of the OB (Graziadei and Monti-Graziadei, 1992; De Carlos et al., 1995; LaMantia et al., 2000). Primary olfactory axons are also implicated in regulating the early neurogenesis within the OB (De Carlos et al., 1995; Gong and Shipley, 1995), although direct demonstration that olfactory afferents are essential for OB neurogenesis is lacking. The genesis and differentiation of OB projection and local circuit neurons are under distinct genetic controls. The projection neurons (mitral and tufted cells) have a pallial origin and are regulated by cortical transcription factors such as *Tbr1* (Bulfone et al., 1998), whereas the local circuit neurons (periglomerular and granule cells) have a subpallial origin and are regulated by transcription factors such as *Dlx1* and *Dlx2* (Qiu et al., 1995; Bulfone et al., 1998).

The *Dlx* genes are homeodomain transcription factors that regulate development of multiple cell-types derived from the subcortical telencephalon (Qiu et al., 1995; Anderson et al., 1997b; Bulfone et al., 1998). *Dlx1, Dlx2, Dlx5* and *Dlx6* are expressed in precursors of telencephalic GABAergic and dopaminergic neurons. Their expression persists at lower levels in postmitotic neurons (Liu et al., 1997; Stuhmer et al., 2002a; Stuhmer et al., 2002b). In the telencephalon, expression of *Dlx5* and *Dlx6* generally occurs after *Dlx1* and *Dlx2* (Eisenstat et al., 1999; Stuhmer et al., 2002a; Stuhmer et al., 2002b).

Local circuit neuron development in the OB is sensitive to all *Dlx* mutations studied to date. Both $Dlx1^{-/-}$ and $Dlx2^{-/-}$ mutants have a reduction in TH^+ neurons (Qiu et al., 1995); Long and Rubenstein, unpublished observations) and $Dlx1\&2^{-/-}$ mutants lack

>95% of $GABA^+$ and TH^+ neurons (Bulfone et al., 1998). *Dlx1&2*$^{-/-}$ mutants also lack *Dlx5* expression in most regions of the forebrain (Anderson et al., 1997b; Zerucha et al., 1997), and therefore, it is possible that the local circuit neuron defects in these mutants arise from DLX5 deficiency. To address this possibility, we have investigated OB development of *Dlx5*$^{-/-}$ mutant animals.

Dlx5 is expressed in multiple components of the olfactory system. It is expressed in the olfactory placode and in the OE; its expression in the olfactory placode is required for morphogenesis of the skeleton of the frontonasal prominence (Acampora et al., 1999; Depew et al., 1999). *Dlx5* is also expressed in the SVZ, GC and PG of the OB. Here we focus on the role of DLX5 regulating the development of the OE and OB.

MATERIALS AND METHODS

Animals and Tissue Preparation

A mouse mutant strain with a null allele of *Dlx5* was used in this study (Depew et al., 1999). This mouse strain was maintained by backcrossing to *C57BL/6J* mice for more than ten generations. For staging of embryos, midday of the vaginal plug was calculated as embryonic day 0.5 (E0.5). Mouse colonies were maintained in accordance with the protocols approved by the Committee on Animal Research at UCSF. Animals expected to contain $Dlx5^{-/-}$ mutant embryos were sacrificed by Cesarean section. PCR was performed as described (Bulfone et al., 1993a; Depew et al., 1999) to genotype offspring resulting from *Dlx5* heterozygous matings. Heterozygous and wild type embryos showed the same phenotype, so both are used as controls throughout this paper. Embryos were anaesthetized by cooling, dissected and immersion fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 4-12 hours. Samples were either cryoprotected in a gradient of sucrose to 30%, frozen in embedding medium (OCT, Tissue-Tek, Torrance, CA) and cut using a cryostat or dehydrated in ethanol, embedded in paraffin and cut using a microtome.

In Situ Hybridization

In situ hybridization experiments were performed using ^{35}S riboprobes on 10 μm frozen sections as described previously (Bulfone et al., 1993a). We generously thank the following people for cDNAs: Drs. Brian Condie (*GAD67*), Peter Gruss (*Pax6*), Tom Curran (*Reelin*), Dona Chickarishi (*Tyrosine Hydroxylase*), and Francois Guillemot

(*Hes5*). The *Dlx1*, *Dlx2* and *Dlx5* plasmids were generated in the Rubenstein lab.

Histochemistry

Samples were sectioned at 10 μm and mounted onto SuperFrost Plus slides (Fisher, Pittsburgh, PA). Sections were stained with either Gimori trichrome (E10.5) on paraffin embedded sections or cresyl violet (E12.5 to postnatal day 0 (P0)) on OCT embedded sections and analyzed.

BrdU Labeling

Pregnant female mice were injected intraperitoneally with 40 mg/kg of bromodeoxyuridine (BrdU, Sigma, St. Louis, MO) and sacrificed either 60 minutes later (Fig. 7) or at E18.5 (Fig. 8) as described (Anderson et al., 1997b).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Marin et al., 2000). We used the following primary rabbit polyclonal antibodies: anti-NPY (diluted 1:3000; Incstar, Stillwater, MN); anti-GAD65 (diluted 1:1000; Chemicon, Temecula, CA); anti-GAD67 (diluted 1:2000; Chemicon, Temecula, CA); anti-TBR1-c (diluted 1:100; kindly provided by Dr. M. Sheng); mouse monoclonal antibodies: anti-ß-III Tubulin (diluted 1:200; Promega, Madison, WI); anti-GAP43 (diluted 1:1000; Chemicon, Temecula, CA); anti-REELIN (diluted 1:500; kindly provided by Dr. A. Goffinet); rat monoclonal antibodies: anti-NCAM (diluted 1:100; Sigma, St. Louis, MO); anti-BrdU (diluted 1:10; Harlan, Crawley Down, Sussex, England); goat polyclonal antibody: anti-OMP (diluted

1:2000, kindly provided by Dr. F. Margolis).

DiI Labeling

P0 *Dlx5*$^{-/-}$ mutants and their wild type littermates were immersion fixed with 4% PFA, their brains were then removed and kept in fixative. Crystals of the axonal tracer, 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR), were placed into the lateral olfactory tract (LOT) to retrogradely label mitral cells of the OB. Crystals of similar shape and size (100-200 μm in diameter) were used in homozygous mutant and wild type littermate embryos. Brains were kept in 4% PFA at room temperature for 2 to 3 weeks to allow the DiI to diffuse. Subsequently, brains were embedded in 5% low melting point agarose (FMC Bioproducts, Rockland, MA) and 100 μm coronal sections were cut with a vibrating microtome (VT1000S, Leica, Nussloch, Germany). Sections were counterstained with 50 μg/mL Hoechst 33342 (Molecular Probes, Eugene, OR) and mounted using Aquamount (Polysciences, Warrington, PA).

Slice Culture

Embryos were removed by Cesarean section from timed pregnant *Dlx5* heterozygous female mice mated with *Dlx5* heterozygous male mice. Brains were dissected in ice-cold Krebs buffer as described previously (Marin et al., 2000), embedded in 5% low melting point agarose (FMC Bioproducts, Rockland, MA) and 250 μm sagittal sections were cut with a vibrating microtome (VT1000S, Leica, Nusslock, Germany). Slices were then placed onto Transwell membranes (8 μm pore size, 24 mm diameter membrane, Costar,

Acton, MA) that were previously coated with 1 mg/mL of Vitrogen (Cohesion, Palo Alto, CA) for 1 hour. The Transwell membranes containing the slices were placed into Neurobasal media containing 2% B-27 supplement (LTI, Gaithersburg, MD), 0.5% glucose, 2% glutamine and 2% penicillin/streptomycin. The slices were then injected with a *LacZ*-encoding defective retroviral vector using a micromanipulator and nitrogen injector (Marin et al., 2000). The slices were placed in a 37°C incubator with 5% CO_2 for 72 hours. The slices were then processed for ß-galactosidase histochemistry as described previously (Marin et al., 2000).

RESULTS

Dlx5$^{-/-}$ Mutants Have A Hypoplastic Olfactory Epithelium That Fails To Form Normal Axonal Connections with the Olfactory Bulb

Dlx5 is expressed throughout the olfactory placode and later, in the olfactory pit, in addition to expression in other regions of the embryo (Fig. 1A). Its expression in the olfactory placode is essential for development of the underlying frontonasal prominence (Acampora et al., 1999; Depew et al., 1999). Further analysis of olfactory placode derivatives shows that the *Dlx5*$^{-/-}$ mutant olfactory pit is small and lacks thickening of the medial epithelium at E10.5 (Fig. 1B,C). At later stages, the OE and vomeronasal organ are greatly reduced in size (Figs. 1D-I; 2A-H; 3A-D; see legend Fig. 3; data not shown). The small neuroepithelium exhibits some normal molecular characteristics of differentiation, including the presence of β-III-Tubulin and NCAM proteins at E12.5 (Fig. 2A-D), expression of the mutant allele, *Dlx5*^{m} (a transcript continues to be produced), *Lhx2*, *Otx1*, *Otx2* and *Pax6* RNA at E13.5 (data not shown) and expression of olfactory marker protein (OMP) at E18.5 (Fig. 2E-H). While some olfactory axons appear to grow from the neuroepithelium and fasciculate (arrowheads in Fig. 2B,D,F,H), these have not been detected contacting the OB in the *Dlx5*$^{-/-}$ mutant. This is further demonstrated by the lack of GAP43 and OMP expression on the surface of the main and accessory OBs at P0 (Fig. 2I-L), analysis of Nissl stained sections at E14.5 and E18.5 (Fig. 3A-D) and DiI axon tracing from the OE at P0 (data not shown). The cribiform plate in the *Dlx5*$^{-/-}$ mutant has a paucity of foramina for the passage of olfactory axons

through the skull on route to the OB (Fig. 3B,D; data not shown), further suggesting that few, if any, OE axons pass through the cribiform plate, or even contact the OB. These results indicate that the *Dlx5*$^{-/-}$ mutation results in a hypoplastic OE that fails to produce axons that contact the OB.

Reduced GABAergic Neuron Production in the Olfactory Bulb of *Dlx5*$^{-/-}$ Mutants

Dlx5 expression is prominent in the subcortical telencephalon and its extension into the OB (Figs. 4C; 5A,C,E). While loss of DLX5 function does not appear to have a major effect on development of the basal ganglia (Figs. 3A-D; 5A-J; Acampora et al., 1999 and data not shown), the growth and histology of the OB are disrupted by this mutation, seen clearly at E14.5 and more dramatic at E18.5 (Figs. 3A-D; 4A-P; 5A-R). Nissl staining at E18.5 shows that *Dlx5*$^{-/-}$ mutants lack the distinct neuronal layers characteristic of the OB (Figs. 3C,D; 4I,J; 7E,F). Coronal sections also suggest that the olfactory ventricle is smaller in the mutant (Figs. 2, 4 and 6).

OB laminar organization was further studied at E18.5 using *in situ* hybridization on cross-sections with RNA probes that define cell-types in distinct layers (Figs. 4, 9A). For the progenitor layers, we examined the expression patterns of the *Dlx* gene family members. *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* are differentially expressed in the progenitor and neuronal cell layers (Fig. 4A-D, data not shown; (Dolle et al., 1992; Bulfone et al., 1998; Stuhmer et al., 2002a)). They are weakly expressed in the ventricular zone (VZ) and strongly expressed in the SVZ. The expression patterns of *Dlx1*, *Dlx2*, *Dlx5*^{m} and *Dlx6* in the *Dlx5*$^{-/-}$ mutant suggest that the size of the SVZ, relative to the rest of the OB, is

increased.

To assess the development of OB local circuit neurons, we analyzed RNA expression of the *Dlx* genes, *GAD67*, *GABA transporter*, *TH* and *Pax6* and protein expression of GAD65 and TH (Fig. 4; data not shown). *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* are expressed in the GABAergic and dopaminergic neurons of the GC and PG. The expression patterns of *Dlx1*, *Dlx2*, *Dlx5*^{m} and *Dlx6* in the *Dlx5*$^{-/-}$ mutant show that the GC and PG are reduced (Fig. 4A-D; Fig. 9; data not shown). Expression of *GAD67*, one of the enzymes which makes GABA, weakly marks the SVZ and strongly labels the GC and PG in the normal OB (Fig. 4E; (Behar et al., 1994)). In the mutant, there is a prominent decrease in *GAD67* expression, specifically, a decrease in the size and intensity of labeling in the GC and a slight reduction in the PG (Fig. 4E,F; Fig. 9). Expression of GAD65 protein is readily detectable in the GC and PG (Fig. 4G; (Feldblum et al., 1993; Esclapez et al., 1994)). In the *Dlx5*$^{-/-}$ mutant, there is a severe reduction of GAD65 granule cell expression, including their projections through the MC and EPL. In addition, the *Dlx5*$^{-/-}$ mutant shows reduced GAD65 expression in the PG (Fig. 4G,H; Fig. 9). Expression of TH marks a subset of granule and periglomerular dopaminergic cells (Gall et al., 1987; Kosaka et al., 1995; Toida et al., 2000; Baker et al., 2001). In the *Dlx5*$^{-/-}$ mutant, expression of *TH* is almost eliminated from the GC and is reduced in the PG (Fig. 4K,L). *Pax6* is co-expressed with *TH* in periglomerular cells, is moderately expressed in the SVZ and strongly expressed in the VZ (Fig. 4M,N; (Gall et al., 1987; Stoykova and Gruss, 1994; Kosaka et al., 1995; Dellovade et al., 1998; Toida et al., 2000)). Its expression in periglomerular cells is reduced similar to the reduction of *TH* (Fig. 4M,N; Fig. 9), consistent with their co-expression. These results demonstrate that the *Dlx5*$^{-/-}$

mutation decreases the number of GAD^+ and TH^+ OB local circuit neurons.

Analysis of the projection neurons of the OB was performed using *Reelin* and *Id2* expression. At E18.5, *Reelin* expression is largely restricted to mitral cells (Bulfone et al., 1998). Although the MC lacks its prominence in Nissl and Hoechst stained sections of the $Dlx5^{-/-}$ mutants (Figs 3C,D; 4I,J; 6A,B; 7E,F), *Reelin* labeling of these cells is robust, and the pattern suggests that this layer is thicker than normal (Fig. 4O,P; Fig. 9). A similar result was observed with *Id2* expression (data not shown; (Neuman et al., 1993)). These results provide evidence that the $Dlx5^{-/-}$ mutation affects multiple populations of OB cells. While it appears not to affect the production of mitral cells, it does alter their morphology. In addition, the $Dlx5^{-/-}$ mutation causes a decrease of the number of GAD^+ and TH^+ OB local circuit neurons.

Subcortical Migrations of Local Circuit Neurons Are Not Appreciably Affected in the $Dlx5^{-/-}$ Mutants

To evaluate potential mechanisms underlying the $Dlx5^{-/-}$ mutant OB phenotype, we studied the production of local circuit and projection neurons during development using *in situ* hybridization on parasagittal sections (Fig. 5). For local circuit neurons, we used *Dlx5* expression to assess progenitors and *GAD67* expression to assess postmitotic GABAergic cells. For projection neurons, we studied the expression of *Reelin*.

At E14.5, there appears to be at least three subcortical SVZ zones that are continuous with the SVZ of the OB; they are the SVZ of the septum, lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE). These zones are revealed by

the expression of *Dlx5* and *GAD67* (Fig. 5A,C,G,I). The lateral side of the OB is continuous with *Dlx5*$^{+}$ and *GAD67*$^{+}$ cells in the LGE and the MGE (Fig. 5A,G). The medial side of the OB is continuous with *Dlx5*$^{+}$ and *GAD67*$^{+}$ cells in the septum (Fig. 5C,I). *Dlx5* and *GAD67* expressing cells enter the ventral side of the OB. These findings suggest that OB local circuit neurons are derived from several subcortical sources, which may generate cells that tangentially migrate into the OB via a ventral route.

The *Dlx5*$^{-/-}$ mutation did not grossly affect SVZ or mantle zone (laminae containing postmitotic neurons and their processes) expression of *Dlx5*^{m} or *GAD67* in the LGE, MGE or septum (Fig. 5B,D,H,J). In addition, cortical *GAD67*$^{+}$ neurons, which are also largely derived from *Dlx*-expressing subcortical sources (Marin and Rubenstein, 2001), were not appreciably reduced (Fig. 5G-J). On the other hand, *GAD67* expression in the mantle zone of the OB was reduced (Fig. 5H,J). This suggests that the early production of OB local circuit neurons is defective. While early production of OB local circuit neurons is reduced, the generation of *Reelin*$^{+}$ projection neurons appears normal at E14.5 (Fig. 5M-P).

In situ hybridization analysis at E18.5 also showed that *Dlx5*^{m} and *GAD67* expression in the OB SVZ appeared normal in *Dlx5*$^{-/-}$ mutants, whereas the number of *Dlx5*$^{+}$ and *GAD67*$^{+}$ OB neurons was reduced in the GC and PG (Fig. 5E,F,K,L). *Reelin* expression in the MC remained robust, but its tight laminar organization was disrupted (Fig. 5Q,R). Thus, the *Dlx5*$^{-/-}$ mutation causes the decreased production of GAD67^{+} local circuit neurons of the OB from early ages and, although projection neuron production appears normal, the lamination of the MC is disrupted.

Non-autonomous Defect in Mitral Cell Morphogenesis in the *Dlx5*$^{-/-}$ Mutants

Although *Dlx5* is not expressed in mitral cells, and is probably not expressed in mitral cell precursors, we observed a non-cell autonomous effect on the MC in the *Dlx5*$^{-/-}$ mutant. As shown by Nissl and Hoechst staining (Figs. 4I,J; 6A,B; 7E,F), and by *Reelin*, *Id2* and *Tbr1* expression (Figs. 4O,P; 5Q,R; 6G-L; data not shown), *Dlx5*$^{-/-}$ mutants have a thickened MC whose contour is irregular. To further evaluate this phenotype, we retrogradely labeled the mitral cells and their dendrites with DiI. DiI labeling in the lateral olfactory tract (LOT) labeled the GC and IPL (consisting of mitral cell axons), mitral cells bodies in the MC and their processes in the EPL and PG. In controls, mitral cells were regularly spaced and had radially oriented dendrites, whereas in the *Dlx5*$^{-/-}$ mutants, the orientation of the mitral cell dendrites was variable (Fig. 6C-F). This result was confirmed by immunofluorescent double labeling of mitral cells for TBR1 (nuclear-labeling, Fig. 6G,H,K,L) and REELIN (proximal dendrite-labeling, Fig. 6I-L). This analysis provides additional evidence that the orientation of proximal dendrites was rotated, the dendritic trees were smaller and that the mitral cells and their axons were in the same layer (Fig. 6G-L). These results further support the idea that the *Dlx5*$^{-/-}$ mutation alters the morphology of the mitral cells. The potential mechanisms underlying this phenotype are considered in the Discussion.

Analysis of Proliferation and Cell Death in the Olfactory Bulbs of *Dlx5*$^{-/-}$ Mutants

The *in situ* hybridization analyses suggest that *Dlx5*$^{-/-}$ mutants have a deficit in the

production of local circuit neurons which begins as early as E14.5. To test whether this is due to a defect in the proliferation of OB progenitors, we used a BrdU incorporation assay at developmental stages when local circuit neurons constitute the majority of neurons being generated (Hinds, 1968a, b, 1972a, b). One hour following a pulse of BrdU at E14.5, E16.5 or E18.5, we identified the location of cells in S-phase of the cell cycle in coronal sections of *Dlx5*$^{-/-}$ mutants and control littermates (Fig. 7A-D,I,J). At each of these ages, the BrdU pulse labeled three sets of cells: 1) OB progenitors in the VZ and SVZ; 2) cells in the ONL; 3) mesenchymal cells surrounding the brain (i.e.: meninges). In the *Dlx5*$^{-/-}$ mutants, the size of the progenitor zone, and the density of S-phase cells within it, appeared roughly normal, although we can not rule out a subtle change in the rate of proliferation in this layer.

In contrast, there was a reduction in the number of proliferating cells in the OB's most superficial layer in the *Dlx5*$^{-/-}$ mutant at E16.5 and E18.5 (Fig. 7C,D,I,J). To confirm this finding, we used *in situ* hybridization with *Hes5*, a bHLH gene that is expressed in proliferating neural progenitors (Akazawa et al., 1992) and in cells of the ONL (Fig. 7G). In controls, *Hes5* expression was high in the VZ, moderate in the SVZ, and high in the ONL, whereas in the *Dlx5*$^{-/-}$ mutant, *Hes5* expression appeared normal in the OB progenitor zones, but was very low in the most superficial area of the OB (Fig. 7G,H). The proliferating cells in the ONL are known to differentiate into a specialized set of glia that express NPY (Ubink et al., 1994; Ubink and Hokfelt, 2000). These cells are derived from the olfactory placode/epithelium (Doucette, 1993; Mallamaci et al., 1996). Indeed, NPY expression in the ONL is greatly reduced at E18.5 in the *Dlx5*$^{-/-}$ mutant (Fig. 7K,L). The residual BrdU-labeled cells surrounding the mutant OB may correspond

to mesenchymal cells, such as the meninges (Fig. 7I,J). Thus, the *Dlx5*$^{-/-}$ mutation reduces the production of olfactory nerve glia, probably due to its function in the development of the OE.

To assess whether the decreased numbers of local circuit neurons could be due to increased levels of cell death, in either the progenitor or postmitotic cell populations, we used the TUNEL apoptosis assay. We found similar levels of TUNEL^{+} cells in *Dlx5*$^{-/-}$ mutants and control littermates at E14 and E18 (data not shown). These results demonstrate that the *Dlx5*$^{-/-}$ mutation does not have a noticeable effect on cell death or proliferation using the methods that we have employed. However, there is a dramatic reduction in the number of BrdU labeled cells in the most superficial layer of the *Dlx5*$^{-/-}$ mutant OB, which likely correspond to olfactory nerve glia.

Analysis of Neuronal Migration in the Olfactory Bulb and Rostral Migratory Stream of *Dlx5*$^{-/-}$ Mutants

To assess whether the reduction in granule and periglomerular cells is due to defects in migration, we used BrdU birthdating and slice culture migration assays. BrdU pulses were performed on E14.5 and E16.5 embryos to label cells going through S-phase at that time. The BrdU-treated embryos were harvested on E18.5, and the positions of BrdU-labeled cells were analyzed in coronal sections of the OB. The majority of mitral cells are born before E14.5 (Hinds, 1968a, b, 1972a, b); therefore, labeled cells outside the progenitor zones in this experiment will tend to correspond to local circuit neurons, tufted cells and glia (Hinds, 1968a, b, 1972a, b). BrdU exposure at E14.5 and E16.5 in

control embryos led to BrdU incorporation into some cells which remained in the progenitor zones and some which were located superficially in the OB (Fig. 8A-D). In the *Dlx5*$^{-/-}$ mutants, fewer BrdU-labeled cells were present outside of the progenitor zone. This is particularly clear for the embryos labeled at E16.5 (Fig. 8C,D). Furthermore, labeling at E14.5 resulted in an accumulation of BrdU^{+} cells in the SVZ (note the increased density of labeled cells in the SVZ and the very small olfactory ventricle; Fig. 8A,B), suggesting that the *Dlx5*$^{-/-}$ mutation reduces the rate at which SVZ progenitors mature into neurons.

The BrdU results suggest that radial migration from the progenitor zone to the local circuit neuron layers is reduced in the *Dlx5*$^{-/-}$ mutants. To assess whether tangential migration along the rostral migratory stream (RMS) is affected by the *Dlx5*$^{-/-}$ mutation, we used a slice culture cell migration assay (Tobet et al., 1996) (Fig. 8E,F). Parasagittal vibrating microtome slices from E18.5 control and *Dlx5*$^{-/-}$ mutant embryos were generated. We labeled the RMS with injections in 5-6 different locations using a *LacZ*-encoding replication incompetent retroviral vector. The slices were grown for 72 hours, and the locations of *LacZ*-expressing cells were assessed by ß-galactosidase activity. The experiment showed that *Dlx5*$^{-/-}$ mutants have tangential migration in their RMS (Fig. 8E,F) (N=5 controls; N=5 *Dlx5*$^{-/-}$ mutants). Similar results were obtained using DiI labeling of migrating cells (data not shown). These results provide evidence that tangential migration still occurs in the *Dlx5*$^{-/-}$ mutants.

DISCUSSION

Herein we show that DLX5 has a central role in the development of the primary structures involved in olfaction. Murine *Dlx5*$^{-/-}$ mutants have a hypoplastic OE that fails to produce axons which innervate the OB. Despite the lack of innervation, the OB still forms, although with a reduced size and altered lamination. The size and lamination defects (Fig. 9) are contributed to by cell-autonomous defects in local circuit neuron production and by a non-cell autonomous effect on the orientation and dendritic branching of the mitral cells.

Loss of Normal Olfactory Input to the Telencephalon Is Not Essential For Olfactory Bulb Neurogenesis

Previous studies have suggested that the olfactory placode and/or epithelium may have a key role in the initiation of OB development and neurogenesis (Graziadei and Monti-Graziadei, 1992; LaMantia et al., 1993; Gong and Shipley, 1995; LaMantia et al., 2000). Although *Dlx5* is expressed in the olfactory placode, *Dlx5*$^{-/-}$ mutants are able to produce an olfactory pit, and differentiate respiratory and neuroepithelial tissues. Thus, DLX5 is not essential for specification of the entire olfactory placode. Perhaps, other *Dlx* family members (e.g. *Dlx6*) compensate for early DLX5 function in this tissue; this possibility can be studied in *Dlx5&6*$^{-/-}$ mutants which have recently been generated (Robledo et al., 2002).

The OE is severely hypoplastic in the *Dlx5*$^{-/-}$ mutants (Figs. 1,2,3). As early as E10.5, *Dlx5*$^{-/-}$ mutants show reduced olfactory neuroepithelial structures (Fig. 1C). At this point, it is uncertain whether the mutation causes a general hypoplasia, or whether specific subdivisions of the OE are preferentially affected (De Carlos et al., 1995).

Loss of *Dlx5* also affects the differentiation of the olfactory neurons. While they can express NCAM, β-III-Tubulin and OMP (Fig. 2B,D,F,H), their axons fail to grow to the olfactory bulb. Although we cannot completely rule out the possibility that a few OE axons do contact the OB, our analyses of Nissl-stained; Hoechst-stained; OMP, GAP43, S100 and Calretinin immunohistochemistry and DiI labeling of OE axons all support the observation that no axons contact the OB. The mechanism(s) underlying this defect could lie either in the olfactory neurons, or in the environment through which they navigate, as DLX5 function is required in the morphogenesis of frontonasal mesenchyme (Acampora et al., 1999; Depew et al., 1999) and in the differentiation of the OB (Figs. 3-9).

Associated with the OE and axonal abnormalities are the deficits in olfactory ensheathing glia in the OB nerve layer and GnRH (LHRH) neurons in the forebrain of the *Dlx5*$^{-/-}$ mutants (Fig. 7L,M; data not shown). These cell types are derived from the olfactory placode and migrate along the olfactory nerve (Doucette, 1993; Tobet et al., 1993). These deficiencies could be due to a role of DLX5 in the differentiation of these cell types, and/or due to a defect in their migration that is secondary to the failure of olfactory nerve growth.

There is evidence that early olfactory axons may regulate cell cycle kinetics in the telencephalic anlage of the OB (Gong and Shipley, 1995). Although we have not directly ruled out this possibility, the fact that in the absence of most olfactory axons, the OB is

induced and its early neurogenesis (mitral cells) appears normal suggests that olfactory axons are not necessary for these processes. A similar result is observed in *Mash1*$^{-/-}$ mutants, which fail to produce olfactory neurons (Guillemot et al., 1993; Casarosa et al., 1999).

While olfactory axons are not essential for the induction and growth of the OB, we provide evidence that they are required for its laminar properties. Nissl and Hoechst staining of *Dlx5*$^{-/-}$ mutant OBs fail to reveal normal laminar properties, despite the laminar expression of several GC, MC and PG markers (Figs. 4,6,7,9). The lack of histological lamination is not entirely due to the deficiencies in local circuit neurons, as *Dlx1&2*$^{-/-}$ mutants, which lack local circuit neurons, exhibit clear OB lamination (Bulfone et al., 1998). DiI labeling of mitral cells in the *Dlx5*$^{-/-}$ mutants shows that these cells lack a radial orientation and have hypoplastic dendritic trees. *Reelin* labeling shows that the mitral cell layer is thicker (Fig. 4O,P; 9), perhaps due to haphazard packing of mitral cell bodies (Fig. 6C-L). These non-autonomous effects, which may result from the lack of OE input, contribute to the defect in OB lamination in the *Dlx5*$^{-/-}$ mutants. Thus, olfactory axons probably have a central role in organizing cellular morphogenesis of OB neurons.

Dlx Genes Regulate the Generation of Olfactory Bulb Local Circuit Neurons

Dlx1, *Dlx2*, *Dlx5* and *Dlx6* are expressed in the progenitors of OB local circuit neurons (Figs. 4,5,9; (Liu et al., 1997; Stuhmer et al., 2002a)). Their expression is maintained in postmitotic granule and periglomerular neurons, albeit at a lower level

(Fig. 4,9; (Stuhmer et al., 2002a; Stuhmer et al., 2002b)). *Dlx2*$^{-/-}$ mutants have greatly reduced numbers of TH^{+} periglomerular cells (~80%; Qui et al., 1995), *Dlx1*$^{-/-}$ mutants less so (~15% reduction) (Long and Rubenstein, unpublished), whereas *Dlx1&2*$^{-/-}$ mutants lack most GABA^{+} and TH^{+} neurons ((Bulfone et al., 1998); Long and Rubenstein, unpublished). *Dlx1&2*$^{-/-}$ mutants fail to express *Dlx5* in the SVZ of most of the telencephalon, however, residual expression is found in part of the septum (Anderson et al., 1997b; Zerucha et al., 1997). Through the analysis of the *Dlx5*$^{-/-}$ mutants, we have demonstrated that the severe reduction of OB local circuit neurons in *Dlx1&2*$^{-/-}$ mutants is not due only to the loss of *Dlx5*. In addition, we have demonstrated that, like *Dlx2*$^{-/-}$ mutants, *Dlx5*$^{-/-}$ mutant mice have a hypomorphic OB local circuit neuron phenotype (Fig. 9). The *Dlx5*$^{-/-}$ mutation appears to preferentially affect granule cells as evidenced by the particularly severe reduction of *GAD67*, GAD65 and *TH* expression in this layer (Fig. 4E-H,K,L). The reduction in *TH* expression could be caused by the lack of olfactory axons (Baker et al., 1983; Baker, 1990; Baker and Farbman, 1993). However, we suggest that a cell autonomous mechanism contributes to this phenotype, as both *Dlx2*$^{-/-}$ and *Dlx1&2*$^{-/-}$ mutants have fewer TH^{+} PG cells, even though the olfactory innervation appears to be normal (Qiu et al., 1995; Bulfone et al., 1998). At this point, it is unclear whether *Dlx2*$^{-/-}$ and *Dlx5*$^{-/-}$ mutants have phenotypic differences in the production of local circuit neurons; ongoing work is aimed at elucidating the individual and combined roles of the *Dlx* genes in OB neurogenesis.

Dlx and *GAD* Expression in the Embryonic Brain Suggests Pathways of Olfactory Bulb Local Circuit Neuron Migration

Based on the expression of *Dlx1*, *Dlx2*, *Dlx5* and *GAD67*, there may be at least three distinct progenitor zones that contribute tangentially migrating precursors to OB local circuit neurons (Fig. 5A,C and data not shown). Cells may migrate from the SVZ of the septum to medial parts of the OB (Fig. 5C), in addition to cells that may migrate from the SVZ of the MGE and LGE into lateral parts of the OB (Fig. 5A). In each case, these progenitors enter the ventral part of the OB (Fig. 5). We propose that the rostral convergence of the three embryonic pathways becomes the RMS. The number of progenitor zones and potential migration pathways suggests that different subtypes of OB local circuit neurons may originate from distinct locations.

However, DLX5 function is not essential for the formation of these progenitor zones or potential migration pathways (Fig. 5B,D,H,J). Indeed, in E18.5 $Dlx5^{-/-}$ mutants, tangential migration along the RMS was similar to controls using the retroviral and DiI-labeling assays (Fig. 8E,F and data not shown). Furthermore, we did not detect gross abnormalities in the proliferation of OB local circuit neuron progenitors in the $Dlx5^{-/-}$ mutants (Fig. 7 and data not shown). Of course, subtle defects in proliferation, migration or survival would not have been detected with our methods.

Thus, why do $Dlx5^{-/-}$ mutants have fewer GAD65^{+}, *GAD67*$^{+}$ and *TH*$^{+}$ neurons in the GC and PG (Figs. 4, 5, 9)? A BrdU pulse-chase (E16.5-E18.5) shows a reduction in the number of cells that populate the OB mantle zone (Fig. 8C,D). This suggests that the reduction in OB local circuit neurons is due to their reduced production. If the defect were due to reduced migration of postmitotic neurons, we would have observed periventricular ectopic collections of postmitotic neurons. Unlike the $Dlx1\&2^{-/-}$ mutants,

where this is a prominent aspect of their phenotype in the basal ganglia (Anderson et al., 1997b; Marin et al., 2000), periventricular ectopic neurons were not found in the *Dlx5*$^{-/-}$ mutants. Therefore, we suggest that DLX5 is necessary for progenitors in the SVZ of the OB to mature into postmitotic local circuit neurons. This defect could cause an expansion of the progenitor zone (Fig. 9). This would be consistent with the apparent accumulation of BrdU-labeled cells in the SVZ in the BrdU pulse chase (E14.5-E18.5) (Fig. 8A,B). Furthermore, while there is not a massive expansion of the SVZ, this defect could explain why the olfactory ventricle is smaller in most mutants (Fig. 2,4,6,7,8). Thus, future studies will focus on establishing how DLX5 regulates the transition from SVZ-type progenitor to neuron.

FIGURES

E10.5
E12.5
+/
+/
−/−
−/−
A
B
C
D
E
F
G
H
I
OV
OP
VNO
mdBA1
BA2
AER
BA3
BA4
LFNP
MFNP
LGE
LV
Se
POB
OE
RE
MGE

Figure 1: Analysis of OE development in E10.5 Gimori-stained (B,C) and E12.5 Nissl-stained (D-I) control and *Dlx5*$^{-/-}$ mutant embryos. (A) Schematic of *Dlx5* expression in an E10.5 embryo (brain expression is not shown). (B,C) The olfactory pit is smaller in the mutant (C). In addition, the size of the LFNP and MFNP are reduced. The hole in the mutant section (C) is a sectioning artifact. (D-I) Series of horizontal sections from rostral (D,E) to caudal (H,I) illustrating the reduction of OE size in the *Dlx5*$^{-/-}$ mutant embryo (E,G,I) compared to control (D,F,H). Note the large distance between the mutant OE and the telencephalon. AER, apical ectodermal ridge; BA2,3,4, branchial arch 2,3,4; LFNP, lateral frontonasal process; LGE, lateral ganglionic eminence; LV, lateral ventricle; mdBA1, mandibular branchial arch 1; MFNP, medial frontonasal process; MGE, medial ganglionic eminence; OE, olfactory epithelium; OP, olfactory pit; OV, otic vesicle; POB, primordial olfactory bulb; RE, respiratory epithelium; Se, septum; VNO, vomeronasal organ. Scale bars: B-I, 400 μm.

E12.5
E18.5
P0
+/
−/−
+/
−/−
β-III TUBULIN
NCAM
GAP43
OMP
OMP
A
B
C
D
E
F
G
H
I
J
K
L
OE
OE
OE
OE
l
m
m
l
ONL
OB
OB
OB
OB
ONL

Figure 2: Molecular analysis of the OE (A-H) and olfactory nerve (I-L) in control and *Dlx5*$^{-/-}$ mutant embryos. Antibody staining of ß-III Tubulin (A,B) and NCAM (C,D) in E12.5 embryos reveals that, despite its small size, the mutant (B,D) OE expresses markers of differentiation. However, development of the lateral OE is dramatically reduced (B,D) compared to controls (A,C). Arrowheads show fasciculated axons leaving the OE. Antibody staining for mature olfactory neuronal markers was performed using an antibody to OMP at E18.5 (E-H). The mutant OE expresses OMP (F,H), but in a much small region of the OE compared to controls (E,G). G and H are high power magnifications of E and F; arrows mark the extent of OMP expression and arrowheads mark fasciculated axon bundles. (H-J) Expression of GAP43 (J) and OMP (L) is absent from the most superficial layer of the OB in the *Dlx5*$^{-/-}$ mutants, suggesting that the olfactory nerve does not reach the OB. l, lateral; m, medial; OB, olfactory bulb; OE, olfactory epithelium; ONL, olfactory nerve layer. Scale bars: A-D,G,H 175 μm; E,F,I-L, 500 μm.

+/
-/-
E14.5
E18.5
A
B
C
D
Cx
AOB
OB
ON
OE
NS
LV
Se
LGE
CP
*

Figure 3: Nissl stained sagittal sections of the telencephalon and OE from E14.5 (A,B) and E18.5 (C,D) control and *Dlx5*$^{-/-}$ mutant embryos. At E14.5, it is already apparent that the *Dlx5*$^{-/-}$ mutant OB (B) is reduced in size when compared to controls (A). In addition, there is also no OE in this embryo, which occurs in 25% of the cases. At E18.5, the reduction in OB size is more apparent in *Dlx5*$^{-/-}$ mutant embryos (D). The mutant OE is greatly reduced in size (D). Additionally, there are few, if any, foramina in the cribiform plate of the mutant (D). The asterisk (*) in B and D indicates the space between the brain and cribiform plate that would normally be occupied by the olfactory nerve. AOB, accessory olfactory bulb; CP, cribiform plate; Cx, cortex; LGE, lateral ganglionic eminence; LV, lateral ventricle; NS, nasal sinus; OB, olfactory bulb; OE, olfactory epithelium; ON, olfactory nerve; Se, septum. Scale bars: A-D, 310 μm.

Nissl
TH
Pax6
Reelin
+/
−/−
+/
−/−
Dlx1
Dlx5/Dlx5m
GAD67
GAD65
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
PG
GC
VZ
SVZ
MC
EPL

Figure 4: *In situ* RNA hybridization and immunofluorescence analysis of the expression of several local circuit neuron (A-H,K-N) and projection neuron (O,P) markers in coronal hemi-sections of E18.5 OBs. In control embryos, several layers are apparent in the OB by Nissl stain, including the PG, MC, GC, SVZ and VZ (I). However, in the *Dlx5*$^{-/-}$ mutant, these layers are not readily visible (J). *In situ* analysis of *Dlx1* and *Dlx5* in the wild type embryo (A,C) shows high expression in the SVZ, weaker expression in the VZ, GC and PG and very low expression in MC. In the mutant embryo (B,D), analysis of these markers (*Dlx1* and *Dlx5*^{m}) reveals laminar organization similar to control embryos, but at slightly reduced levels in the GC and PG. *GAD67* expression in control embryos clearly demarcates the GC and PG with strong expression in these local circuit neurons (E). In the mutant embryo (F), we see a reduction in the thickness of *GAD67*$^{+}$ cells in the GC in addition to a reduction in levels of the GC and PG. GAD65 immunofluorescence labels the PG and GC in controls (G). The *Dlx5*$^{-/-}$ mutant has a severe reduction in granule cell somal and granule cell dendritic expression and a moderate reduction in the PG (H). *TH* expression shows a dramatic reduction in the GC and PG in the mutant embryo (L) when compared to controls (K). Another marker expressed by PG neurons, *Pax6*, shows a slight reduction in the mutant (N) when compared to the control (M). *Pax6* expression is also slightly reduced in the SVZ (M,N). *Reelin*, a projection neuron marker, demonstrates a thickening of the MC in *Dlx5*$^{-/-}$ mutant embryos (P). EPL: external plexiform layer; GC, granule cell layer; MC, mitral cell layer; PG, periglomerular layer; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: A-F,I-P, 590 μm; G,H, 215 μm.

E14.5
E18.5
+/
−/−
+/
−/−
+/
−/−
Dlx5/Dlx5m
GAD67
Reelin
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Cx
LGE
MZ
MGE
SVZ
Se
OB
RMS
MC

Figure 5: Expression of *Dlx5, GAD67* and *Reelin* at E14.5 and E18.5 in sagittal sections through the forebrain. In panels A, B, G and H, expression in the LGE and MGE is continuous into the OB. In the $Dlx5^{-/-}$ mutant, there is a slight reduction in $Dlx5^{m}$ and *GAD67* expression in the OB (A,B,G,H). *Dlx5* and *GAD67* expression is also continuous from the septum to the OB (C,D,I,J). Analysis of *Reelin*, a marker of the projection neurons of the OB, shows virtually normal expression despite the mutant OBs small size (M,N,O,P). At E18.5, the decreased expression of $Dlx5^{m}$ and *GAD67* further demonstrate the reduction of GC and PG in the $Dlx5^{-/-}$ mutant (E,F,K,L). However, at E18.5, *Reelin* expression is more diffuse in the $Dlx5^{-/-}$ mutant (R) than in control (Q). Cx, cortex; LGE, lateral ganglionic eminence; MC, mitral cell layer; MGE, medial ganglionic eminence; OB, olfactory bulb; RMS, rostral migratory stream; Se, septum. Scale bars: A-R, 870 μm.

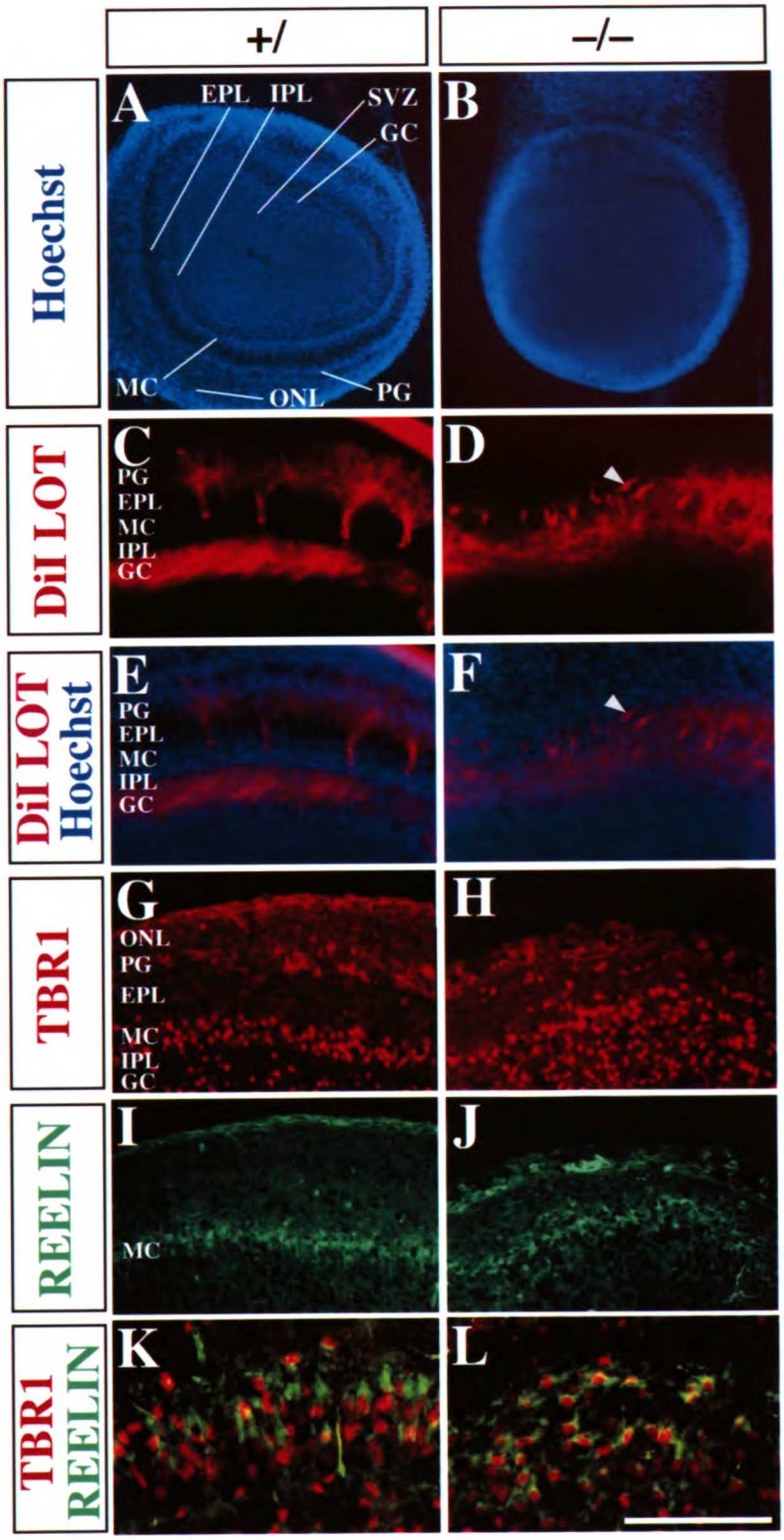

+/
−/−
Hoechst
DiI LOT
DiI LOT Hoechst
TBR1
REELIN
TBR1 REELIN
A
B
C
D
E
F
G
H
I
J
K
L
EPL
IPL
SVZ
GC
MC
ONL
PG

Figure 6: Cell non-autonomous defect in mitral cells revealed by DiI retrograde tracing and immunofluorescence. Hoechst staining of P0 embryos reveals a defect in laminar organization in the *Dlx5*$^{-/-}$ mutants (B). DiI placed into the LOT retrogradely labels mitral cells of the OB. In control embryos (C,E), radially oriented mitral cell bodies and their radially extended dendritic processes are visible. In the *Dlx5*$^{-/-}$ mutants (D,F), however, the orientation of the mitral cells is disorganized and their dendritic processes are small and disoriented (as shown by arrowheads). TBR1 (nuclear, G) and REELIN (proximal dendrite, I) immunofluorescence shows the MC in controls, which is disorganized in the mutant (H,J). Co-labeling of TBR1 (red) and REELIN (green) in mitral cells is shown in (K,L). EPL, external plexiform layer; GC, granule cell layer; IPL, internal plexiform layer; MC, mitral cell layer; ONL: outer nerve layer; PG, periglomerular layer; SVZ, subventricular zone. Scale bars: A,B, 775 μm; C-J; 320 μm; K,L, 155 μm.

BrdU - 1 Hour
Hes5
NPY
E16.5
E14.5
E18.5
+/–
–/–
A
B
C
D
E
F
G
H
I
J
K
L
VZ
SVZ
Mz
PG
ML
GC
ONL
BrdU - 1 Hour
Nissl
BrdU - 1 Hour

Figure 7: Proliferation assays reveal a loss of ONL cells. A one hour pulse of BrdU given at E14.5 and E16.5 (A,B,C,D) labels mitotically active cells in the VZ, SVZ, ONL and mesenchyme (Mz) in and surrounding the OB. The density of BrdU-labeled cells appeared normal in the VZ, SVZ and Mz, whereas by E16.5, a decrease is apparent in the *Dlx5*$^{-/-}$ mutants most superficial layer of the OB (arrowhead). A one hour pulse of BrdU at E18.5 (I,J) shows a large reduction in the number of BrdU positive cells in the *Dlx5*$^{-/-}$ mutants most superficial layer of the OB. *Hes5* expression, which marks many types of dividing cells, is also reduced in the *Dlx5*$^{-/-}$ mutants most superficial layer of the OB (G,H). NPY expression in olfactory nerve ensheathing glia is lost in the *Dlx5*$^{-/-}$ mutant (K,L). GC, granule cell layer; MC, mitral cell layer; Mz, mesenchyme; ONL, olfactory nerve layer; PG, periglomerular layer; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: A,B, 220 μm; C-F,I-L, 500 μm; G,H, 614 μm.

+/
–/–
BrdU at E14.5
Chase to E18.5
BrdU at E16.5
Chase to E18.5
Slice Migration
A
B
C
D
E
F
SVZ
PG
ONL
GC
Cx
OB

Figure 8: Migration assays in the *Dlx5*$^{-/-}$ mutant. A pulse of BrdU given at E14.5 with survival until E18.5 shows a reduction in the number of BrdU^{+} cells at the periphery of the OB in the *Dlx5*$^{-/-}$ mutant, and an increase in the density of BrdU^{+} cells within the SVZ (A,B). A BrdU pulse at E16.5 with survival until E18.5 shows a clear reduction in the number of BrdU^{+} cells in the mantle zone of the OB (C,D). A *LacZ*-encoding replication incompetent retrovirus was injected into parasagittal slices from an E18.5 telencephalon into multiple positions along proximal positions of the RMS and then stained for *β-galactosidase* activity to identify the positions of *LacZ*-expressing migratory cells (E,F; shown by arrowheads). Cx, cortex; GC, granule cell layer; OB, olfactory bulb; ONL, olfactory nerve layer; PG, periglomerular layer; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: A-D, 445 μm; E,F, 1150 μm.

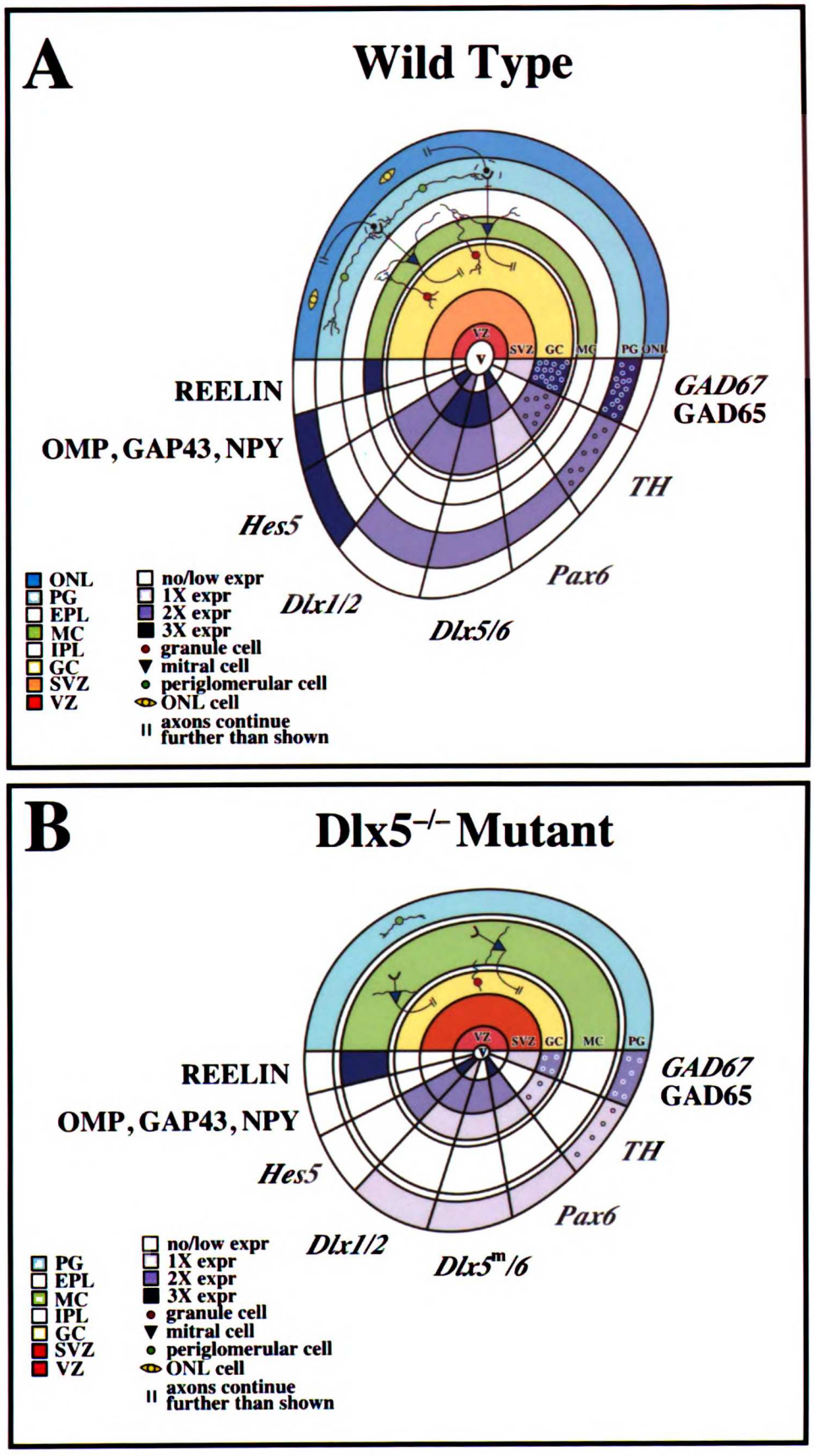

A
Wild Type
REELIN
OMP, GAP43, NPY
Hes5
Dlx1/2
Dlx5/6
Pax6
TH
GAD67
GAD65
VZ
SVZ
GC
MC
PG
ONL
ONL
PG
EPL
MC
IPL
GC
SVZ
VZ
no/low expr
1X expr
2X expr
3X expr
granule cell
mitral cell
periglomerular cell
ONL cell
axons continue further than shown
B
Dlx5−/− Mutant
REELIN
OMP, GAP43, NPY
Hes5
Dlx1/2
Dlx5m/6
Pax6
TH
GAD67
GAD65
VZ
SVZ
GC
MC
PG
PG
EPL
MC
IPL
GC
SVZ
VZ
no/low expr
1X expr
2X expr
3X expr
granule cell
mitral cell
periglomerular cell
ONL cell
axons continue further than shown

Figure 9: Schemas showing OB laminar organization and molecular expression in E18.5 wild type (A) and *Dlx5*$^{-/-}$ mutants (B). The top half of each OB shows the laminar organization (each color representing a different layer) and principal cell types. The bottom half of each OB shows the expression of various genes, where increasing color density represents increasing levels of expression. The top half of the mutant schema illustrates that *Dlx5*$^{-/-}$ mutants lack an ONL (blue), have a thicker MC (light green) and appear to have a defect in the migration of SVZ cells born at E14.5 (darker orange; see Fig. 8A,B). In addition, the mutation disrupts the radial orientation of the mitral cells (triangles) and their processes, reduces the numbers and alters the processes of periglomerular and granule cells (red and green circles), and virtually eliminates glia in the ONL (yellow spindles). The lower half of the *Dlx5*$^{-/-}$ mutant schema illustrates the lack of an ONL, emphasized by the loss of OMP, GAP43, NPY and *Hes5* expression; shows the reduced expression of *Dlx1*, *Dlx2*, *Dlx5*^{m}, *Dlx6*, *TH*, GAD65 and *GAD67* in the GC and PG, and the reduced expression of *Pax6* in the PG. See figure for color and shape definitions. EPL, external plexiform layer; IPL, internal plexiform layer; GC, granule cell layer; MC, mitral cell layer; ONL, olfactory nerve layer; PG, periglomerular layer; SVZ, subventricular zone; VZ, ventricular zone; v, olfactory ventricle

CHAPTER TWO

Dlx1 and *Dlx2* Are Required For Olfactory Bulb Local Circuit Neuron Migration

ABSTRACT

The olfactory bulb (OB) is a complex structure, generated from cells derived from several sources. One of the most widely studied sources of OB neurons is the rostral migratory stream (RMS). In the adult, these neurons migrate tangentially from the anterior horn of the SVZ to the OB via a unique process called chain migration. This chain migration is in stark contrast to the well-known glia-guided mechanisms of cell migration. The *Dlx* family of homeodomain containing transcription factors has been implicated in the development and migration of several types of neurons. The *Dlx1&2*$^{-/-}$ mutation has been shown to cause a severe decrease in the numbers of GABAergic local circuit neurons in late stages of OB development. Here, we demonstrate that virtually all of the OB local circuit neuron markers are absent and that this effect begins as early as embryonic day 14.5. Additionally, we show that the loss of OB local circuit neuron marker expression is a result of a cell autonomous migration defect. Furthermore, the loss of DLX1&2 function results in abnormal Slit/Robo expression, which may be the underlying cause of the migration defect.

INTRODUCTION

The tangential migration of neurons from distant sources has been a well studied method of creating the complex brain structure of the telencephalon (Pearlman et al., 1998; Parnavelas, 2000; Marin and Rubenstein, 2001). Neurons born in one location migrate vast distances and via diverse pathways to reach their final destination in various places in the telencephalon. The telencephalon is generally subdivided into two domains, the dorsal, or pallial domain, and the ventral, or subpallial domain. Most of these tangentially migrating neurons originate in two structures of the subpallial domain, the medial and lateral ganglionic eminences (MGE and LGE, respectively) (Anderson et al., 2001; Marin and Rubenstein, 2001). The most well studied tangential migratory pathway is the rostral migratory stream (RMS) of the adult mouse (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Luskin and Boone, 1994; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996). Neurons of the RMS originate in the anterior subventricular zone (SVZ) and migrate tangentially towards the olfactory bulb (OB). Upon reaching the OB, these neurons then migrate radially to populate the granule cell and periglomerular layers. They then differentiate further to become postmitotic local circuit neurons.

These RMS neurons have several unique characteristics. The first is that they continue to divide and migrate throughout the lifetime of the animal (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1997). A second is that they migrate as closely apposed chains of neurons ensheathed by a meshwork of astrocytes (Lois et al., 1996; Thomas et al., 1996; Doetsch et al., 1997; Peretto et al., 1997; Garcia-Verdugo et al., 1998; Wozniak and Bruska, 1999). Upon reaching the OB, these neurons then migrate as single neurons along a radial trajectory to

reach their target layers (Peretto et al., 1999). And lastly, these RMS neurons continue to proliferate despite expressing markers of differentiation (Luskin, 1993; Menezes et al., 1995).

The molecules necessary for the RMS neurons are just beginning to be elucidated. Some of these molecules function as long-range guidance cues and others function in cell-cell interactions. There are even others that function as transcriptional regulators to control the gene expression necessary for the migration of the RMS neurons.

One set of molecules involved in the migration of RMS neurons is thought to act via long-range mechanisms. An interesting hypothesis is that of a chemorepulsive factor(s) in the septum which repels RMS neurons away from the anterior SVZ towards the olfactory bulb (Hu and Rutishauser, 1996). This factor(s) has been identified to be members of the *Slit* family (Itoh et al., 1998; Li et al., 1999; Wu et al., 1999; Chen et al., 2001). *Slit* proteins have been shown to have a chemorepulsive effect on axonal growth (Rothberg et al., 1990; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Brose and Tessier-Lavigne, 2000) and promote axon branching (Wang et al., 1999; Brose and Tessier-Lavigne, 2000). These results suggest that Slit proteins may be involved in guiding neurons of the RMS.

Some of the molecules that have been shown to be important for chain migration are involved in cell-cell interactions between the neurons of the RMS. Several studies have suggested that the polysialic acid (PSA) moiety of the neural cell adhesion molecule (NCAM) is required for this process (Ono et al., 1994; Rousselot et al., 1995; Hu et al., 1996). PSA has been shown to promote homotypic interactions between migrating RMS neurons (Rousselot et al., 1995; Hu et al., 1996; Bruses and Rutishauser, 2001), results

which were contrary to the original belief that PSA was thought to have "anti-adhesive" properties (Rutishauser, 1996). Recent studies however, have cast doubt on the absolute requirement for PSA-NCAM in the promotion of chain formation (Chazal et al., 2000).

A last set of molecules which has been implicated in the process of chain migration is a family of transcription factors. The *Dlx* gene family are homeodomain containing transcription factors shown to be important for the development of OB, cerebral cortex and basal ganglia neurons (Qiu et al., 1995; Anderson et al., 1997a; Anderson et al., 1997b; Bulfone et al., 1998; Anderson et al., 1999; Marin et al., 2000; Marin and Rubenstein, 2001). Single mutations in *Dlx1*, *Dlx2* and *Dlx5* result in abnormalities of the OB local circuit neurons (Qiu et al., 1995; Long and Rubenstein, unpublished observations). Furthermore, simultaneous mutations in both *Dlx1* and *Dlx2* result in a dramatic loss of virtually all γ-amino butyric acid (GABA) expressing local circuit neurons of the OB (Anderson et al., 1997b; Bulfone et al., 1998). From these studies, it is clear that the *Dlx* gene family is important for the proper development of the OB. However, it is less clear how this family functions in this process. Here, we focus on the role of *Dlx1* and *Dlx2* in the process of OB local circuit neuron formation.

MATERIALS AND METHODS

Animals and Tissue Preparation

A mouse mutant strain with a null allele of *Dlx1&2* was used in this study (Anderson et al., 1997b; Qiu et al., 1997). This mouse strain was maintained by backcrossing to *C57BL/6J* mice for more than ten generations. For staging of embryos, midday of the vaginal plug was calculated as embryonic day 0.5 (E0.5). Mouse colonies were maintained in accordance with the protocols approved by the Committee on Animal Research at UCSF. Animals expected to contain *Dlx1&2*$^{-/-}$ mutant embryos were sacrificed by Cesarean section. PCR was performed as described (Bulfone et al., 1993a; Anderson et al., 1997b; Qiu et al., 1997; Depew et al., 1999) to genotype offspring resulting from *Dlx1&2* heterozygous matings. Heterozygous and wild type embryos showed the same phenotype, so both are used as controls throughout this paper. Embryos were anaesthetized by cooling, dissected and immersion fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 4-12 hours. Samples were either cryoprotected in a gradient of sucrose to 30%, frozen in embedding medium (OCT, Tissue-Tek, Torrance, CA) and cut using a cryostat or dehydrated in ethanol, embedded in paraffin and cut using a microtome.

In Situ Hybridization

In situ hybridization experiments were performed using ^{35}S riboprobes on 10 μm frozen sections as described previously (Bulfone et al., 1993a). We generously thank the following people for cDNAs: Drs. Brian Condie (*GAD67*), Peter Gruss (*Pax6*), Tom

Curran (*Reelin*) and Francois Guillemot (*Hes5*). The *Dlx2* and *Dlx5* plasmids were generated in the Rubenstein lab.

Histochemistry

Samples were sectioned at 10 μm and mounted onto SuperFrost Plus slides (Fisher, Pittsburgh, PA). Sections were stained with cresyl violet (E14.5 to postnatal day 0 (P0)) on OCT embedded sections and analyzed.

Slice Culture

Embryos were removed by Cesarean section from timed pregnant *Dlx1&2* heterozygous female mice mated with *Dlx1&2* heterozygous male mice. Brains were dissected in ice-cold Krebs buffer as described previously (Marin et al., 2000), embedded in 5% low melting point agarose (FMC Bioproducts, Rockland, MA) and 250 μm sagittal sections were cut with a vibrating microtome (VT1000S, Leica, Nusslock, Germany). Slices were then placed onto Transwell membranes (8 μm pore size, 24 mm diameter membrane, Costar, Acton, MA) that were previously coated with 1 mg/mL of Vitrogen (Cohesion, Palo Alto, CA) for 1 hour. The Transwell membranes containing the slices were placed into Neurobasal media containing 2% B-27 supplement (LTI, Gaithersburg, MD), 0.5% glucose, 2% glutamine and 2% penicillin/streptomycin. A crystal of the axonal tracer, 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR), was placed into the rostral migratory stream (RMS). The slices were placed in a 37°C incubator with 5% CO_2 for 72 hours. The slices were then analyzed with fluorescence microscopy for DiI labeled cells that had migrated beyond the diffusion

range of the crystal.

Matrigel Assay

Embryos were collected and processed as above for slices. A tungsten needle was used to dissect out a small piece from the subventricular zone of the lateral ganglionic eminence. This small piece was then placed onto a 25 μL pad of Matrigel (Becton Dickinson, Bedford, MA). Then 30 μL of Matrigel was placed on top of the explant and cultured in Neurobasal media containing 2% B-27 supplement (LTI, Gaithersburg, MD), 0.5% glucose, 2% glutamine and 2% penicillin/streptomycin for 72 hours. The explants were fixed for 2 hours in 4% PFA, immersed in 50 μg/mL Hoechst 33342 (Molecular Probes, Eugene, OR) and mounted using Vectashield (Vector Labs, Burlingame, CA).

Transplantations

Embryos were removed by Cesarean section from timed pregnant *Dlx1&2* heterozygous female mice mated with *Dlx1&2* heterozygous male mice. Brains were dissected in ice-cold Krebs buffer as described previously (Marin et al., 2000). *Dlx1&2*$^{-/-}$ mutant and wild type cells to be used for transplantation were removed and processed as previously described (Herrera et al., 1999).

RESULTS

Dlx1&2$^{-/-}$ Mutants Have Enlarged Ventricles and Severely Reduced Expression of Local Circuit Neuron Markers of the Olfactory Bulb

Dlx1&2$^{-/-}$ mutant animals have previously been shown at E18.5 to have a severe reduction in the number of GABA^{+} cells of the OB (Anderson et al., 1997b; Bulfone et al., 1998). In order to further understand this finding, we investigated the presence of this phenotype at earlier developmental stages. *In situ* analysis of sagittal sections shows a clear reduction of local circuit neuron markers at E14.5. *Dlx2*^{m} (there is still a *Dlx2* transcript expressed in the *Dlx1&2*$^{-/-}$ mutant animals) expression is reduced in the *Dlx1&2*$^{-/-}$ mutant embryo (Fig. 1B,H). This is further demonstrated by the severe reduction of *Dlx5* (Fig. 1C,I) and *GAD67* (Fig. 1D,J) in the OB. Expression of *Pax6*, a marker of proliferating cells, and *Reelin*, a marker of OB projection neurons, remain normal in the *Dlx1&2*$^{-/-}$ mutant embryos (Fig. 1E,F,K,L).

Further analysis at E18.5 supports the findings at E14.5. In the *Dlx1&2*$^{-/-}$ mutant embryos, markers of local circuit neurons are virtually absent from the OB. There is a continued severe reduction of *Dlx2*^{m}, *Dlx5* and *GAD67* in the OB of *Dlx1&2*$^{-/-}$ mutant embryos (Fig. 1N-P,T-V). In addition, unlike at E14.5, *Pax6* expression is altered. It is reduced in the PG and has a sharper boundary between the SVZ and GC (Fig. 1Q,W). *Reelin* expression remains normal (Fig. 1R,X). These *in situ* analyses suggest that the *Dlx1&2*$^{-/-}$ mutation causes a defect in local circuit neurons that begins as early as E14.5.

In Vitro Analyses Reveal a Migration Defect in the *Dlx1&2*$^{-/-}$ Mutant Embryos

In order to understand whether the lack of local circuit neuron marker expression in the *Dlx1&2*$^{-/-}$ mutant embryo is due to a lack of differentiation or due to a absence of the neurons, we employed an *in vitro* slice culture migration assay. Parasagittal vibrating microtome slices from E17.5 control and *Dlx1&2*$^{-/-}$ mutant embryos were generated. The RMS was labeled with a crystal of DiI and slices cultured for 72 hours. The locations of the labeled cells were visualized using fluorescence microscopy. The experiment clearly showed a lack of migration in the *Dlx1&2*$^{-/-}$ mutant embryos, with virtually no labeled cells outside of the DiI crystal as compared to control (Fig. 2A,B).

An additional migration analysis was utilized to assess not only the migratory ability of cells, but their capacity to 'chain migrate' as in the adult. This experiment was performed by placing small explants from the telencephalon into Matrigel and culturing for 72 hours. At E14.5, *Dlx1&2*$^{-/-}$ mutant explants show a reduced ability to migrate into the Matrigel compared to control (Fig 2C,D). At E18.5, the reduced ability of cells to migrate into the Matrigel was more pronounced (Fig. 2E,F). In addition, the cells were unable to form chains as in control (Fig. 2E inset). Both of these experiments suggest that *Dlx1&2*$^{-/-}$ mutant cells have a defect in their migratory ability.

In Vivo Analysis Confirms a Migration Defect in the *Dlx1&2*$^{-/-}$ Mutant Embryos

The *in vitro* migration analyses suggest that the *Dlx1&2*$^{-/-}$ mutant cells are unable to migrate and form chains. However, it is possible that the environment of the mutant is

disruptive to the cells and their ability to migrate. To test this possibility, we dissociated and labeled both E15.5 and E18.5 control and *Dlx1&2*$^{-/-}$ mutant cells. These cells were then transplanted into adult mice. After 6 days of survival, both E15.5 and E18.5 control cells were located well within the RMS, migrating towards the OB (Fig. 3A,C; data not shown). However, both E15.5 and E18.5 *Dlx1&2*$^{-/-}$ mutant cells were not found either migrating to the OB or within the RMS (Fig. 3B,D; data not shown). The *Dlx1&2*$^{-/-}$ mutant cells were primarily located surrounding the injection site. This result further demonstrates the migration defect of *Dlx1&2*$^{-/-}$ mutant cells.

Regulatory Molecules Are Disrupted in the *Dlx1&2*$^{-/-}$ Mutant Embryos

In order to understand what factors might contribute to the migration defect of *Dlx1&2*$^{-/-}$ mutant cells, we examined several regulatory molecules by *in situ* hybridization. Most of these molecules are known to play a role in controlling cell or axonal movements. Beginning at E14.5, we examined the expression of *vesicular GABA transporter* (*VGAT*) (Evans et al., 1996). We used *VGAT* expression to assess the location of GABAergic cell populations in relation to the regulatory molecules we tested. In the *Dlx1&2*$^{-/-}$ mutant embryo, it is clear that there is a severe reduction in *VGAT* (Fig. 4A,B). This is expected, due to the observation that *GAD67* expression is severely reduced and *VGAT* should mark the same or similar populations. The expression patterns of the regulatory molecules will be compared to *VGAT* at E14.5 in the *Dlx1&2*$^{-/-}$ mutant embryo.

It has been previously shown that there is an upregulation of Notch signaling molecules in the basal ganglia of *Dlx1&2*$^{-/-}$ mutant embryos (Yun and Rubenstein, unpublished observations). Analysis of *Hes5*, a transcriptional component of the Notch pathway, reveals an increase in *Hes5* expression throughout the LGE, MGE and along the pathway to the OB in the *Dlx1&2*$^{-/-}$ mutant embryo at E14.5 (Fig. 4C,D). This finding suggests that the disruption of the Notch signaling pathway in the *Dlx1&2*$^{-/-}$ mutant embryos may result in the inability of neuronal cells to migrate to the OB.

Previous studies have suggested the *Robo/Slit* family of regulatory molecules is important in the migration of neurons to the OB (Hu and Rutishauser, 1996; Hu, 1999). Expression of some members of this family is disrupted in *Dlx1&2*$^{-/-}$ mutant embryos. The two *Robo* family members are normally expressed in the projection neurons of the OB, with additional *Robo2* expression in migrating and newly differentiated local circuit neurons. In E14.5 parasagittal sections, *Robo1* expression is slightly increased in the *Dlx1&2*$^{-/-}$ mutant embryos when compared to control (arrowheads; Fig. 4G,H). The expression of *Robo2* in the *Dlx1&2*$^{-/-}$ mutant embryo is lost at the rostral most extension of the LGE (arrowhead; Fig. 4I,J). A ligand for these two receptors, *Slit1*, shows an increase in regions of the MGE and LGE in the *Dlx1&2*$^{-/-}$ mutant embryo (Fig. 4E,F). The altered expression pattern is similar to *Hes5*, but at lower expression levels. The results suggest that the disruption of Robo/Slit expression may cause the defect in migration of the *Dlx1&2*$^{-/-}$ mutant cells.

A rostral to caudal coronal analysis at E14.5 of these same regulatory molecules further accentuates the altered expression patterns. The increase in *Hes5* expression in the *Dlx1&2*$^{-/-}$ mutant embryo is very clear in the LGE (Fig. 5E-H), however, it is less obvious

in the more rostral sections, where the expression domain is smaller (Fig. 5A-D). *Robo1* expression in the coronal plane reveals interesting expression patterns. In the caudal sections, *Robo1* is expressed in the mantle of the LGE in the wild type embryo (Fig. 5O,P). In the *Dlx1&2*$^{-/-}$ mutant embryo, *Robo1* is expanded into the SVZ of the LGE, consistent with the previous observations of increases in some differentiation markers in the SVZ of the mutant. Further rostrally, *Robo1* expression is lost from the mantle in the wild type and *Dlx1&2*$^{-/-}$ mutant embryos (Fig. 5M,N). The most rostral sections show virtually no difference in *Robo1* expression (Fig. 5I-L). *Robo2* expression is expressed in the SVZ and mantle of the LGE in wild type embryos (Fig. 5W). In *Dlx1&2*$^{-/-}$ mutant embryos, *Robo2* expression is lost from the SVZ and a majority of the mantle of the LGE (arrowheads; Fig. 5X). *Robo2* is reduced or absent in lateral aspects of rostral sections of the *Dlx1&2*$^{-/-}$ mutant embryo (arrowheads; Fig. 5Q-V). *Slit1* expression is slightly increased in the LGE of the *Dlx1&2*$^{-/-}$ mutant embryo (Fig. 5F'). In other areas, its expression in the *Dlx1&2*$^{-/-}$ mutant embryo is similar to control (Fig. 5Y-D').

At E18.5, these regulatory molecules show a greater disruption in expression than at E14.5. The expression of *ErbB4* clearly labels the RMS in the control embryo (Fig. 6A,C,E,G,I). However, in the *Dlx1&2*$^{-/-}$ mutant embryo, its expression is lost from the OB (Fig. 6A,B). A medial flange of expression is also lost in rostral sections (arrowheads; Fig. 6C,D). Additionally, *ErbB4* expression in the *Dlx1&2*$^{-/-}$ mutant embryo appears to increase and expand laterally as you progress towards the striatum (arrowheads; Fig. 6E-J). *Robo1* expression in the *Dlx1&2*$^{-/-}$ mutant embryo remains similar to control levels up until the area surrounding the striatum (Fig. 6K-R). In the striatum, *Robo1* expression is increased in the SVZ of the *Dlx1&2*$^{-/-}$ mutant embryo

(arrowheads; Fig. 6S,T). *Robo2* expression in the local circuit neurons of the OB is severely reduced, while its projection neuron expression remains unchanged (Fig. 6U,V). A little further caudal to the OB, a medial flange of *Robo2* expression is lost from the *Dlx1&2*$^{-/-}$ mutant embryo (arrowheads; Fig. 6W,X). Even further caudal, towards the striatum, virtually all *Robo2* expression is lost (arrowheads; Fig. 6Y-D'). Again, the *Robo* ligand, *Slit1*, has relatively normal expression in rostral sections of the *Dlx1&2*$^{-/-}$ mutant embryo (Fig. 6E'-H'). However, its expression caudally in the *Dlx1&2*$^{-/-}$ mutant embryo is increased in the SVZ and mantle zones (Fig. 6K'-N'). This evidence further supports the hypothesis that the disruption of Robo/Slit signaling may cause the disruption of neuronal migration in the *Dlx1&2*$^{-/-}$ mutant animals.

DISCUSSION

These results shown here demonstrate that the *Dlx1&2*$^{-/-}$ mutation causes a severe decrease in the expression of OB local circuit neuron markers. This virtual absence of OB local circuit neuron markers is a result of lack of migratory ability of the *Dlx1&2*$^{-/-}$ mutant cells. In addition, the *Dlx1&2*$^{-/-}$ mutant cells are unable to migrate in a wild type environment, further suggesting a cell autonomous effect. Expression patterns of several known regulatory molecules are disrupted in *Dlx1&2*$^{-/-}$ mutant embryos, which may be the cause of the *Dlx1&2*$^{-/-}$ mutant cells' inability to migrate.

Dlx1&2$^{-/-}$ Mutant Embryos Show a Loss of Several Local Circuit Neuron Markers

The *Dlx* genes are expressed in the progenitors of the OB local circuit neurons (Liu et al., 1997; Stuhmer et al., 2002a). Previous studies have shown that the *Dlx1&2*$^{-/-}$ mutation causes a severe reduction of TH and GABA expression in the E18.5 OB (Anderson et al., 1997b; Bulfone et al., 1998). However, the extent and onset of this effect were unknown. Investigations at E14.5 and E18.5 reveal a dramatic reduction in virtually all local circuit neuron markers of the OB (Fig. 1A-X; data not shown). *Dlx2*^{m}, *Dlx5* and *GAD67* show a near absence of expression in the OB of E14.5 and E18.5 *Dlx1&2*$^{-/-}$ mutant embryos (Fig. 1B-D;H-J;N-P;T-V). In addition, at E18.5, expression of Calretinin and Calbindin is undetectable (data not shown). *Pax6* expression is reduced in the PG at E18.5 (Fig. 1Q,W), However, *Pax6* expression at E14.5 and *Reelin*, a marker of OB projection neurons, are similar to controls (Fig. 1E,F,K,L,R,X). All of these data

support the hypothesis that the *Dlx1&2*$^{-/-}$ mutation results in a virtual absence of OB local circuit neuron markers, an effect which begins as early as E14.5.

Dlx1&2$^{-/-}$ Mutant Cells Have Reduced Migratory Abilities

The OB local circuit neurons of the adult mouse are generated vast distances from the OB and migrate tangentially via a pathway called the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996). This pathway is well studied in the adult mouse, however, its embryonic counterpart has not been characterized or shown to involve similar mechanisms. Therefore, the understanding of how the OB is formed and local circuit neurons of the OB generated are still under investigation at embryonic stages.

The small size of the OB and the loss of OB local circuit neuron marker expression at E14.5 and E18.5 suggests that the local circuit neurons are absent from the OB due to a migration deficiency of the *Dlx1&2*$^{-/-}$ mutant embryos. To test this hypothesis, we performed *in vitro* migration assays. In the first assay, a crystal of DiI inserted into the putative RMS of both *Dlx1&2*$^{-/-}$ mutant and wild type E17.5 slices revealed several labeled cells in the OB of wild type and none or very few labeled cells in the mutant (Fig. 2A,B). This suggests that the *Dlx1&2*$^{-/-}$ mutant has a migration defect. To further test the migratory ability of the *Dlx1&2*$^{-/-}$ mutant cells, small explants from the SVZ of the LGE were placed into Matrigel and cultured. After 72 hours, the locations of cells that have migrated away from the explant were assessed. In the E15.5 *Dlx1&2*$^{-/-}$ mutant explants, few cells had migrated away from the explant compared to the control

(Fig. 2C,D). The same was also true at E18.5 (Fig. 2E,F). In addition, the *Dlx1&2*$^{-/-}$ mutant cells were unable to form characteristic chains of cells as well (Fig. 2E inset). All of these data confirm the hypothesis that there is a migration defect in the *Dlx1&2*$^{-/-}$ mutant embryos.

The migration defect in the *Dlx1&2*$^{-/-}$ mutant embryos might be a result of the mutation causing a non-cell autonomous disruption in the environment in which the cells migrate to the OB. To test this hypothesis, we performed an *in vivo* transplantation of *Dlx1&2*$^{-/-}$ mutant cells into an adult host. After 6 days of survival, the wild type transplanted cells were found all along the RMS from the injection site to the OB (Fig. 3A,C). However, there were no *Dlx1&2*$^{-/-}$ mutant cells anywhere along the RMS and most of the mutant cells were located very close to the injection site (Fig. 3B,D). These findings further confirm that the migration defect in the *Dlx1&2*$^{-/-}$ mutant embryos is a cell autonomous effect.

Regulatory Molecules are Disrupted in the *Dlx1&2*$^{-/-}$ Mutant Embryos

Neuronal migration is crucial for the development of the OB. The factors which control this migration are just beginning to be discovered. Members of the Slit/Robo family of molecules have been implicated in the tangential migration of neurons (Wu et al., 1999), radial migration of neurons (Hu, 1999), axon guidance (Brose et al., 1999; Kidd et al., 1999) and axon branching (Wang et al., 1999). Furthermore, members of the Notch signaling pathway (Ikeya and Hayashi, 1999; Lindner et al., 2001; Wilson et al., 2001; Schuurmans and Guillemot, 2002) and Erb/NRG family (Perroteau et al., 1998;

Perroteau et al., 1999; Steiner et al., 1999; Kornblum et al., 2000) have been implicated in differentiation and neuronal migration. To test whether these molecules may be responsible for the disruption of neuronal migration in the *Dlx1&2*$^{-/-}$ mutant embryos, RNA *in situ* hybridization was performed on sections.

Expression of the Notch intracellular signaling molecule, *Hes5*, is increased in the SVZ and mantle in E14.5 *Dlx1&2*$^{-/-}$ mutant embryos (Fig. 4C,D; Fig. 5A-H). This altered expression pattern is also seen with *Robo1* (Fig. 4G,H; Fig. 5I-P; Fig. 6K-T) and its ligand, *Slit1* (Fig. 4E,F; Fig. 5Y-F'; Fig. 6E'-N') in both E14.5 and E18.5 *Dlx1&2*$^{-/-}$ mutant embryos. The increase in expression of all these molecules may result in the inability of the OB local circuit neurons and their progenitors to migrate to the OB.

The increase in expression of the previous molecules is complemented by the loss of expression of several molecules which are expressed in the migrating local circuit neurons. Expression of *GAD67*, *Dlx2*, *Dlx5* and *VGAT* are all lost in the migrating local circuit neurons in the *Dlx1&2*$^{-/-}$ mutant embryo (Fig. 1D,J,P,V; Fig. 1B,H,N,T; Fig. 1C,I,O,U; Fig. 4A,B). Expression of other regulatory molecules is lost in a similar fashion. *ErbB4* (Fig. 6A-J) and *Robo2* (Fig. 4I,J; Fig. 5Q-X; Fig. 6U-D') expression are both lost in the OB. However, while *Robo2* expression is lost throughout the rostral-caudal axis of the lateral ganglionic eminence and its derivatives, *ErbB4* expression increases along the rostral-caudal axis. This suggests that the migration defect in the *Dlx1&2*$^{-/-}$ mutant embryos is a true migration defect of OB local circuit neuron precursors, not in their proliferation. It also suggests that *ErbB4* expression is upstream of *Robo2*, *GAD67*, *VGAT*, *Dlx2* and *Dlx5* expression in migrating OB local circuit neurons.

Further investigation into the role of these regulatory molecules in migration is underway.

FIGURES

Nissl
Dlx2
Dlx5
GAD67
Pax6
Reelin
E14.5
E18.5
+/
−/−
+/
−/−
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
LGE
Cx
MGE
Se
OB

Figure 1: *In situ* RNA hybridization analysis of *Dlx1&2*$^{-/-}$ mutant and control E14.5 and E18.5 embryos. At E14.5, Nissl staining of sagittal sections reveals an enlarged OB ventricle in the *Dlx1&2*$^{-/-}$ mutant embryos (A,G), which is further expanded at E18.5 (M,S). Expression of *Dlx2*^{m} (a transcript is still expressed in the *Dlx1&2*$^{-/-}$ mutant) is greatly reduced in the OB at both E14.5 (B,H) and E18.5 (N,T). The same is true for *Dlx5* (C,I,O,U) and *GAD67* (D,J,P,V). Expression of *Pax6* is similar to control at E14.5 (E,K) in the *Dlx1&2*$^{-/-}$ mutant embryo, but has reduced PG expression at E18.5 (Q,W). A marker of projection neurons of the OB, *Reelin*, is similar to controls at all stages (F,L,R,X). Cx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; OB, olfactory bulb; Se, septum. Scale bar: A-X, 720 μm.

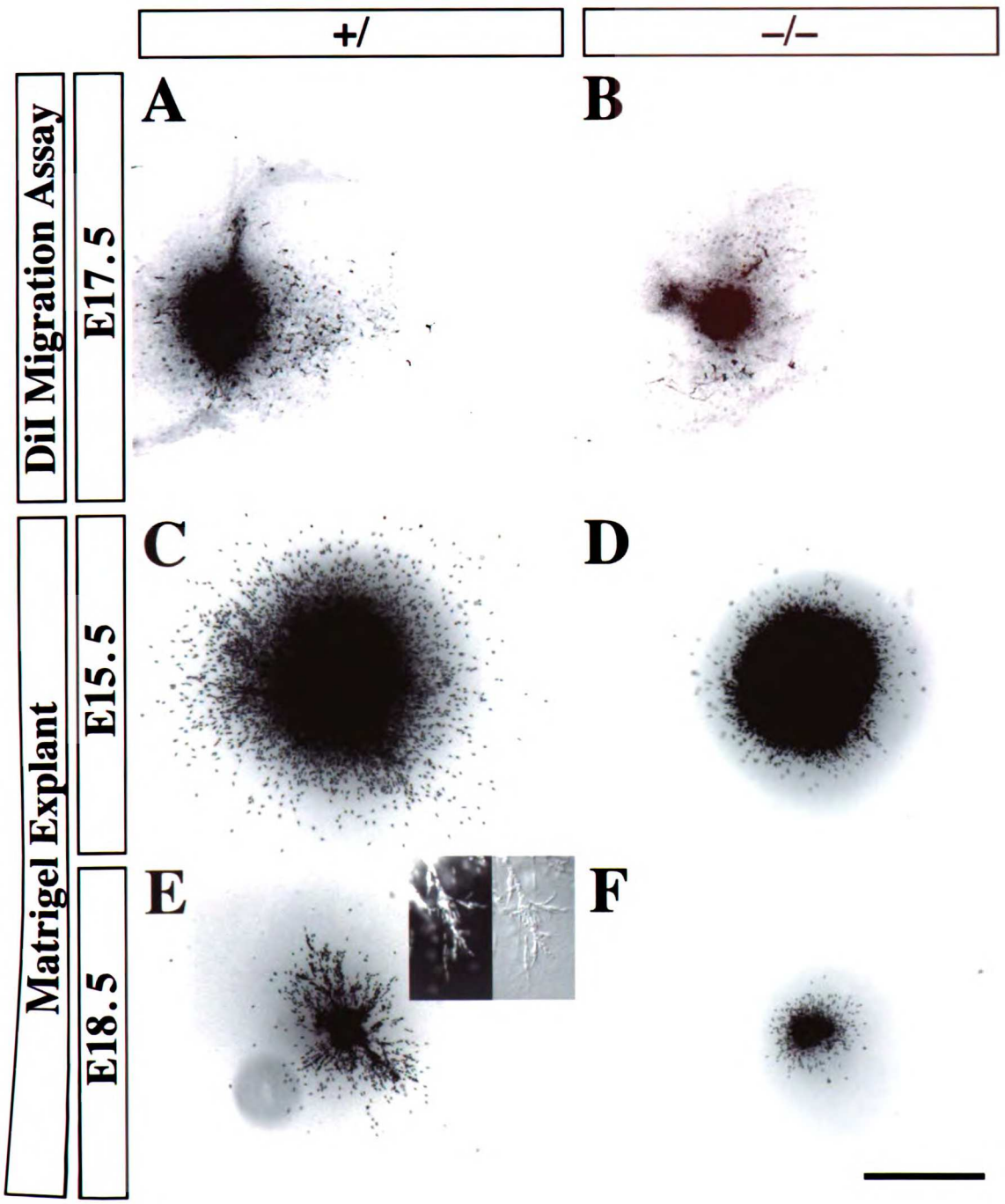
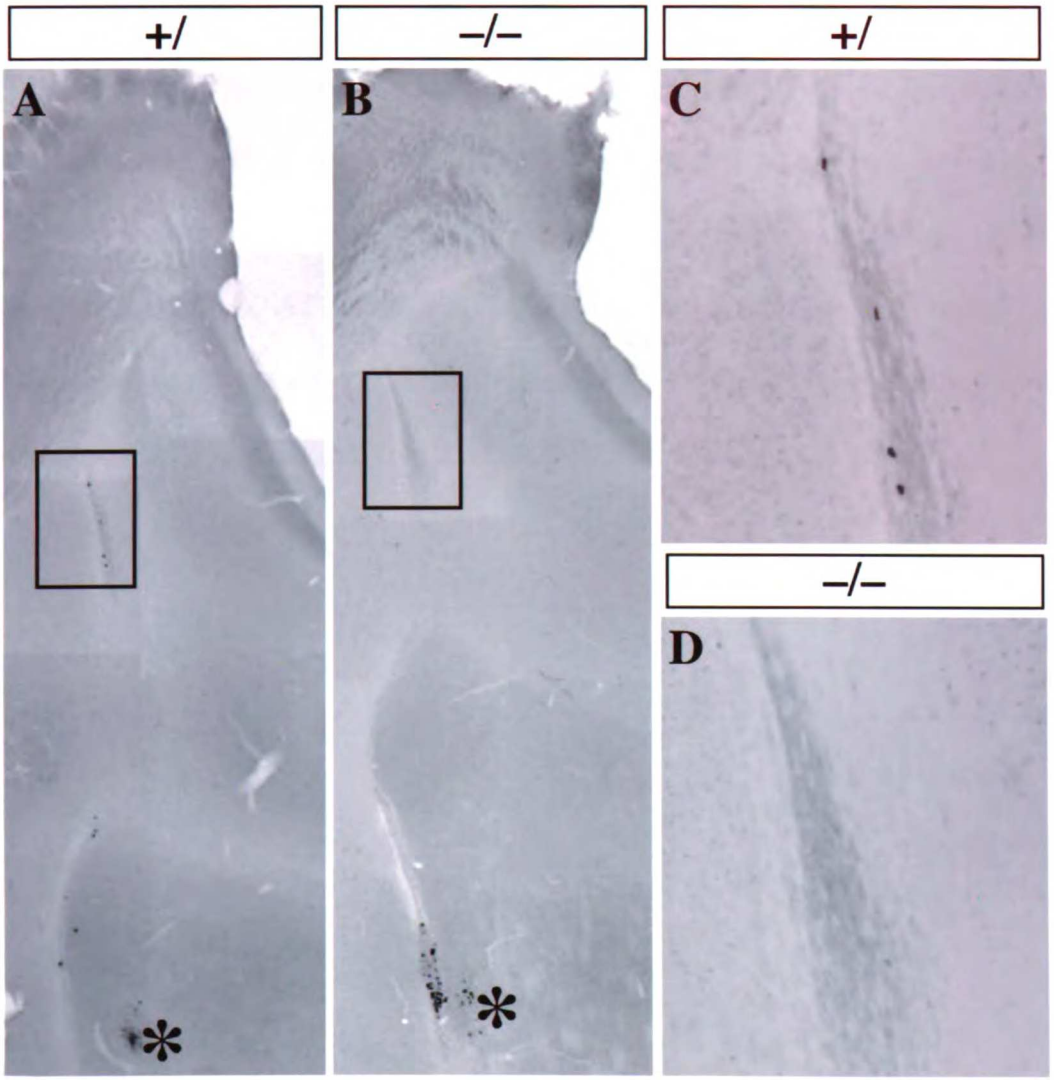

+/
–/–
DiI Migration Assay
E17.5
A
B
Matrigel Explant
E15.5
C
D
E18.5
E
F

Figure 2: *In vitro* migration analysis of *Dlx1&2*$^{-/-}$ mutant and control embryos. A DiI crystal was placed into the RMS of E17.5 *Dlx1&2*$^{-/-}$ mutant (B) and control (A) embryos and allowed to survive for 72 hours. Immunofluorescence analysis reveals a virtual absence of DiI labeled cells outside of the DiI crystal or in the OB of the *Dlx1&2*$^{-/-}$ mutant slice (B). Small explants of SVZ cells taken from the LGE of *Dlx1&2*$^{-/-}$ mutant and control embryos were placed into Matrigel and cultured for 72 hours (C-F). At E15.5, *Dlx1&2*$^{-/-}$ mutant explants lacked migration of Hoechst stained cells away from the explant when compared to controls (C,D). At E18.5, not only were the *Dlx1&2*$^{-/-}$ mutant cells again unable to migrate away from the explant (E,F), but they were also unable to form chains similar to controls (inset in E). Scale bar: A,B 450 μm; C,D 100μm; E,F 350 μm; inset 50 μm.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–/–
D

Figure 3: *In vivo* transplantation of *Dlx1&2*$^{-/-}$ mutant and control cells into adult mice. BrdU labeled SVZ explants were taken from the striatum of E18.5 *Dlx1&2*$^{-/-}$ mutant and control embryos and injected into adult mice with survival for 6 days. Control cells integrated into and along the host RMS and migrated away from the injection site (asterisk) towards the OB (A). A higher magnification view of the boxed area in (A) is provided in (C). *Dlx1&2*$^{-/-}$ mutant cells were not found anywhere outside of a very short distance away from the injection site (asterisk, B). A higher magnification view of the boxed area in (B) is provided in (D). Scale bar: A,B 400 μm; C,D 100 μm.

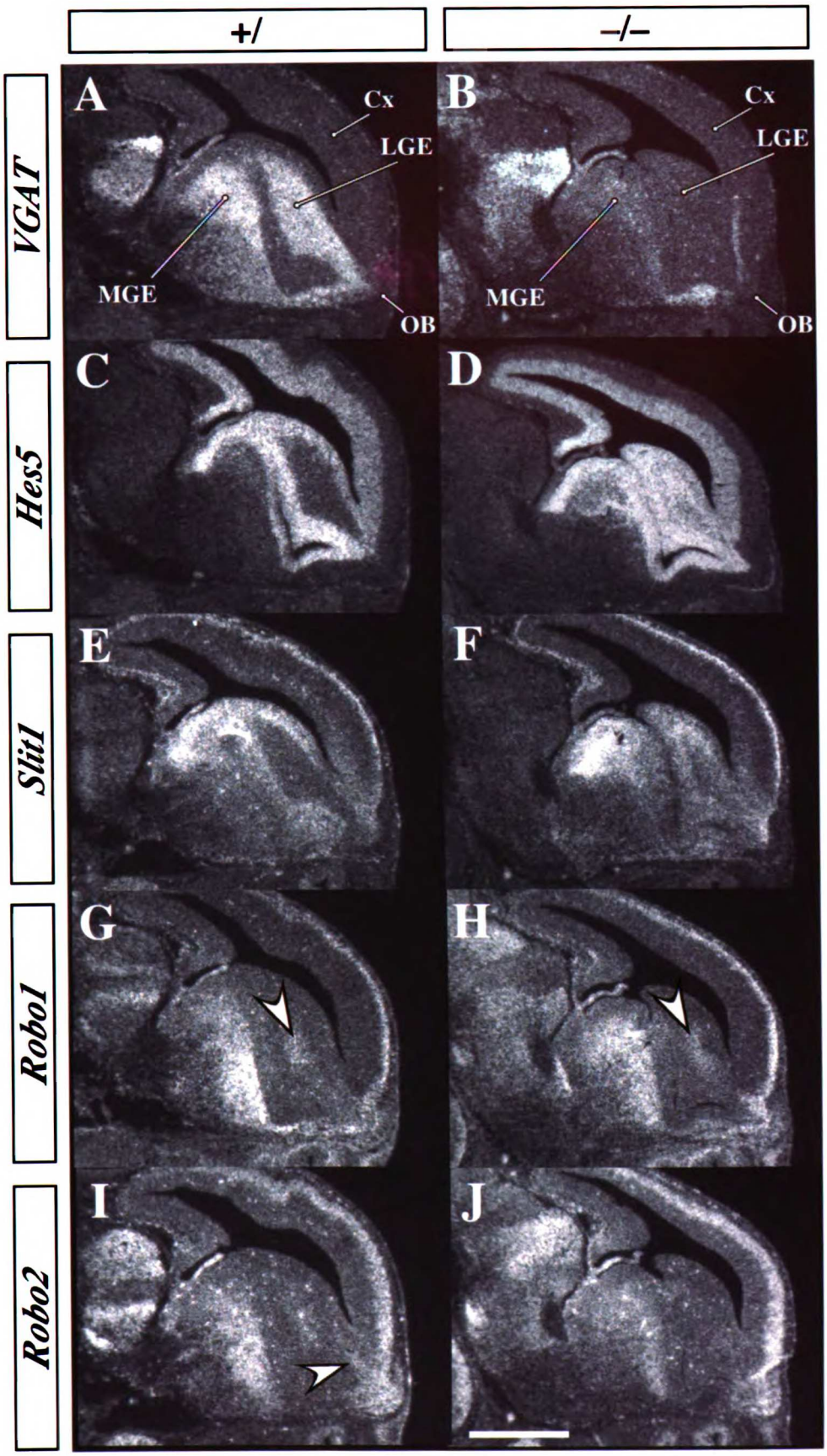
+/
–/–
VGAT
Hes5
Slit1
Robo1
Robo2
A
B
C
D
E
F
G
H
I
J
Cx
LGE
MGE
OB

Figure 4: *In situ* RNA hybridization of regulatory molecules in E14.5 parasagittal *Dlx1&2*$^{-/-}$ mutant and control embryos. Expression of *VGAT* is nearly lost from the LGE and MGE of the *Dlx1&2*$^{-/-}$ mutant embryo (A,B). Vertical stripe in cortex of (B) is a tissue fold and is not real expression. *Hes5* expression, which is normally restricted to the VZ, is increased in the SVZ of the LGE and MGE of the *Dlx1&2*$^{-/-}$ mutant embryo (C,D). The same is true for *Slit1*, although the overall expression levels in these zones are less than that of *Hes5* (E,F). Expression of *Robo1* in the LGE is slightly increased (G, arrowhead in H), while *Robo2* expression is lost from the rostral extension of the LGE near the OB (arrowhead in I, J). Scale bar: A-J 300 μm.

E14.5
Slit1
Robo2
Robo1
Hes5
+/+
-/-
OB
LGE

Figure 5: *In situ* RNA hybridization of regulatory molecules in E14.5 coronal *Dlx1&2*$^{-/-}$ mutant and control embryos. Expression of *Hes5* in coronal section reveals an increase in the SVZ and mantle of the LGE of the *Dlx1&2*$^{-/-}$ mutant embryo (E-H). *Robo1* expression also shows an increase in the SVZ of the LGE in *Dlx1&2*$^{-/-}$ mutant embryos (arrowheads O,P). In contrast, *Robo2* expression is lost from both the lateral aspect of the OB and the SVZ of the LGE in *Dlx1&2*$^{-/-}$ mutant embryos (arrowheads Q,R,W,X). *Slit1* expression is increased in the SVZ and mantle of the LGE in the *Dlx1&2*$^{-/-}$ mutant embryo (E',F'). LGE, lateral ganglionic eminence; OB, olfactory bulb. Scale bar: A-F' 150 μm.

E18.5
Slit1
Robo2
Robo1
ErbB4
+/+
-/-
+/+
-/-
+/+
-/-
+/+
-/-
OB
Str
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
A'
B'
C'
D'
E'
F'
G'
H'
I'
J'
K'
L'
M'
N'

Figure 6: *In situ* RNA hybridization of regulatory molecules in E18.5 coronal *Dlx1&2*$^{-/-}$ mutant and control embryos. Expression of *ErbB4* marks a population of migrating local circuit neurons and is lost from the OB of the *Dlx1&2*$^{-/-}$ mutant embryos (A-D). Further caudal sections show an increase and expansion of *ErbB4* expression in the striatum of *Dlx1&2*$^{-/-}$ mutant embryos (arrowheads G-J). *Robo1* expression is increased in the SVZ of the striatum of the *Dlx1&2*$^{-/-}$ mutant embryo (arrowhead S,T). *Robo2* expression is severely reduced throughout the rostral-caudal axis in the migrating OB local circuit neuron population (compare wild type *Robo2* expression (U-C') to wild type *ErbB4* expression (A-I)), while the projection neuron population of the OB remains similar to control (arrowheads U-D'). *Slit1* expression is expanded in the SVZ and mantle of the striatum (arrowheads K'-N'). OB, olfactory bulb; Str, striatum. Scale bars: A-N' 150 μm.

PERSPECTIVES

The development of the telencephalon is a complex process involving thousands of molecules, whose expression is coordinated both temporally and spatially. This coordinated effort generates the complex structures responsible for our cognitive and essential life processes. Understanding how the mechanisms function in the generation of these structures is crucial to the health of individuals afflicted with neurological disorders or disease. The knowledge gained from studying these developmental processes can one day be applied to the treatment of these conditions.

The work presented in this thesis is neither the beginning nor the end of the long task in understanding these developmental processes. The *Dlx* gene family is one of many families which are involved in the development of the telencephalon. Specifically, the *Dlx* genes are involved in the development of neurons which express GABA, acetylcholine and dopamine (Anderson et al., 1999; Marin and Rubenstein, 2001; Marin et al., 2001). The loss of the inhibitory GABAergic neurons in the cortex may result in defects in the functional circuits of the cortex. This could lead to hyperactive states, such as seizures. Similarly, the loss of GABAergic, cholinergic and dopaminergic neurons in the basal ganglia may cause defects in the functional circuits of the basal ganglia. This could lead to learning impairment, the loss of complex motor actions and the disruption of cognitive behavior.

In order to understand the role of the *Dlx* genes, a simple system must be analyzed. One set of neurons which are particularly sensitive to the *Dlx* mutations are the local circuit neurons of the OB. Due to their sensitivity, these neurons of the OB provide a unique system in which to study the genetic programs of proliferation, migration and differentiation. The work presented here has attempted to clarify the role of the *Dlx* genes

in these OB processes. Evidence has been presented which suggests that the *Dlx1&2* genes are required for the migration of local circuit neurons to the OB. In addition, the evidence presented suggests that the *Dlx5* gene is important not only in the differentiation of local circuit neurons of the OB, but also the development of another component of the olfactory pathway, the olfactory epithelium. These findings are not surprising given our hypothesis of how the *Dlx* genes function, with *Dlx1&2* expressed in early progenitors and important for early specification and with *Dlx5&6* expressed in differentiating neurons and important for the differentiation process.

Although some insight has been gained into how the *Dlx* genes function, much more work needs to be done. All of the *Dlx* mutations result in the death upon birth of homozygous mutants. The cause of this has yet to be resolved, but is thought to lie in *Dlx* function in the enteric nervous system of the animals. In addition, there are numerous craniofacial defects in all the *Dlx* mutant animals and limb defects in some of the compound *Dlx* mutant animals, which are *Dlx* functions most likely evolutionarily conserved from *Dll* in Drosophila.

From the many studies across species, it appears that the *Dlx* genes have several functions (Anderson et al., 1999; Bendall and Abate-Shen, 2000; Panganiban, 2000; Young et al., 2000). These functions depend on what tissue or structure in which the *Dlx* genes are expressed. A major challenge in the study of *Dlx* genes is to understand both how a single gene can have multiple functions depending on the cell context and how compensation by other family members is accomplished. Teasing out these functions will require careful analysis of conditional mutant animals, upstream and downstream targets and biochemical analysis of the proteins themselves.

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