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Dlx1&2 and Mash1 Transcription Factors Control Striatal Patterning and Differentiation Through Parallel and Overlapping Pathways

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Abstract

Here we define the expression of ~100 transcription factors in progenitors and neurons of the developing basal ganglia. We have begun to elucidate the transcriptional hierarchy of these genes with respect to the *Dlx* homeodomain genes, which are essential for differentiation of most GABAergic projection neurons of the basal ganglia. This analysis identified *Dlx*-dependent and *Dlx*-independent pathways. The *Dlx*-independent pathway depends in part on the function of the *Mash1* b-HLH transcription factor. These analyses define core transcriptional components that differentially specify the identity and differentiation of the striatum, nucleus accumbens and septum.

Keywords

Dlx; Mash; Neuron; Basal Ganglia; Mouse; Forebrain; GABA

Introduction

The basal ganglia have fundamental roles within cortical-basal ganglia-thalamic networks that control progressively higher-order types of learning: limbic, associative and sensorimotor (Yin and Knowlton, 2006). The principal telencephalic constituents of the basal ganglia include the striatum (caudate, putamen, nucleus accumbens), and the globus pallidus, whose embryonic anlage are the lateral and medial ganglionic eminences (LGE, MGE), respectively (Campbell, 2003; Puelles et al., 2000). The progenitor zone of the LGE is the source for striatal projection neurons and olfactory bulb interneurons, whereas the progenitor zone of the MGE is the source for pallidal projection neurons and cortical and striatal interneurons (Campbell, 2003; Marin and Rubenstein, 2003). The septal anlage (Se) lies adjacent to the LGE and MGE, and is the source of septal projection neurons and is thought to generate some olfactory bulb interneurons (Kohwi et al., 2007; Long et al., 2007; Long et al., 2003). The progenitor domain of the nucleus accumbens is poorly defined.

Current efforts are aimed at elucidating the genetic circuits that regulate the specification, differentiation and function of LGE-, MGE- and Se-derived cells, with particular emphasis on defining the transcription factors and relevant signaling pathways. Sonic hedgehog (Shh)

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and fibroblast growth factor (FGF) signaling coordinately are essential for specification and patterning the basal ganglia (Gutin et al., 2006; Storm et al., 2006) - both pathways converge on expression of *Nkx2.1*, a homeobox transcription factor that is essential for MGE specification (Sussel et al., 1999). For instance, severe *Fgf8* hypomorphs fail to establish *Nkx2.1* and *Shh* expression in the anlage of the MGE. However, these mutants express *Dlx* homeobox transcription factors (Storm et al., 2006), whose function is essential in perhaps all differentiating basal ganglia neurons including striatal projection neurons (Anderson et al., 1997a; Anderson et al., 1997b; Lobo et al., 2006). The vast majority of basal ganglia neurons are GABAergic and the *Dlx* genes are sufficient to promote GABAergic differentiation (Anderson et al., 1999; Stuhmer et al., 2002). Herein we provide evidence that *Dlx1&2* work in concert with other transcription factors to specify GABAergic fate.

Specification of the striatum depends on the function of the *Gsh1&2* homeobox genes, which are expressed in the LGE ventricular zone (VZ) (Corbin et al., 2000; Toresson and Campbell, 2001; Toresson et al., 2000; Yun et al., 2003; Yun et al., 2001); there is evidence that these genes drive LGE expression of *Mash1* and *Dlx1&2*. *Mash1* encodes a bHLH transcription factor that autonomously promotes neurogenesis and non-autonomously represses differentiation of adjacent progenitors through Notch-signaling (Casarosa et al., 1999; Horton et al., 1999; Yun et al., 2002). Furthermore, it forms a complex with Brn1, a POU-homeobox protein, which promotes neural differentiation (Castro et al., 2006). *Mash1* also promotes GABAergic fate (Fode et al., 2000).

Dlx1&2 repress *Mash1* expression and Notch signaling, thereby driving later steps in LGE development (Yun et al., 2002). *Dlx1&2*$^{-/-}$ mutants have reduced LGE expression of the *Arx* homeobox gene (Cobos et al., 2005a). *Arx* is required for migration of late-born striatal projection neurons (Colombo et al., 2007) and interneurons destined for the olfactory bulb (Yoshihara et al., 2005). These phenotypes are also found in the *Dlx1&2*$^{-/-}$ mutants (Anderson et al., 1997b; Long et al., 2007). However, striatal development is not fully blocked in the *Dlx1&2*$^{-/-}$ mutants, demonstrating that parallel and/or redundant pathways continue to promote the generation and migration of some striatal neurons. Thus, we have sought to identify other transcription factors that control LGE specification and differentiation.

Herein, we describe a comprehensive analysis of transcription factors that are expressed at various stages of differentiation in the embryonic LGE and the effect of loss of *Dlx1&2* function on their expression. Thereby, we define transcription factors that are genetically downstream of *Dlx1&2*, as well as transcription factors that are candidates to function upstream, redundantly and in parallel.

Mash1 is a key candidate to function with *Dlx1&2* to promote striatal differentiation. Through analysis of *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ triple mutants we demonstrate that most striatal differentiation depends on their combined function. Furthermore, we have defined the unique and combined function of *Dlx1&2* and *Mash1* in regulating development of distinct dorsoventral domains with the LGE and adjacent parts of the septum - this provides novel insights in development of the accumbens nucleus. Together, this study forms the foundation to decipher the transcription factor circuitry that controls development of the basal ganglia.

Materials and Methods

RNA preparation and gene expression array analysis

RNA was isolated from both the cortex and the lateral and medial ganglionic eminences and their mantle of E15.5 mouse basal ganglia by dissection with fine forceps. We paid

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particular attention to avoiding contamination from the adjacent ventrolateral cortex in the basal ganglia samples. We identified *Dlx1&2*$^{-/-}$ mutants based on their cleft palate and subsequently by PCR genotyping. RNA was pooled independently from the cortex and the subpallium of two *Dlx1&2*$^{-/-}$ and two *Dlx1&2*$^{+/-}$ mutants (~20 μg). The sex of the specimens was not determined.

RNA was purified from forebrain tissue by first homogenizing in 1mL TRIzol Reagent (Invitrogen, Carlsbad, CA) using a Teflon homogenizer and then incubated at room temperature for 5 minutes. 200μL of chloroform (Fisher Scientific, Pittsburgh, PA) was then added, samples shaken vigorously and spun in a microcentrifuge at 12,000 × g for 15 minutes at 4°C. The colorless upper phase was removed and RNA precipitated at room temperature for 10 minutes after the addition of 0.5mL of isopropyl alcohol (Fisher Scientific). The samples were spun in a microcentrifuge at 12,000 × g for 10 minutes, washed with 70% ethyl alcohol (Fisher Scientific) and resuspended in 10μL of nuclease-free water. The purified total RNA was shipped to the NINDS/NIMH Microarray Consortium (http://arrayconsortium.tgen.org/) where biotin-labeled cRNA hybridization probes were generated using the Affymetrix's GeneChip IVT Labeling Kit (Santa Clara, CA), which simultaneously performs in vitro transcription (a linear ~20-60-fold amplification) and biotin-labeling. Briefly, the provided RNA was added to 4μL of 10X IVT Labeling Buffer, 12μL of IVT Labeling NTP Mix, 4μL of IVT Labeling Enzyme Mix and nuclease-free water and incubated for 16 hours at 37°C. The samples were then stored at −80°C until use in hybridization. Amplifications and hybridizations (in triplicate) using the Affymetrix Mouse Genome 430 2.0 array (which has coverage for 39,000 transcripts) were performed. cRNA was fragmented into 35-200-bp fragments using a magnesium acetate buffer (Affymetrix). 10μg of labeled cRNA was hybridized to Mouse Genome 430 2.0 array for 16 hours at 45°C. The GeneChips were washed and stained according to the manufacturer's recommendations using the GeneChips Fluidics Station (Model 450; Affymetrix). Each expressed gene sequence is represented by 11 probe pairs on the array and each oligonucleotide probe is a 25mer. TGEN uses GeneChip Operating Software (GCOS) to scan the arrays and to perform a statistical algorithm that determines the signal intensity of each gene. The data was presented using two different primary analyses: Iterative comparisons and analyses performed in Genespring v6.2. For more in-depth analysis, we considered two populations of genes; the first being those genes obtained from the array that showed at least a 2-fold change in expression between the BG of control and *Dlx1&2*$^{-/-}$ mutants with a P value of < 0.05, and the second those genes that we hypothesized were important based on our knowledge and literature searches.

Animals and Tissue Preparation

Mice were maintained in standard conditions with food and water *ad libitum*. All experimental procedures were approved by the Committee on Animal Health and Care at the University of California, San Francisco (UCSF). Mouse colonies were maintained at UCSF, in accordance with National Institutes of Health and UCSF guidelines. Mouse strains with a null allele of *Dlx1&2* and *Mash1* were used in this study (Anderson et al., 1997b; Guillemot et al., 1993; Qiu et al., 1997). These strains were maintained by backcrossing to C57BL/6J mice. For staging of embryos, midday of the *vaginal* plug was calculated as embryonic day 0.5 (E0.5). PCR genotyping was performed as described (Anderson et al., 1997b; Parras et al., 2004). Since no obvious differences in the phenotypes of *Dlx1&2*$^{+/+}$ and *Dlx1&2*$^{+/-}$ and *Mash1*$^{+/+}$ and *Mash1*$^{+/-}$ brains have been detected, they were both used as controls. Embryos were anaesthetized by cooling, dissected and immersion fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 8.0) for 4-12 hours. Samples were either cryoprotected in a gradient of sucrose to 30%, frozen in embedding

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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 digoxigenin riboprobes on 20μm frozen sections cut on a cryostat. The sections were subsequently postfixed in 4% paraformaldehyde (PFA; Fisher Scientific) for 15 min. After three washes in 1X PBS, sections were treated with 10μg/ml proteinase K (Roche, Indianapolis, IN) in 1X PBS for 15 minutes, transferred to 4% PFA for 5 minutes, and then washed three time for 5 minutes each in 1X PBS. Subsequently, sections were acetylated for 10 minutes (1.3% triethanolamine, 0.25% acetic anhydride, 17.5mM HCl). Slides were then transferred to a hybridizing chamber (Thermo-Shandon, Pittsburgh, PA) where they were incubated for 1 hour at room temperature with 500μl of hybridization solution [50% formamide (Ambion, Austin, TX), 10% dextran sulfate, 0.2% tRNA (Invitrogen), 1X Denhardts solution (from a 50X stock; Sigma, St. Louis, MO), 1X salt solution (from a 10X stock containing 2M NaCl, 0.1M Tris, 50mM NaH_2PO_4, 50 mM Na_2HPO_4, 50 mM EDTA, pH 7.5)]. Digoxigenin (DIG)-labeled RNA probes were heated to 80°C for 10 minutes, cooled in ice, and added to prewarmed (62°C) hybridization solution to a final concentration of 200-400ng/ml (typically 0.2μl of probe in 100μl of hybridization solution). 200μl of hybridization solution containing the appropriate probe was added to each slide, which was subsequently covered with a coverslip and incubated overnight at 62°C. The next day, the coverslips were gently removed and the slides were washed three times for 20 minutes each with 50% formamide (Ambion), 0.5X SSC, and 0.1% Tween 20 at 62°C. Slides were then washed three times in MABT (0.1M maleic acid, 0.2M NaOH, 0.2M NaCl, 0.01% Tween 20, incubated for 1 hour in blocking solution [10% blocking solution (Roche) and 10% sheep serum (Sigma) in MABT]. Slides were then incubated overnight with anti-DIG antibody (1:5000; Roche) diluted in a solution containing 1% sheep serum and 1% blocking solution in MABT. Slides were next washed three times for 60 minutes each in MABT. The slides were then washed three times for 5 minutes each in reaction buffer (0.1M Tris, pH 9.5, 0.1M NaCl, and 50mM $MgCl_2$) and incubated in the dark in nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution [3.4μl/ml from NBT stock and 3.5μl/ml from BCIP stock in reaction buffer (100mg/ml NBT stock in 70% dimethylformamide; 50mg/ml BCIP stock in 100% dimethylformamide; Roche)]. Slides were checked periodically and the reaction was stopped in 10mM Tris pH 8.0, 0.05M EDTA buffer. Finally, slides were dried overnight, dehydrated, and coverslipped with Permount (Fisher Scientific). Statements about the levels of expression are based on interpretation by at least two independent observers; whenever possible domains of expression outside of the *Dlx* expression zone are used as internal controls. Images of stained sections were captured using a Zeiss AxioCam MR (Thornwood, NY) and saved as TIFF files. These files were then opened with Adobe Photoshop (San Jose, CA) and adjusted using the auto contrast menu item and compiled into Adobe Illustrator to create the figures. Table 1 lists the genes and the nucleotide positions used for the probes in the *in situ* analysis.

Results

Identification of Transcription Factors Differentially Expressed in the Embryonic Basal Ganglia and Cortex

To understand the mechanisms that regulate patterning and differentiation of the mouse embryonic basal ganglia and cortex, we used gene expression array analysis and informative methods to identify the transcription factors (TFs) expressed at E15.5. Table 2 lists TFs identified by the microarray analysis on RNA prepared from E15.5 basal ganglia and cortex. We have generally restricted our analysis to TFs whose expression was above the level of 30 units (we can not usually detect expression by *in situ* hybridization of most genes below 30

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units). We compared TF expression in basal ganglia and cortex (Table 2). Using these results, we have defined three classes of TFs:

Class 1—The expression of these TFs is largely restricted to the E15.5 basal ganglia; they are indicated in green: *ATBF1, Brn4, Dlx1/2/5/6, Ebf1, ESRG, Gbx1/2, Gsh1/2, Ikaros, Islet1, Lhx6/7, Lim1, Med6, Meis1, Nkx2.1, Nkx2.2, Nkx5.1, Nkx6.2, Nolz1, Npas1, Otx2, Pbx3, Peg3, Prox1, RARβ, RXRγ, Six3, Vax1 and Zic1.* These TFs are likely to be responsible for regulating regional identity or phenotypes specific to basal ganglia neurons, such as gene programs responsible for making GABAergic medium spiny neurons of the striatum or GABAergic local circuit neurons of the cortex and olfactory bulb.

Class 2—These TFs are expressed in both E15.5 cortical and basal ganglia cells, but show at least a 2-fold bias towards basal ganglia expression; they are indicated in aqua: *Arx, Asb4, Brn5, COUP-TFII, Egr3, ER81, Evi3, FoxP1, FoxP2, Lmo4, MafB, Mash1, Oct6, Olig1/2, Sox1, Sox10, Sp8, Sp9 and TCF4.* These TFs may share similar functions within the cortical and subcortical telencephalon, but can also influence processes specific to the basal ganglia.

Class 3—These TFs show are expressed at roughly equal levels in the E15.5 cortex and basal ganglia, or are expressed at higher levels in the cortex and are indicated in yellow: *BF1, Brn2, COUP-TF1, Ctip1, Ctip2, Cux2, antisense Dlx6, Emx1, Emx2, Erm, FoxG1, FoxP4, Gli1, Hes1, HesR1, Hes5, Id4, Lhx2, Lmo1, Lmo3, Mef2c, Meis2, Nex1, NHLH2, Nur77, Otx1, Pax6, Pbx1, RORβ, Sall3, Sox4, Sox11, Tlx, TLE4.* These TF are likely to have general roles in regulating developmental processes common to both parts of the telencephalon.

In addition to regional specificity, we have sorted some of these TFs according to their expression within proliferative zones. Table 3 lists TFs that are expressed in the LGE, and defines their expression in primary and secondary progenitors (VZ and SVZ) and in postmitotic neurons of the striatum (MZ). For instance, *Dlx1&2* and *Mash1* are expressed in progenitors, whereas *Ikaros* and *RXRγ* are expressed in postmitotic neurons. Below we describe how loss of either *Dlx1&2* or *Mash1* function affects the expression of many of these TFs.

Identification of Transcription Factors Whose Expression Is Altered in the Subcortical Telencephalon of *Dlx1&2*$^{-/-}$ Mutants

The *Dlx* family of transcription factors is preferentially expressed in the basal ganglia at E15.5 (Table 2) (Bulfone et al., 1993a; Bulfone et al., 1993b; Eisenstat et al., 1999; Liu et al., 1997; Porteus et al., 1994). Analysis of mice with targeted null mutations in both *Dlx1* and *Dlx2* (*Dlx1&2*$^{-/-}$) show that the *Dlx* genes are necessary for differentiation and migration of basal ganglia GABAergic neurons (Anderson et al., 1997a; Cobos et al., 2007; Yun et al., 2002). To identify *Dlx*-regulated TF genes, we used gene expression microarray analysis to compare TF expression in the basal ganglia of E15.5 control and *Dlx1&2*$^{-/-}$ mutants. Of the genes listed in Table 2, 15 genes showed greater than 2-fold reduced expression, 8 genes showed greater than 2-fold increased expression, and the expression of 72 genes did not change significantly in the *Dlx1&2*$^{-/-}$ basal ganglia (Table 2).

The microarray data do not indicate how the TF expression changed within the different cellular subtypes of the basal ganglia. For example, changes in expression could reflect alterations in progenitors and/or postmitotic cells. To obtain spatial resolution of TF gene expression, we performed *in situ* hybridization on E15.5 control and *Dlx1&2*$^{-/-}$ mutant coronal sections (Figures 1-6). Because of the complexity of the basal ganglia, we have

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concentrated this study's analysis on rostral telencephalic regions that contain the LGE and Septum. Table 3 summarizes the expression patterns of 60 TFs in the LGE, and defines how their expression changes in primary and secondary progenitors (VZ and SVZ, respectively) and in postmitotic neurons of the striatum (MZ) of *Dlx1&2*$^{-/-}$ mutants. Details of the *in situ* hybridization analysis will be described below.

Dlx1&2 Specify the Molecular Identity of the SVZ in the dLGE by Positively Regulating Expression of a Set of Transcription Factors

Dlx1&2 are expressed in a dorsoventral gradient in progenitor cells of the LGE at E12.5 and E15.5 (Fig. 1a,b; Supplementary Fig. 1). Their expression is particularly high in the dorsal LGE (dLGE) where they are detected in most cells in both the ventricular and subventricular zones beginning around E10.5 (Yun et al., 2002). They also show a similar dorsoventral gradient in the septum (Fig. 1a,b). *Dlx1&2*$^{-/-}$ mutants have a clear defect in LGE development, whereas the septal deficits are subtle (Fig.1a-r′) (Anderson et al., 1997a; Anderson et al., 1997b). *Dlx5* and *Dlx6* expression is lost in the LGE and maintained or increased in septal neurons (Fig. 1c-d′; Table 3) (Anderson et al., 1997a; Anderson et al., 1997b). Thus, in contrast with the septum, the LGE of *Dlx1&2*$^{-/-}$ mutants lack expression of all DLX proteins expressed in the brain (DLX1,2,5&6) (Figure 1) (Eisenstat et al., 1999).

Truncated *Dlx1* and *Dlx2* transcripts, that do not encode functional proteins, are produced in *Dlx1&2*$^{-/-}$ mutants (Fig. 1a-b′) (Long et al., 2007; Zerucha et al., 2000). Using *in situ* probes to the truncated *Dlx1* and *Dlx2* transcripts, we investigated the population of *Dlx*-lineage cells that persist in *Dlx1&2*$^{-/-}$ mutants. *Dlx1* RNA expression continues at low levels throughout the SVZ of the subpallium in the *Dlx1&2*$^{-/-}$ mutants. Thus, we conclude that *Dlx1* expression is, at least in part, independent of *Dlx* function and cells in the *Dlx* lineage are present in primary and secondary progenitor populations (Fig. 1a′; Supplementary Fig. 1). However, *Dlx1* expression in the mantle zone is not detectable in the mutant, suggesting that mantle neurons generated from the LGE progenitors fail to activate and/or maintain *Dlx1* RNA expression.

Unlike *Dlx1, Dlx2* RNA expression is not detectable in the dorsal LGE (dLGE) and dorsal Septum (dSe). However, its expression is maintained, albeit at low levels, in the SVZ of the ventral LGE (vLGE) and ventral Septum (vSe) (Fig. 1b′; Supplementary Fig. 1). Lack of *Dlx2* RNA in the dLGE and dSe suggests that these progenitor zones are the most severely affected by loss of *Dlx1&2* function. This hypothesis is supported by the greatly reduced expression of several TFs in the dLGE: *ATBF1, Brn4, ER81, ESRG, Meis1, Meis2, Oct6, Pbx1, Six3, Sp8 and Vax1* at E15.5 (Fig. 1e-i′, 1l-r′; Table 2) (E12.5 analysis of a subset of these TFs support this conclusion; Supplementary Fig. 1).

While the dLGE shows the greatest reduction in TF gene expression, the vLGE also is defective in the *Dlx1&2*$^{-/-}$ mutants, as exemplified by reduced *Brn4*, *Gli1*, and Oct6, expression (Fig. 1f-f′, 1j-j′, 1n-n′). Similar to the LGE, dorsal parts of the septum are preferentially affected by loss of *Dlx1&2* function, as exemplified by reduced *ATBF1, Brn4, Ctip1, ER81* and *Pbx1* expression in the dSe (Figures 1e-e′, 1f-f′, 5i-i′, 1h-h′, 1o-o′).

Ectopic Cortical Marker Expression in the dLGE of *Dlx1&2*$^{-/-}$ Mutants

Disruption of *Dlx1&2* function, perhaps through the loss of DLX1,2,5&6 expression, has a profound effect on specification of dLGE SVZ cells. Another TF expressed in the developing basal ganglia, *Gsh2*, has been shown to be important for specifying dorsoventral fate in the LGE (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). To test whether *Dlx1&2* have a similar function as *Gsh2* in specifying dLGE identity, we studied whether there is ectopic expression of ventrolateral cortical markers in the dLGE. In the *Dlx1&2*$^{-/-}$

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mutants, we previously observed ectopic expression of *Neuropilin2* in the dLGE, which is normally strongly expressed in the ventral cortex (Fig. 6q,q′) (Le et al., 2007; Marin et al., 2001). Consistent with this, the TFs *Ebf3, Id2* and *NHLH2*, which ordinarily mark cells of ventrolateral cortex, all show ectopic expression in the SVZ of the dLGE (Fig. 2a-c′; Tables 1, 3). This further suggests that some dLGE SVZ cells have shifted from a subcortical identity towards a cortical one. *Gsh2* expression is maintained in the *Dlx1&2*$^{-/-}$ mutants (Table 2), suggesting that the shift in subcortical identity occurs through a *Dlx1&2*-dependant and *Gsh2*-independant pathway.

To more fully explore the extent of the subcortical to cortical shift in gene expression, we examined expression of other cortical markers, including both TFs (*Dbx1, Emx1, Emx2, Otx1, Pax6, Tbr1 and Tbr2*) and non-TFs (vesicular glutamate transporters: *Vglut1* and *Vglut2*). None of these genes were ectopically expressed in the dLGE (Fig. 2d-e′ and data not shown). Furthermore, analysis at E12.5 failed to show ectopic *Ebf3, Id2* and *NHLH2*, providing evidence that this phenotype appeared during further maturation of the dLGE/striatum (Supplementary Fig. 1). Thus, although the E15.5 *Dlx1&2*$^{-/-}$ dLGE SVZ has ectopic expression of some ventrolateral cortical TFs, it has not fully shifted its fate to produce cortical neurons with glutamatergic features (*Vglut1&2*), and it maintains expression of *GAD67*, although at a lower level (Fig. 6gg,gg#x2032;). These results suggest that the *Dlx1&2*$^{-/-}$ dLGE maintains its subpallial identity likely through the expression of subcortical TFs necessary for dLGE specification.

dLGE Molecular Identity Is Partially Maintained in the *Dlx1&2*$^{-/-}$ Mutant: *Dlx1&2* Negatively Regulate a Subset of Transcription Factors Expressed in the VZ and SVZ of the LGE

To identify TFs that might compensate for loss of *Dlx1&2* function and maintain dLGE identity, we further characterized TFs that are upregulated in *Dlx1&2*$^{-/-}$ basal ganglia. Loss of *Dlx1&2* function leads to increased SVZ expression of some TFs that mark LGE progenitors. This includes TFs that normally are expressed in the VZ of the LGE (*COUPTFI, Erm, Lhx2, Otx2, Pax6, RORβ* and *Tlx*) and TFs that are expressed in both the VZ & SVZ, or SVZ, of the LGE (*ESRG, Foxg1, Gsh2, Hes5, Lim1, Lmo1, Mash1, Sall3, Sox11* and *Sp9*) (Figs. 1,3; see Supplementary Fig. 1 for E12.5 data) (Yun et al., 2002). Thus, while loss of *Dlx* expression results in down-regulation of some TFs in the dLGE SVZ (Fig. 1e-r′), a separate class of TFs may be responsible for maintaining dLGE molecular properties (Fig. 3), explaining why the dLGE does not fully take on cortical properties (Fig. 2).

It merits mention that the expression of some TFs, whose expression marks VZ cells throughout the telencephalon (*Hes1 and Hesr1*), do not appear to be altered in the *Dlx1&2*$^{-/-}$ mutant (data not shown) (Yun et al., 2002). Thus, while *Dlx1&2* function is required to repress expression of several VZ TFs and maintain LGE identity, expression of a distinct set of progenitor cell regulators is not under *Dlx* control, thereby contributing to the *Dlx* independent specification of the LGE.

Ectopic Expression of Selected MGE and Diencephalic TFs in the SVZ of the LGE

Our data suggest that *Dlx1&2* promote LGE differentiation through repression of LGE progenitor TFs (*COUP-TFI, Erm, Foxg1, Gsh2, Hes5, Lhx2, Lmo1, Mash1, Otx2, Pax6, ROR-β, Sall3, Sp9* and *Tlx*), and ventral cortical TFs (*Ebf3, Id2* and *NHLH2*). To determine if *Dlx1&2* might regulate LGE identity through repression of TFs expressed in other forebrain domains, we examined the expression of TFs that are restricted to the MGE and diencephalon. Indeed, *Dlx1&2* repress TFs that are normally restricted to the E12.5 and E15.5 MGE (*Gsh1, Gbx1* and *Gbx2*), and the progenitor cells of a small domain of the amygdala and diencephalon (*Otp*) (Fig. 4 and Supplementary Fig. 1). *Dlx* repression is

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specific to these ventral genes, as other ventral telencephalic TFs are not ectopically expressed (*Nkx2.1, Nkx5.1, Nkx6.2, Lhx6* and *Lhx7/8*; Supplementary Fig. 1 and data not shown). Thus, *Dlx1&2* have a fundamental role in specifying the properties of LGE SVZ progenitors, through repressing certain MGE TFs, a diencephalic TF, ventrolateral cortical TFs and selected TFs expressed in the VZ of the LGE.

Expression of Some TFs Is Partially Maintained in LGE SVZ and in Differentiating Striatal Cells in the *Dlx1&2*$^{-/-}$ Mutant

To determine which TFs might act in parallel with *Dlx1&2* for LGE formation, we next examined which aspects of LGE differentiation are maintained in the *Dlx1&2*$^{-/-}$ mutants. Although progenitor cells of the *Dlx1&2*$^{-/-}$ mutant LGE have ectopic expression of cortical and MGE TFs, they still express *Gsh2* and *Mash1*, TFs that are essential for LGE development (Casarosa et al., 1999; Corbin et al., 2000; Horton et al., 1999; Toresson and Campbell, 2001; Toresson et al., 2000; Yun et al., 2002; Yun et al., 2003; Yun et al., 2001). Thus, to address what aspects of LGE development are preserved in *Dlx1&2*$^{-/-}$ mutants, we studied the expression of TFs that mark the E15.5 LGE SVZ and mantle zone (MZ; striatum and olfactory tubercle) (Fig. 5). This analysis identified two types of TFs: 1) those whose expression is strongly reduced in the SVZ and/or MZ (particularly in the dLGE), and 2) those whose expression is mildly reduced and/or maintained.

There were several TFs whose expression was either eliminated (*Dlx5&6*) or greatly reduced in the SVZ/MZ of the dLGE (*Egr3, Evi3, Ikaros, Mef2c, RARβ* and *RXRγ*) (Figs 1c-d′, 5a-f′). Note that *ATBF1, Egr3, Ikaros, RARβ* and *RXRγ* expression are nearly specific for striatal and septal cells. The striatal expression of these TFs is reduced particularly in parts related to the dLGE (*ATBF1, Meis2, Pbx3*) (Fig. 5h,h′,r-s′). Furthermore, expression of several TFs in striatal-related structures, such as the olfactory tubercle (OT), appears to be lost (*ATBF1, FoxP1, FoxP2, FoxP4, Islet1, Lmo4, RXRγ, Six3* and *Sox1*) (Fig. 5e,e′,h,h′,k-m′,p,p′,s-t′).

A larger set of LGE TFs continue to be expressed to varying degrees in the LGE SVZ and/or MZ: *Arx, ATBF1, Ctip1, Ebf1, ESRG, Evi3, FoxG1, FoxP1, FoxP2, FoxP4, Islet1, Lmo3, Lmo4, Meis1, Meis2, Pbx3, Six3, Sox1, Sox4, Sox11* and *Tle4* (Figs 1i,i′,l,l′; 3f,f′,m,m′; 5b,b′,g-v′) (Cobos et al., 2005a). Expression of some TFs remains strong in both the dLGE and vLGE, such as *Ctip1, Ebf1, FoxP1, FoxP2, FoxP4, Islet1, Lmo3, Lmo4, Sox1, Sox4* and *Tle4* (Fig. 5i-p′,u-v′).

Expression of Some Effector Genes Is Partially Maintained in the LGE SVZ and in Differentiating Striatal Cells in the *Dlx1&2*$^{-/-}$ Mutant

Thus, based on TF expression in *Dlx1&2*$^{-/-}$ mutants, some programs of striatal differentiation are attenuated, others appear to be partially maintained. To characterize which aspects of striatal differentiation are affected in *Dlx1&2*$^{-/-}$ mutants, we examined expression of genes that mark the striatal differentiated state: *dopamine receptor 1 (D1R), dopamine receptor 2 (D2R), glutamic acid decarboxylase 67 (GAD67* or *GAD1), preprotachykinin (Substance P)* and *vesicular GABA transporter*, as well as other markers of the developing striatum (*Cad8, Golf, Gucy1a3, Neurexin3, PK2, PKR1, Robo1, Robo2, Sema3a, Tiam2* and *TrkB*) (Fig. 6 and data not shown) (Long et al., 2007). In some cases, expression of striatal genes was not detectable in the *Dlx1&2*$^{-/-}$ mutant (i.e. *Cad8* and *Tiam2;* Fig. 6a,a′,m,m′). However, in most cases, residual expression was seen in superficial parts of the striatal mantle (*D1R, D2R, Enk, GAD67, Golf, Gucy1a3, SubP, Robo2, Sema3a* and *TrkB*), demonstrating that the TF programs for striatal histogenesis are partially preserved (Fig. 6). Note, however, the expression of these genes is particularly

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attenuated in parts of the striatum related to the dLGE, highlighting the evidence that *Dlx1&2* function is critical for this region.

Loss of *Dlx1&2* function does not strongly affect some genes (*Robo1*) and in some cases leads to over-expression of other genes (*CyclinD2, Pak3, PK2, PKR1, Slit1*) (Fig. 6) (Cobos et al., 2007). These findings further support the idea that aspects of LGE identity are maintained by TFs, such as *Mash1*, which continue to be expressed in the *Dlx1&2*$^{-/-}$ mutants (Figs. 1,3,5) (Cobos et al., 2007; Long et al., 2007). Next, we tested this hypothesis by studying the LGE and striatal phenotype of *Dlx1&2*$^{-/-}$*;Mash1*$^{-/-}$ triple mutants.

Dlx1&2$^{-/-}$*;Mash1*$^{-/-}$ Compound Mutants Define Genes Epistatic Only to *Dlx1&2* or Epistatic to Both *Dlx1&2* and *Mash1*

While many aspects of LGE/striatal differentiation are lost in the *Dlx1&2*$^{-/-}$ mutants, many aspects are also maintained (Figs. 1,3,5,6). The maintained characteristics may be regulated by the expression of TFs whose expression persists in mutant LGE progenitors (Figs. 1,3, 5). A good candidate of this type of TF is *Mash1*, due to its over-expression in the *Dlx1&2*$^{-/-}$ mutants (Fig. 3j,j′). As MASH1 and DLX2 proteins are co-expressed in progenitors of the dLGE (Porteus et al., 1994; Yun et al., 2002), they have the potential to regulate the developmental programs of these cells in parallel and/or in series. Here, we explored the hypothesis that *Mash1* has a critical role in maintaining certain aspects of LGE/striatal differentiation in the *Dlx1&2*$^{-/-}$ mutants.

We studied the expression of TFs and selected other genes in the LGE and striatum in *Dlx1&2*$^{-/-}$*, Mash1*$^{-/-}$ and *Dlx1&2*$^{-/-}$*;Mash1*$^{-/-}$ mutants at E15.5, concentrating on genes whose expression persists in *Dlx1&2*$^{-/-}$ mutants (Fig. 7; Table 4).

Expression of these genes fell into two general classes (Supplementary Table 2): I) epistatic only to *Dlx1&2*$^{-/-}$ or II) epistatic to both *Dlx1&2*$^{-/-}$ and *Mash1*$^{-/-}$. Expression of Class I genes (*ER81, Gli1, Gsh1, Sp8*) is reduced or lost in the *Dlx1&2*$^{-/-}$ mutants, is not overtly affected in the *Mash1*$^{-/-}$ mutants and the triple mutant phenocopies the *Dlx1&2*$^{-/-}$ mutant. Expression of Class II genes is altered in both the *Dlx1&2*$^{-/-}$ and *Mash1*$^{-/-}$ mutants, and in most cases these phenotypes are exacerbated in the triple mutants. There are six subtypes of Class II genes based on their expression changes (described in Supplementary Table 2).

Finally, several transcription factors continue to be expressed, albeit generally at lower levels, in the LGE of *Dlx1&2*$^{-/-}$*;Mash1*$^{-/-}$ mutants (*Arx, Gsh1, Gsh2, Islet1, Lmo4, Olig2, Pax6* and *Pbx1*) (Figure 7; Table 4), demonstrating that some fundamental aspects of LGE specification are independent of *Dlx* and *Mash1*. Some of these genes may be responsible for maintenance of the remaining striatal differentiation in these mutants.

Mash1 and *Dlx1&2* Regulation of Septum and vLGE Differentiation

While dLGE development is more dependent on *Dlx1&2* than *Mash1*, the septum and the vLGE are more dependent on *Mash1* (Fig. 7; Table 4). The septum in *Dlx1&2*$^{-/-}$ mutants is relatively normal, most likely preserved through the continued expression of *Mash1* and *Dlx5&6* (Fig. 1c′,d′; 3j,j′). However, in *Mash1*$^{-/-}$ mutants, the septum (particularly the ventral septum) is hypoplastic and lacks almost all expression of *Er81, Hes5, Islet1, Olig2, Pbx1, Sp8* and *Sp9* (Fig. 7; Supplementary Fig. 2). Despite these dramatic reductions, *Mash1*$^{-/-}$ mutants maintain expression of *Arx, Dlx1, Dlx5* and *GAD67* (Fig. 7). Septal size in *Dlx1&2*$^{-/-}$*;Mash1*$^{-/-}$ mutants is further reduced, and *Arx* and *GAD67* expression are substantially decreased (Fig. 7h′′,l′′), showing that this aspect of septal identity is determined by *Dlx* function. Also note in the *Mash1*$^{-/-}$ mutants that vLGE expression of *Arx, Islet1, Lmo4, Pbx1, Preprotachykinin, Six3* and *Sp9* is attenuated (Fig. 7, Supplementary Fig. 2).

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Discussion

In this study, we have provided a foundation for defining the transcription factor (TF) circuitry that controls development of the LGE and its product, the striatum. Table 2 lists all of the TFs, that we could reliably identify, that are expressed at E15.5 in the developing mouse basal ganglia (factors that are part of the core transcriptional machinery are not listed). Based on gene expression array and *in situ* hybridization we have identified 53 TFs that have higher expression levels in the basal ganglia than in the cortex (TFs colored in green and aqua in Table 2); these are likely to have roles in defining features that are specific to basal ganglia neurons, such as GABAergic fate.

Among these TFs, *Dlx1&2* and *Mash1* are known to have central roles in basal ganglia differentiation (Anderson et al., 1997b; Casarosa et al., 1999; Fode et al., 2000; Horton et al., 1999; Yun et al., 2002). We systematically defined the role of *Dlx1&2* in regulating the expression of TFs listed in Table 2, identifying TFs whose expression is dependent and independent of *Dlx1&2* function (Table 3). We provide evidence that some of the *Dlx1&2* independent TFs depend on *Mash1* function, and *vice versa* (Table 4). Based on analysis of *Dlx1&2*$^{-/-}$*, Mash1*$^{-/-}$ *and Dlx1&2*$^{-/-}$*;Mash1*$^{-/-}$ mutants we propose epistatic relationships between these TFs (Table 4; Supplementary Table 2).

Subdivisions of the Basal Ganglia

The progenitor domains of the embryonic basal ganglia consists of the septum, LGE, MGE and preoptic area, each of which has multiple subdivisions (Campbell, 2003; Flames et al., 2007; Long et al., 2007; Yun et al., 2001). In this paper, we have focused on rostral parts of the LGE and the septum, each of which has dorsal and ventral progenitor domains (Fig. 1). In a subsequent paper, we will report on our analysis of the caudal LGE (which includes most of the CGE), MGE and preoptic area.

The dLGE contains progenitors for both the striatum and olfactory bulb interneurons (Corbin et al., 2000; Stenman et al., 2003; Toresson et al., 2000; Yun et al., 2001), whereas vLGE progenitors are currently thought to produce primarily striatal neurons (Toresson and Campbell, 2001; Yun et al., 2003). Given its proximity to the septum, we suggest that rostral parts of the vLGE produce accumbens neurons.

Dlx1&2 are expressed in a dorsoventral gradient in both the LGE and septal progenitor domains (Fig. 1a,b; Supplementary Fig. 1) (Eisenstat et al., 1999; Yun et al., 2002). In the dLGE, *Dlx1&2* are expressed in most cells of the VZ. Previously, we demonstrated that DLX2 and MASH1 are co-expressed in most dLGE progenitors (VZ and SVZ), whereas in the vLGE there is much less DLX2 expression, particularly in the VZ (Yun et al., 2002). Here, we present evidence that *Dlx1&2* function is more important in the dLGE than the vLGE, whereas *Mash1* function is more important in the vLGE and the septum.

Dlx1&2 Specify the Fate and Differentiation of dLGE Neurons

Previous analyses of the *Dlx1&2*$^{-/-}$ mutants demonstrated that these TFs regulate differentiation and migration of LGE-derived progenitors in part through repressing expression of *Mash1* and the *Notch*-signaling pathway (Anderson et al., 1997b; Yun et al., 2002). Here, we demonstrate more profound defects in the dLGE - the SVZ of the *Dlx1&2*$^{-/-}$ mutants ectopically express ventral pallial (*Ebf3, Id2*), MGE (*Gbx1&2; Gsh1*) and diencephalic (*Otp*) TFs (Fig. 2,4). The neurons generated in the *Dlx1&2*$^{-/-}$ mutant dLGE express low levels of *GAD67* and *vesicular GABA transporter* (Fig. 6) (Long et al., 2007). Thus, *Dlx1&2* are essential for repressing both dorsal (pallial) and ventral (MGE) TFs from the dLGE, in addition to promoting GABAergic fate. This is consistent with previous evidence that the *Dlx genes* are sufficient to induce expression of GABAergic

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markers as a result of ectopic expression of *Dlx2* and *Dlx5* in cortical progenitors (Anderson et al., 1999; Stuhmer et al., 2002).

Loss of *Dlx1&2* greatly reduces dLGE expression of *Arx, ATBF1, Brn4, Dlx5, Dlx6, ER81, Meis1, Meis2, Oct6, Pbx1, Six3, Sp8* and *Vax1* (Figs. 1,5; Table 3). Currently, the function of only *Arx, Sp8* and *Vax1* has been defined in the dLGE. *Arx, Sp8* and *Vax1* promote development of interneurons that migrate rostrally from this zone to the olfactory bulb (Soria et al., 2004; Waclaw et al., 2006; Yoshihara et al., 2005). *Dlx1&2*$^{-/-}$ mutants fail to produce olfactory bulb interneurons due to a combination of molecular specification and migration defects, which include reduced expression of *Arx, Sp8* and *Vax1* (Figs. 1,5) (Bulfone et al., 1998; Long et al., 2007).

Dlx1&2$^{-/-}$ mutants also show severe defects in striatal and olfactory tubercle development. Previously, we provided evidence that early LGE differentiation and migration to the striatum were preserved (E11.5-E12.5) relative to those processes at E15.5 (Anderson et al., 1997b; Yun et al., 2002). However, early LGE development is not normal; most of the molecular defects observed at E15.5 can be appreciated at E11.5 and E12.5 (Supplementary Fig. 1) (Cobos et al., 2005b; Long et al., 2007). Furthermore, there is a reduction in the numbers of neurons that express markers of both the striatonigral (dopamine receptor 1; *D1R* and *preprotachykinin*) and striatopallidal (dopamine receptor 2; *D2R* and *enkephalin*) medium spiny neurons (Fig. 6; data not shown).

Reduced expression of several TFs is likely to contribute to the *Dlx1&2*$^{-/-}$ striatal hypoplasia and molecular defects. Reduced *Arx* expression (Fig. 5g,g′) (Cobos et al., 2005b) may result in migration defects of LGE-derived cells, as *Arx*$^{-/-}$ mice have a related phenotype (Colombo et al., 2007). Likewise, reduced expression of retinoid nuclear receptors (*RAR*β and *RXR*γ) could contribute to the striatal phenotype (Fig. 5e,e′, f,f′). Retinoid signaling through these receptors is implicated in regulating striatal differentiation (Toresson et al., 1999; Waclaw et al., 2004) and the expression of *D1R* and *D2R* (Krezel et al., 1998; Wang and Liu, 2005).

Despite the severe molecular defects in the LGE of the *Dlx1&2*$^{-/-}$ mutants, some features of the LGE and even the striatum are preserved (Figs. 3, 5, 6,7; Tables 2,3). This is most likely due to a set of TFs that continue to be expressed in LGE progenitors (Figs. 3,5; Tables 2,3). For instance, expression of the neurogenic TFs *Sox4* and *Sox11* (Bergsland et al., 2006) is preserved (Figs. 3m,m′; 5u,u′), consistent with the preservation of core features of neurogenesis in the LGE, such as MAP2 and β-III-tubulin expression (Anderson et al., 1997b; Cobos et al., 2007). Furthermore, partial LGE identity may be maintained in *Dlx1&2*$^{-/-}$ mutants by virtue of *Gsh1, Gsh2, Mash1* and *Tlx* expression in progenitor cells. These TFs contribute to striatal development (Casarosa et al., 1999; Corbin et al., 2000; Horton et al., 1999; Stenman et al., 2003; Toresson and Campbell, 2001; Toresson et al., 2000; Yun et al., 2002; Yun et al., 2003; Yun et al., 2001).

Indeed, striatal expression of certain TFs is maintained at relatively high levels (Fig. 5). This includes *Ebf1*, a TF that regulates prenatal striatal development (Garel et al., 1999). In the postnatal brain, *Ebf1* is preferentially expressed in striatonigral neurons. Consistent with this, the *Ebf1*$^{-/-}$ mutant mouse shows defects in gene expression (*preprotachykinin*) and projections of striatal neurons to the substantia nigra (Lobo et al., 2006). The continued expression of *Ebf1* in the *Dlx1&2*$^{-/-}$ mutants suggests that differentiation of striatonigral neurons may be preserved. However, other molecular features of these neurons [*D1R, preprotachykinin*, and *Evi3* (also known as *Zfp521*)] are more reduced than *Ebf1* expression (Figs. 5b,b′; 6c,c′,j,j′). This suggests that *Dlx1&2* differentially regulate expression of distinct sets of genes within immature striatonigral neurons.

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Expression of a separate set of genes is repressed by *Dlx1&2* and is promoted by *Mash1* (*Hes5, Olig2* and *Sp9*) (Fig. 7; Table 4; Supplementary Table 2). Previously, we provided evidence that elevations in *Mash1* expression in the *Dlx1&2*$^{-/-}$ mutants leads to increased Notch-signaling that increases *Hes5* expression (Yun et al., 2002). Current work provides evidence that *Dlx1&2* repression of *Olig2* is central to promoting neurogenesis and blocking oligodendrogenesis in the telencephalon (Petryniak et al., 2007).

Mash1 Has a Prominent Role in Differentiation of vLGE and Septal Neurons

While the dLGE development is severely derailed by loss of *Dlx1&2*, vLGE and septal development are relatively preserved (Figs. 1,5,6,7; Supplementary Fig. 2; Table 4), perhaps because *Dlx1&2* are not as strongly expressed in the VZ of these progenitor domains (Fig. 1a,b) (Eisenstat et al., 1999; Yun et al., 2002) and because *Dlx5&6* expression are maintained (Fig. 1c′,d′). On the other hand, vLGE and septal differentiation and growth are strongly affected in the *Mash1*$^{-/-}$ mutant (Fig. 7; Supplementary Fig. 2; Table 4). In particular, expression of *Islet1, Lmo4, Meis2, Pbx1, Six3, Sp9*, and *Vax1* are greatly reduced in these progenitor domains (Fig. 7; Supplementary Fig. 2; Table 4). Despite these defects in the septum, expression of *Arx, Dlx1* and *Dlx5* are preserved, which may explain why *GAD67* continues to be expressed in the septum of *Mash1*$^{-/-}$ mutants. Thus, while the dLGE and vLGE/septum express many of the same TFs, their development has distinct dependence on them. Furthermore, this predicts divergent programs for development of the striatum (regulated by the dLGE) and the nucleus accumbens (regulated by the vLGE).

Dlx and *Mash1* Have Parallel and Overlapping Functions in LGE/Striatal Differentiation

DLX2 and MASH1 are co-expressed in VZ and SVZ cells of the dLGE - this has allowed us to test whether they cooperate in regulating differentiation of these cells by comparing the phenotype of single and compound mutants. Based on *in situ* hybridization analysis of *Dlx1&2*$^{-/-}$, *Mash1*$^{-/-}$ and *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ mutants, we have identified genes that are epistatic only to *Dlx1&2*$^{-/-}$ (Class I) or epistatic to both *Dlx1&2*$^{-/-}$ and *Mash1*$^{-/-}$ (Class II) (Figure 7; Table 4; Supplementary Table 2). We have defined six subtypes of Class II genes based on their expression changes (described in Supplementary Table 2). It is striking that LGE progenitors in the triple mutant continue to express *Arx, Gsh, Islet1, Lmo4, Olig2* and *Pbx1* (Fig. 7). Thus, LGE specification (and *GAD67* expression) has not been fully eliminated in the triple mutants, which implicates one or several of these TF genes in maintaining aspects of LGE identity.

In addition to regulating overlapping transcriptional pathways in the LGE, we propose that *Dlx1&2* and *Mash1* regulate parallel transcriptional pathways. For example, whereas *Mash1* promotes expression of genes involved in neurogenic differentiation such as expression of general neural markers such as *Sox1* and *Map2* (Supplementary Fig. 2, data not shown) (Yun et al., 2002), *Dlx1&2* are not required for induction of these genes (Supplementary Fig. 2) (Cobos et al., 2007; Yun et al., 2002).

Our results set the stage for defining the transcription factor network that regulates LGE and striatal differentiation. While this work will require analysis at the level of individual regulatory elements, our study provides a foundation for: 1) performing computational analyses of gene expression networks; 2) designing enhancer analyses; 3) further *in vivo* genetic analyses of single and compound mutants; 4) deciphering transcriptional codes that can be used to drive immature progenitor cells to differentiate into striatal medium spiny neurons.

Finally, we have begun to elucidate how the *Dlx* genes regulate the fate and function of LGE-derived neurons by identifying changes in the expression of effector genes (Fig. 6). For

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instance, *Dlx1&2* have a profound role in defining the GABAergic fate through promoting expression of *GAD67* and *vGAT* (Fig. 6g,g′; Supplementary Figure 1) (Anderson et al., 1999; Long et al., 2007; Stuhmer et al., 2002). *Dlx1&2* also regulate neuronal migration and neurite morphogenesis; recently we presented evidence that this is in part mediated through *Dlx1&2* repression of *Pak3* (Cobos et al., 2007). However, as shown in Fig 6, there are major changes in the expression of other genes that regulate the cytoskeleton through modulating intracellular signaling (*Cad8, CXCR4, Golf, Gucy1a3, PK2, PKR1, RDC, Robo1, Robo2, Sema3a, Slit1, Tiam2, TrkB*). Finally, the *Dlx* genes regulate receptors and neuropeptides that are central modulators of striatal function (*D1R, D2R, enkephalin* and *preprotachykinin*). Therefore, by virtue of their expression in progenitors (VZ & SVZ), immature neurons and mature neurons, the *Dlx* genes are likely to have central roles in transcriptional hierarchies that specify the differentiation and function of striatal neurons and in initiating and maintaining the GABAergic state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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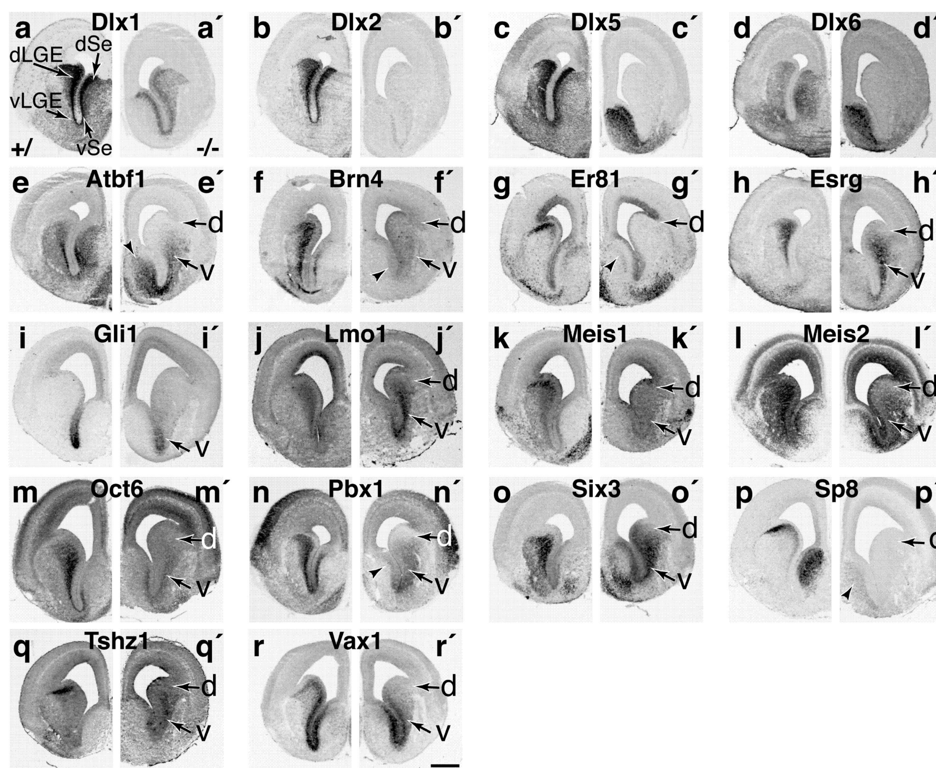

Figure 1. *Dlx1&2*-Dependent Expression of Transcription Factors in LGE Progenitors

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control and *Dlx1&2*$^{-/-}$ mutant animals demonstrates *Dlx1&2*-dependent expression in the SVZ and MZ of the LGE. For some genes the effect is either exclusive, or predominant, to the dLGE, leaving expression in the vLGE relatively normal (indicated by arrows). In other cases, however, vLGE expression is reduced. (a-d′) Note the loss of *Dlx5&6* expression in the LGE, but not in the septum. Arrowheads mark the defects in the dSe, despite the maintenance of several transcription factors. dLGE, dorsal lateral ganglionic eminence; dSe, dorsal septum; vLGE, ventral lateral ganglionic eminence; vSe, ventral septum. Scale bars a-r′, 500 μm

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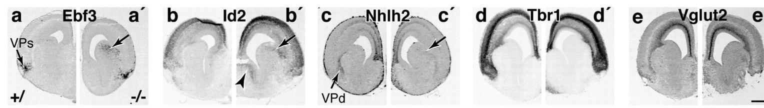

Figure 2. Ectopic Expression of Ventrolateral Cortical Markers in the dLGE

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control and *Dlx1&2*$^{-/-}$ mutants. (a-c′) Arrows mark the ectopic expression of three ventrolateral cortical markers (*Ebf3, Id2, NHLH2*) in the dLGE of the *Dlx1&2*$^{-/-}$ mutant. (d-e′); *Id2* is also ectopically expressed in the septum (arrowhead). Two cortical markers (*Tbr1, Vglut2*) do not show ectopic expression. VPs, ventral pallidum superficial; VPd, ventral pallidum deep. Scale bars a-e′, 500 μm.

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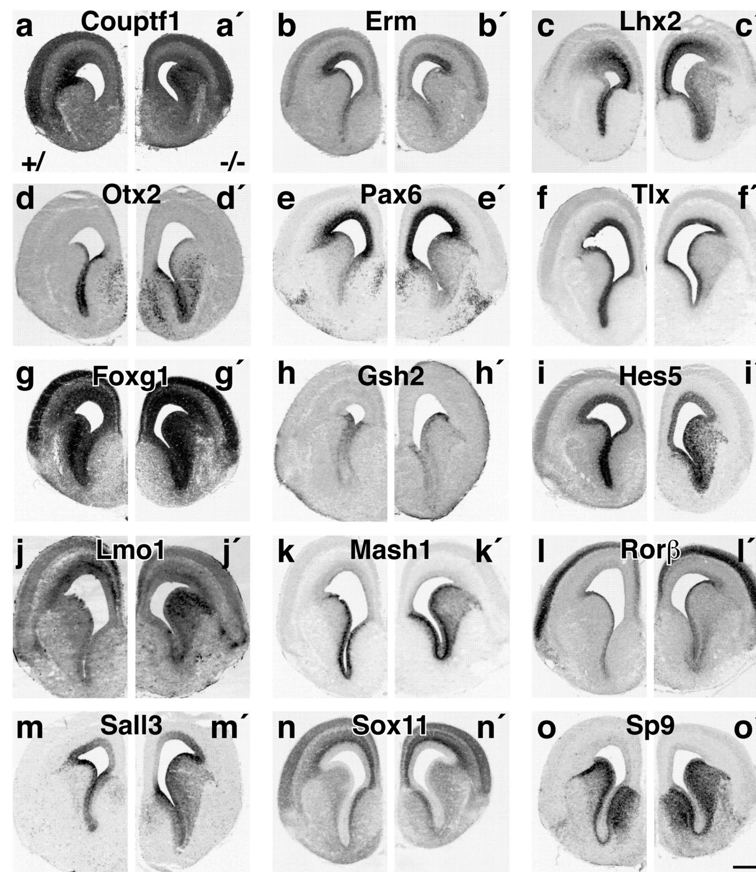

Figure 3. *Dlx1&2* Repress Expression of Transcription Factors in LGE Progenitors

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control and *Dlx1&2*$^{-/-}$ mutants show transcription factors whose expression is up-regulated in the LGE, particularly in the SVZ. (a-f′): Genes whose expression is normally detected only in the VZ. (g-o′): Genes whose expression is normally detected in the VZ and/or SVZ of the LGE. Scale bars a-o′, 500 μm.

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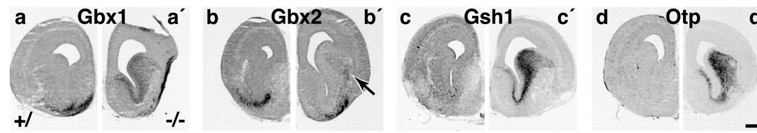

Figure 4. *Dlx1&2*$^{-/-}$ Mutants Have Ectopic LGE and Septal Expression of Transcription Factors That Normally Mark the MGE and/or Diencephalon

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control and *Dlx1&2*$^{-/-}$ mutants. (a-e′) Several transcription factors that are normally not expressed in the LGE or Septum are ectopically expressed in the *Dlx1&2*$^{-/-}$ mutant animals. This includes markers of the MGE (*Gbx1, Gbx2*, and *Gsh1*) and diencephalon (*Otp*). Scale bars a-d′, 500 μm.

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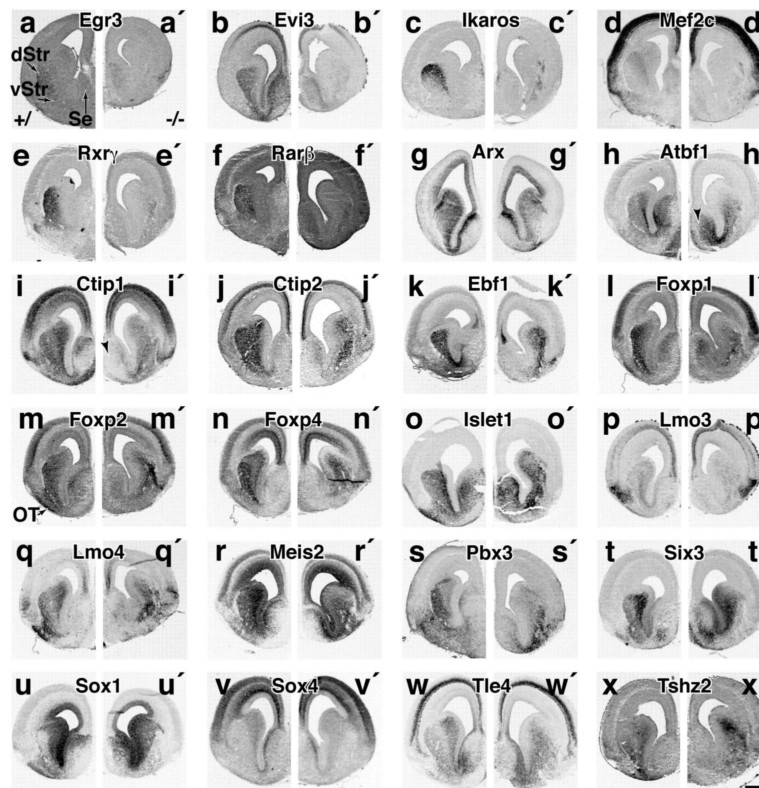

Figure 5. *Dlx*-Dependent and Independent Transcription Factor Expression in Maturing Striatal Neurons

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control and *Dlx1&2*$^{-/-}$ mutants. (a-f′) Transcription factors whose expression is severely reduced in the *Dlx1&2*$^{-/-}$ mutants. (g-x′) Transcription factors whose expression is partially reduced or maintained. (h′,i′) Arrowheads show reduced expression in the dorsal septum. dStr, dorsal striatum; OT, olfactory tubercle, Se, septum; vStr, ventral striatum. Scale bars a-x′, 500 μm.

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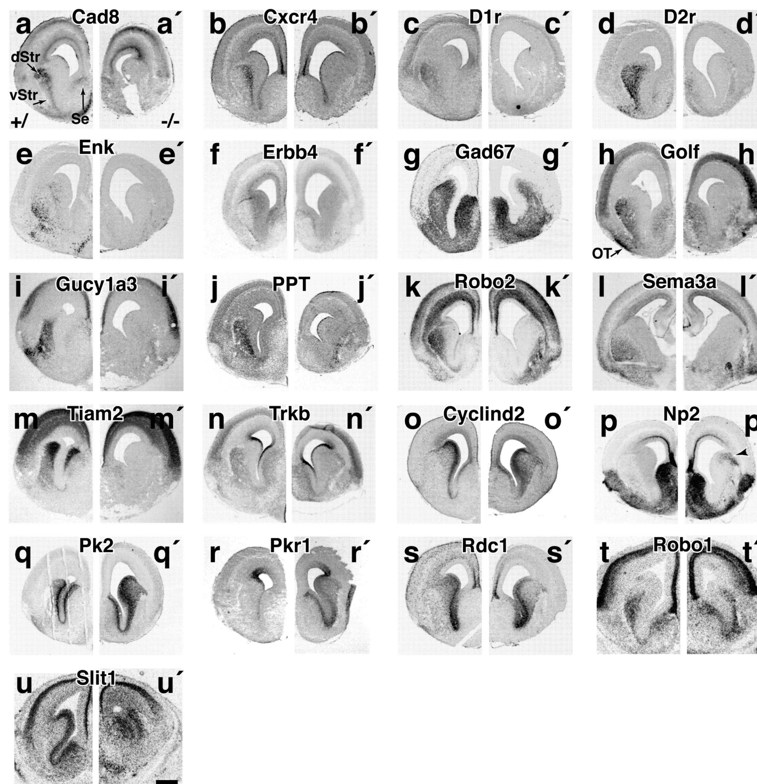

Figure 6. *Dlx*-Dependent and Independent Expression of Non-Transcription Factor Markers of Striatal Differentiation and Migration

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control and *Dlx1&2*$^{-/-}$ mutants. (a-n′) Non transcription factors whose expression is decreased in the striatum. (o-u′) Non transcription factors whose expression is maintained or slightly increased in the striatum. dStr, dorsal striatum; OT, olfactory tubercle; PPT, preprotachykinin; Se, septum; vStr, ventral striatum. Scale bars a-u′, 500 μm.

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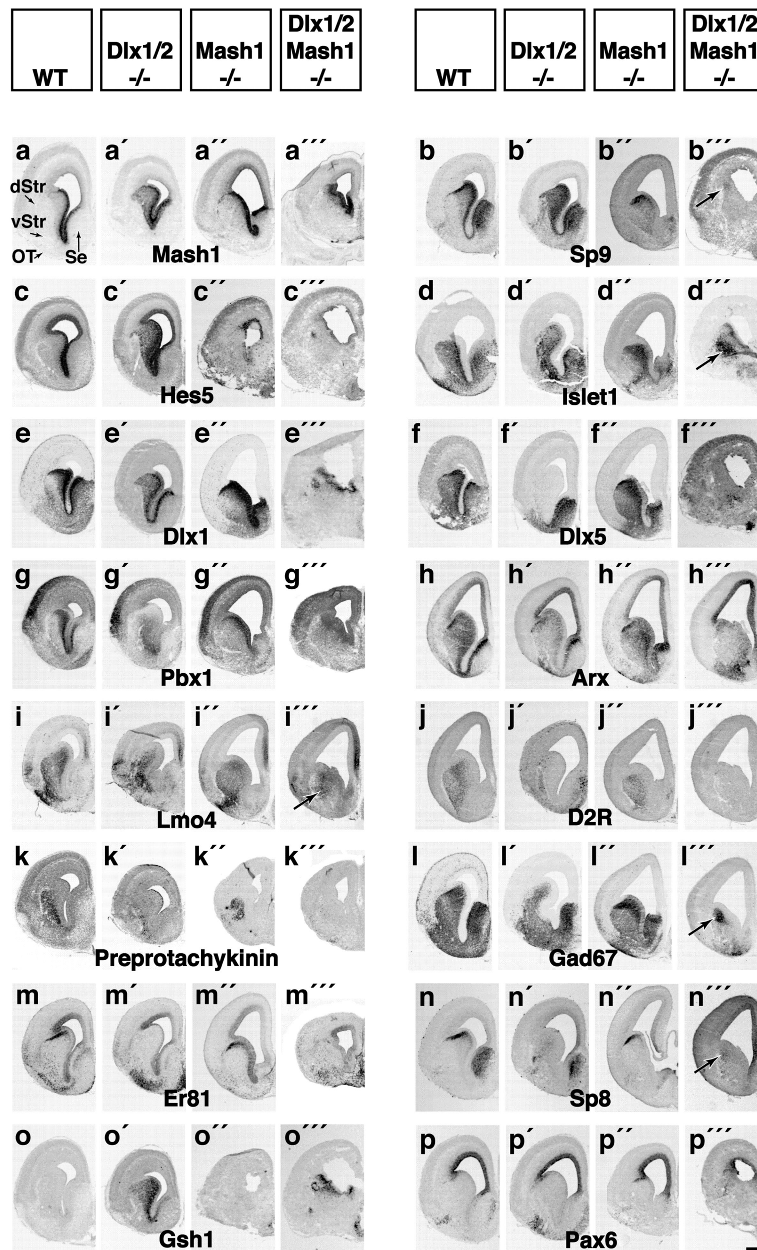

Figure 7. *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ Compound Mutants Define Epistatic Relationships in dLGE, vLGE and Septal Differentiation

In situ hybridization of coronal hemisections through the E15.5 telencephalon of control, *Dlx1&2*$^{-/-}$, *Mash1*$^{-/-}$ mutants and *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ mutants. (a′-p′) The *Dlx1&2*$^{-/-}$ mutant shows severe dLGE differentiation defects and mild vLGE and septum defects (e.g. *Sp8* n, n′). (a″-p″) The *Mash1*$^{-/-}$ mutant animal shows severe septal vLGE defects and mild dLGE defects (e.g. *Sp9* b,b″). (a‴-p‴) The *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ mutant animal demonstrates the cooperative roles of by *Dlx1&2* and *Mash1* in dLGE, vLGE and septal development due to the aggravation of the individual mutant phenotypes. Arrows mark remnants of normal LGE gene expression in the *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ mutants. dStr, dorsal striatum; OT, olfactory tubercle, Se, septum; vStr, ventral striatum. Scale bars a-ppp‴, 500 μm.

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E12 ISH	E15 ISH	TF Name	Basal Ganglia / Cortex	Cortex	BG	BG -/-	BG- / BG+	BG+ / BG-
	*	Arx	4.64	487	2262	618	0.27	3.66
	*	Asb4	4.81	16	77	125	1.62	0.62
	*	ATBF1 (ZFHX3)	30.44	18	548	503	0.92	1.09
	*	BF1 (FoxG1)	0.75	5121	3856	3116	0.81	1.24
	*	Brn2 (POU3F2)	0.56	149	83	93	1.12	0.89
	*	Brn4 (POU3F4)	7.18	28	201	114	0.57	1.76
	*	Brn5 (POU6F1)	0.16	19	3	10	3.33	0.30
	*	CoupTF1 (NR2F1)	1.31	2414	3154	3561	1.13	0.89
	*	CoupTFII (NR2F2)	3.38	102	345	339	0.98	1.02
	*	Ctip1 (Bcl11a, Evi9)	1.06	1522	1616	1052	0.65	1.54
	*	Ctip2 (Bcl11b, Rit-1b)	1.43	1106	1579	792	0.50	1.99
	*	Cux2	*					
	*	Dbx1	1.83	18	33	27	0.82	1.22
	*	Dlx1	9.84	120	1181	7	0.01	168.71
*	*	Dlx2	7.06	50	353	15	0.04	23.53
	*	Dlx5	7.76	96	745	80	0.11	9.31
	*	Dlx6	9.80	15	147	21	0.14	7.00
	*	Dlx6 Antisense (Evf1, Evf2)	4.00	11	44	4	0.09	11.00
	*	Ebf1	21.00		576	132	0.23	4.36
*	*	Ebf3	0.66	74	49	198	4.04	0.25
	*	Egr3	2.05	21	43	18	0.42	2.39
	*	Emx1	0.31		100	75	0.75	1.33
	*	Emx2	0.43	356	152	177	1.16	0.86
*	*	ER81 (Etv1)	3.23	66	213	137	0.64	1.55
	*	Erm (Etv5)	*					
	*	ESRG (ESRRG, NR3B3)	1.57	21	33	26	0.79	1.27
	*	Evi3 (Zfp521, EHZF)	*					
	*	Fez; not LGE	*					
	*	Fez-l ; Zfp312	*					
	*	FoxP1	2.60	149	388	122	0.31	3.18
	*	FoxP2	2.53	34	86	90	1.05	0.96
	*	FoxP4 (mFKHLA)	1.30		56	43	0.77	1.30
*	*	Gbx1; not LGE	*					
	*	Gbx2; not LGE	5.33	9	48	63	1.31	0.76
	*	Gli1 (Zfp5)	*					
*	*	Gsh1	7.57	7	53	156	2.94	0.34
	*	Gsh2	14.77	13	192	205	1.07	0.94
*	*	Hes5	1.36	167	227	290	1.28	0.78
	*	HesR1 (Hey1)	1.10	106	117	144	1.23	0.81
*	*	Id2	0.12	1870	230	686	2.98	0.34
	*	Id4	0.98	197	194	196	1.01	0.99
	*	Ikaros (ZNFN1A1)	2.27	11	25	25	1.00	1.00
	*	Isl1	254.00	6	1524	1318	0.86	1.16

	*	Lhx1 (Lim1); not LGE	0.36	14	5	22	4.40	0.23
	*	Lhx2	0.31	2120	650	774	1.19	0.84
*	*	Lhx6; not LGE	2.67	46	123	98	0.80	1.26
	*	Lhx7; not LGE	252.50	2	505	224	0.44	2.25
	*	Lmo1; not LGE	0.62	569	351	362	1.03	0.97
	*	Lmo3 (Rbtn3)	*					
	*	Lmo4	2.19	360	789	581	0.74	1.36
	*	MAF B; not LGE	1.07	122	131	167	1.27	0.78
	*	Mash1 (Ascl1)	4.51	63	284	330	1.16	0.86
	*	Med6	6.00		386	229	0.59	1.69
	*	Mef2c	0.44	398	174	151	0.87	1.15
	*	Meis1	4.67	49	229	154	0.67	1.49
	*	Meis2 (MRG1B)	1.23	1489	1827	1275	0.70	1.43
	*	Nex1 (NeuroD6)	0.12	5163	620	881	1.42	0.70
	*	NHLH2 (Hen2, NSCL2); not LGE	*					
	*	Nkx2.1 (Ttf1); not LGE	0.46	65	30	47	1.57	0.64
	*	Nkx2.2; not LGE	7.00	3	21	30	1.43	0.70
	*	Nkx5.1 (Hmx1); not LGE	1.67	3	5	3	0.60	1.67
	*	Nkx6.2	1.24	100	124	112	0.90	1.11
	*	Nolz1 (Zfp503)	*					
	*	NPas1; not LGE	0.50	12	6	2	0.33	3.00
	*	Nur77 (NR4A1)	1.18	65	77	51	0.66	1.51
*	*	Oct6 (POU3F1)	1.30	74	96	25	0.26	3.84
	*	Olig1	5.73	79	453	638	1.41	0.71
	*	Olig2	5.66	29	164	281	1.71	0.58
*	*	Otp	0.88	8	7	16	2.29	0.44
	*	Otx1	0.31		40	53	1.33	0.75
*	*	Otx2	0.46		35	71	2.03	0.49
	*	Pax6	0.42	200	83	103	1.24	0.81
*	*	Pbx1	1.03	180	186	126	0.68	1.48
	*	Pbx3	7.28	76	553	671	1.21	0.82
	*	Peg3 (End4, Gcap4, Pw1, Zfp102)	1.22	381	463	818	1.77	0.57
	*	Prox1	3.07	14	43	56	1.30	0.77
	*	RARß	14.00		271	40	0.15	6.78
	*	RORb	0.70		42	83	1.98	0.51
	*	RXRg (NR2B3)	21.29	14	298	48	0.16	6.21
*	*	Sall3 (Msal1, Spalt)	0.84	68	57	145	2.54	0.39
	*	Six3	9.70	33	320	153	0.48	2.09
	*	Sox 1	3.60	15	54	35	0.65	1.54
	*	Sox 4	0.73	827	602	865	1.44	0.70
		Sox 5	0.17	1023	172	223	1.30	0.77
		Sox 6	1.13	55	62	77	1.24	0.81
	*	Sox10	4.30		123	85	0.69	1.45
	*	Sox11	0.77	6142	4716	3765	0.80	1.25
*	*	Sp8 (Btd)	2.40		152	81	0.53	1.88
	*	Sp9	*					
	*	Tbr1	0.33	646	215	270	1.26	0.80
	*	Tbr2	0.16	1030	169	111	0.66	1.52
	*	TCF4	0.30	2771	818	1245	1.52	0.66
	*	Tle4 (Grg4)	*					
	*	Tlx	1.44	16	23	18	0.78	1.28
	*	Trp53	0.82	124	102	80	0.78	1.28
	*	Tshz1	*					
	*	Tshz2	*					
*	*	Vax1	2.69	13	35	22	0.63	1.59
	*	Zic1	1.45	593	859	1592	1.85	0.54
		Zhfx1b	0.44	89	39	32	0.82	1.22

TABLE 2. Expression Levels of Transcription Factors Studied in the *Dlx1&2*$^{-/-}$ Mutants

Table alphabetically lists the transcription factor gene name, basal ganglia/cortex (BG/Ctx) ratio of expression in wild type E15.5 embryos, the expression levels in the wild type cortex (Ctx) and basal ganglia (BG), and the expression level in the BG of the *Dlx1&2*$^{-/-}$ mutants (BG −/−) (expression levels are in arbitrary units generated by the analysis of gene expression array data). Also shown are the investigators from who we received the plasmid used for *in situ* hybridization (Origin of Plasmid). Genes shown in green are expressed primarily in the basal ganglia. Genes shown in aqua are expressed both in the basal ganglia and cortex, but have a 2-fold bias towards the basal ganglia. Genes shown in yellow are expressed in both the basal ganglia and cortex at roughly the same level. Genes shown in lavender are expressed primarily in the cortex. The gene in orange is expressed in the diencephalon and only a small part of the amygdala. The columns on the left indicate whether we performed in situ hybridization (ISH) at E12.5 and E15.5. NR1H4 (FXR),

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FoxO1, NR4A1, and Nolz1 are genes expressed in the striatum at E18.5 (Chang et al., 2004; Gray et al., 2004); our in situ analysis did not detect E15.5 expression.

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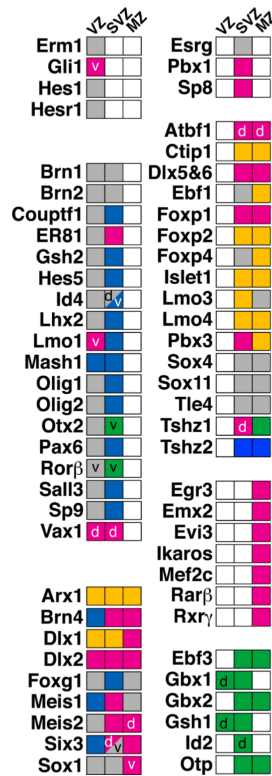

TABLE 3. Expression of Transcription Factors in the VZ, SVZ and MZ of the LGE in the *Dlx1&2*$^{-/-}$ Mutants

Table depicts the ventricular zone (VZ), subventricular zone (SVZ) and mantle zone (MZ) of the LGE of an E15.5 embryo as discrete boxes. The effect of the *Dlx1&2*$^{-/-}$ mutation on gene expression in each box is indicated using a color code: Gray represents unchanged gene expression. White represents no detectable expression. Magenta represents severe reduction in expression. Orange represents moderate/mild reduction in expression. Blue represents an increase of gene expression. Green represents ectopic expression. The genes are ordered as follows: left column are genes expressed in the proliferative zones (VZ and SVZ); right column are genes expressed at later developmental stages (SVZ, SVZ&MZ, MZ). The genes are arranged alphabetically within each grouping. A 'd' represents the effect is primarily in the dorsal part of the LGE and a 'v' represents the ventral part.

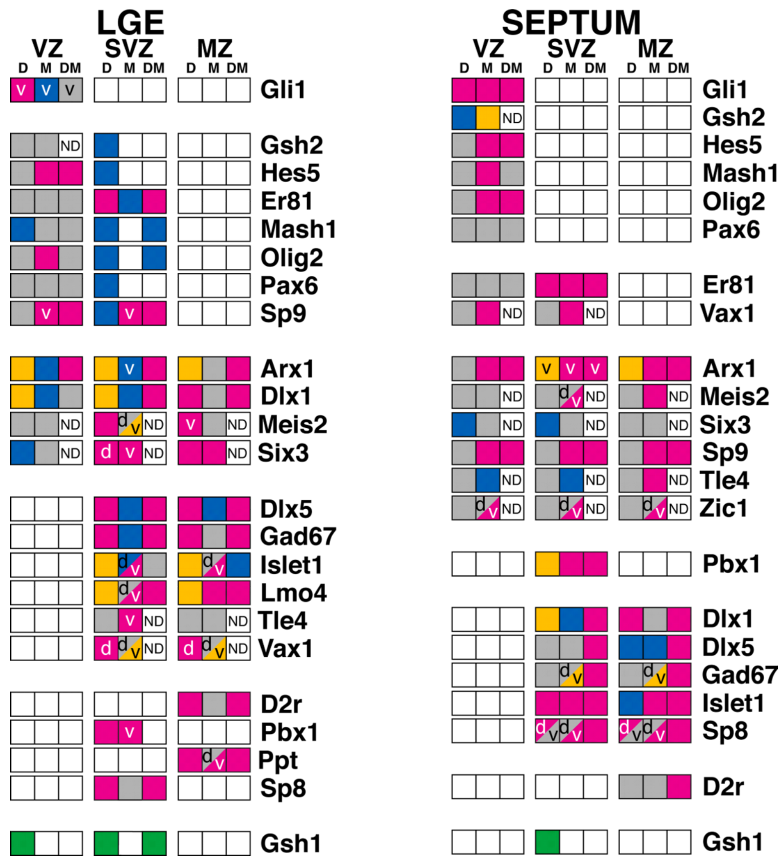

TABLE 4. Expression of Genes in the VZ, SVZ and MZ of the LGE and Septum in *Dlx1&2*$^{-/-}$, *Mash1*$^{-/-}$ and *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ Mutants

Table depicts the ventricular zone (VZ), subventricular zone (SVZ) and mantle zone (MZ) of the LGE and Septum of an E15.5 embryo as discrete boxes. The effect of the *Dlx1&2*$^{-/-}$ (*D*), *Mash1*$^{-/-}$ (*M*) and *Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$ (*DM*) mutations on gene expression in each box is indicated using a color code: Gray represents unchanged gene expression. White represents no detectable expression. Magenta represents severe reduction in expression. Orange represents moderate/mild reduction in expression. Blue represents an increase of gene expression. Green represents ectopic expression. The genes are ordered according to when their expression begins; genes at the beginning are expressed in the VZ, whereas genes at the end are expressed only in the MZ. The genes are arranged alphabetically within each generalized grouping. The left columns correspond to the LGE and the right columns correspond to the septum. A ‘d’ represents the effect is primarily in the dorsal part of the structure and a ‘v’ represents the ventral part.

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TABLE 1

Nucleotide Sequences of In Situ Hybridization Probes Table provides the name of the plasmid donor and nucleotide sequence range for each in situ hybridization probe used in our analysis.

Plasmid Name	Source	Insert Size in Base Pairs	cDNA Nucleotides of Probe or Nucleotides from 3′ End
Arx	Kunio Kitamura	1000	1000 from 3′ end
ATBF1	Dino Leone	464	11887-11423
Brn4	Bryan Crenshaw	573	513-1086
Cad8	Chris Redies	460	1251 to 1711
CoupTF1	Ming Tsai	1500	full coding sequence
Ctip1	Mark Leid	766	full coding sequence
CXCR4	Dan Littman	580	full coding sequence
CyclinD2	A. Malamacci	475	1329-1804
D1R	Josh Corbin	534	3604-4138
D2R	Josh Corbin	804	517-1321
Dlx1	John Rubenstein	2800	2800 from 3′ end
Dlx2	John Rubenstein	1700	1700 from 3′ end
Dlx5	John Rubenstein	1600	1600 from 3′ end
Dlx6	John Rubenstein	210	210 from 3′ end
Ebf1	R. Grosschedl	737	193-930
Ebf3	Sonia Garel	1750	1750 from 3′ end
Egr3	J.D. Powell	1416	full coding sequence
Enk	Josh Corbin	1441	full coding sequence
ER81	Thomas Jessell	2000	full coding sequence
ErbB4	Cary Lai	920	Amino Acids 326-633
Erm; Etv5	Anne Chotteau-Lelievre	1998	full coding sequence
ESRG; NR3B3	Paul Gray	720	302-1022
Evi3; Zfp521	RZPD; ImaGenes	3936	full coding sequence
FoxG1	E.Lai	1563	2094-3657
FoxP1	Russell Ferland	1900	full coding sequence
FoxP2	Russell Ferland	2000	full coding sequence
FoxP4	Russell Ferland	2058	2058 from 3′ end
GAD67	Bryan Condie	2000	full coding sequence
Gbx1	Mike Frohman	550	334-884
Gbx2	Mike Frohman	650	650 from 3′ end
Gli1	Alex Joyner	1683	945-2628
Golf; Gna1	Richard Axel	1540	1-1540
Gsh1	Steve Potter	2200	full coding sequence
Gsh2	Steve Potter	460	1038-1498
Gucy1a3	ATCC 9841394	2000	full coding sequence

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Plasmid Name	Source	Insert Size in Base Pairs	cDNA Nucleotides of Probe or Nucleotides from 3′ End
Hes5	Francois Guillemot	548	73-621
Id2	Mark Israel	260	546-806
Ikaros	Katia Georgopoulos	1093	243-1336
Islet1	Tom Jessell	1510	31-1541
Lhx2	Juan Botas	585	1172-1757
Lhx6	Vassilis Pachnis	1342	1208-2550
Lmo1, human	Gordon Gill	469	61-530
Lmo3	TH Rabbitts	2000	full coding sequence
Lmo4	Gordon Gill	500	full coding sequence
Mash1	Francois Guillemot	2100	full coding sequence
Mef2c	Eric Olson	416	1-416
Meis1	Kenny Campbell	2300	full coding sequence
Meis2	Kenny Campbell	2400	full coding sequence
NHLH2	Debora Good	1900	full coding sequence
Nkx2.1	John Rubenstein	2200	full coding sequence
Nkx6.2	John Rubenstein	1300	1300 from 3′ end
Neuropilin2, NP2	M Tessier-Lavigne	1200	Amino Acids 536-911
Oct6; Pou3f1	M.G. Rosenfeld	2300	full coding sequence
Otp	Antonio Simeone	500	500 from 3′ end
Otx2	John Rubenstein	2100	2100 from 3′ end
Pax6	Peter Gruss	302	656-958
Pbx1	Heike Pöpperl	1294	358-1652
PK2	QY Zhou	509	1-509
PKR1	QY Zhou	975	975 from 3′ end
Preprotachykinin	Invitrogen	1034	Image Clone 1166182
RAR-beta	Kenny Campbell	2025	1036-3061
RDC1	ATCC MGC-18378	1897	63-1960
Robo1	M Tessier-Lavigne	1000	1000 from 3′ end
Robo2	M Tessier-Lavigne	1700	1700 from 3′ end
ROR-beta	Dennis O'Leary	2007	638-2645
RXR-gamma	Kenny Campbell	1226	907-2133
Sall3	P Monaghan	870	2883-3753
Sema3a	M Tessier-Lavigne	1182	1-1182
Six3	Peter Gruss	650	650 from 3′ end
Slit1	M Tessier-Lavigne	746	2353-3099
Sox1	R Lovell Badge	940	1851-2791
Sox11	John Rubenstein	2592	2592 from 3′ end
Sox4	John Rubenstein	1900	1900 from 3′ end
Sp8	Kenny Campbell	880	1622-2502

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Plasmid Name	Source	Insert Size in Base Pairs	cDNA Nucleotides of Probe or Nucleotides from 3′ End
Sp9	Kenny Campbell	2400	full coding sequence
Tbr1	John Rubenstein	264	264 from 3′ end
Tiam2	RZPD; ImaGenes	5148	full coding sequence
Tle4	Johan Ericson	2300	full coding sequence
Tlx	Paula Monaghan	1700	1700 from 3′ end
TrkB	FR Klein	487	1270-1757
Tsh1	X. Caubit	827	2844-3671
Tsh2	X. Caubit	1152	1-1152
Vax1	Peter Gruss	950	950 from 3′ end
VGAT	Brian Condie	730	1295-2025
Vglut2	Robert Edwards	305	305 from 3′ end
Zic1	Jun Aruga	333	333 from 3′ end