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Defective neuronogenesis in the absence of Dlx5

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Dlx genes play an important role in the control of the development of the central nervous system (CNS). Single or compound inactivation of Dlx1, Dlx2, or Dlx5 in the mouse causes defects of neuronal migration and differentiation. Dlx5, in particular, is essential for the correct development of the olfactory system. Targeted inactivation of Dlx1 and Dlx2 in the mouse results in abnormal neuronal differentiation in the embryonic subcortical forebrain and is associated to the loss of Dlx5 and Dlx6 expression. So far, however, it has been impossible to investigate the role of Dlx genes on late neurogenesis, as their inactivation leads to perinatal death. We have now generated cultures of neural stem cells (NSCs) derived from embryonic and newborn Dlx5-null mice, and we have compared their capacity to differentiate in vitro to that of equivalent cells derived from normal littermates. We show here that in the absence of Dlx5, NSCs derived from newborn animals have a severely reduced capacity to generate neurons. This is not the case for cells derived from E12.5 embryos. Forced expression of Dlx5 in cultures of newborn mutant NSCs fully restores their neuronogenic potential. Our data suggest that Dlx5 is essential for secondary (postnatal) neuronogenesis. © 2003 Elsevier Inc. All rights reserved.

Introduction

The fate of neural progenitor cells (NPCs) results from the integration of multiple environmental signals with intrinsic genetic programs (Okano, 2002). Some of these molecular mechanisms, such as, for example, those underlying the generation of spinal motorneurons (Wichterle et al., 2002) or of dopaminergic mesen-

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cephalic neurons (Wagner et al., 1999), are beginning to be revealed (Schuurmans and Guillemot, 2002). Despite these important results, most of the intrinsic and extrinsic determinants of cell fate in the developing central nervous system (CNS) remain to be elucidated. In this respect, it is essential to identify which genes are involved in the initiation of neuronal differentiation of uncommitted NPCs (see for example Nieto et al., 2001; Sun et al., 2001). One approach is to analyze the properties of NPCs derived from mice carrying targeted mutations of genes potentially important for the control of neural differentiation.

Dlx genes encode for homeobox-containing transcription factors related to *Drosophila Distal-less (Dll)*. In mammals, there are six *Dlx* genes arranged in three pairs of closely linked transcription units (Simeone et al., 1994; Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Robinson and Mahon, 1994; Quinn et al., 1997; McGuinness et al., 1996). *Dlx1, Dlx2, Dlx5*, and *Dlx6* mRNAs are expressed in the subcortical forebrain mainly in those territories where GABAergic differentiation occurs and in precursors of the GABAergic lineage (Panganiban and Rubenstein, 2002).

As a direct evidence of a role of *Dlx* genes in the initiation of the GABAergic differentiation program, exogenous expression of *Dlx2* or *Dlx5* in cortical neurons leads to the appearance of molecular markers specific for the GABAergic phenotype (Stuhmer et al., 2002). Furthermore, *Dlx1* and *Dlx2* genes are required for the differentiation of projection neurons in the basal ganglia since the maturation of striatal cells that synthesize GABA, acetylcholine, and dopamine is impaired in mice lacking both *Dlx1* and *Dlx2* (Anderson et al., 1997a; Marin et al., 2000; Yun et al., 2002). Notably in single *Dlx1-*, *Dlx2-*, or *Dlx5-*null mice, defects in basal ganglia formation have not been reported.

In the ganglionic eminences (GE), *Dlx* genes appear to be expressed in a hierarchical order of transcriptional regulation (Zerucha et al., 2000), which is reflected in a patterned expression in the neurogenic ventricular–subventricular zone (VZ–SVZ): *Dlx2* is found in very early progenitors, capable of initiating the neuronal, astrocytic, and oligodendrocytic lineages (He et al., 2001; Marshall and Goldman, 2002), while *Dlx1*, *Dlx5*, and *Dlx6* are found in progressively restricted subset of *Dlx2*-positive cells.

Abbreviations: GE, ganglionic eminence; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NPC, neural progenitor cells; NSC, neural stem cells; OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone; VZ, ventricular zone.

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Fig. 1. Expression of *Dlx5* and *Dlx6* in the neonatal mouse brain. Coronal (frontal) sections were used for X-gal staining of the β – galactosidase reporter expressed in *lacZ*–*Dlx5*^{+/-} and *lacZ*–*Dlx5*^{-/-} mice (C, D, H, and J) for in situ hybridization with *Dlx6* probe (in E, F, and G) or for immunohistochemical staining (A, anti- β -galactosidase; B, anti-Emx2). In A and B, sections correspond to the area indicated in C. Note the high level of expression of *Dlx5* (A, C) but not of *Dlx6* (red asterisk in E) in a region just under the SVZ. Inactivation of *Dlx5* did not eliminate the population of cells in which the *Dlx5* and *Dlx6* promotor is active in the CP and in the RMS (C and D; F–J). Abbreviations: CP, caudate–putamen; RMS, rostral migratory stream (red arrows).



Fig. 2. *lacZ* expression in cultured NSCs. (Top) Undifferentiated NPCs after 3 h culture; β -gal staining is visible in a subpopulation of both *Dlx5^{+/-}* (B) and *Dlx5^{-/-}* (C) cells. (Bottom) NSCs after 24 h culture in differentiating conditions, no staining is seen in astrocytes (black arrows) independently from their genotype. In cultures of *Dlx5^{+/-}* cells, expression of the *lacZ*-*Dlx5* allele is clearly visible in differentiated neurons (arrowheads in E) that can be recognized by their distinctive morphology. No neuronal differentiation and not β – gal positive cells could be found after 24 h in cultures of *Dlx5^{-/-}* NSCs (F). The weak blue staining seen in F is an artefact due to the conditions of illumination of the sample.



Fig. 3. Defective neurogenesis in NPCs from P0 brains. Micrographs of cultured NPCs after 7 days of differentiation, stained with anti-GFAP (red), and TuJ1 anti- β -tubulin (green). NPCs obtained from E12.5 (top) and P0 (bottom) animals are compared. The genotype at the *Dlx5* locus is indicated on the top right of each panel. Inserts in A and B show an example of differentiated neuron (TuJ1 positive cells) from the corresponding culture. Note the strongly reduced number of TuJ1-positive cells in cultures of *Dlx5^{-/-}* neonatal NSCs.

Thus, in the VZ–SVZ of the embryonic GE Dlx gene expression follows a temporal, positional, and functional sequence: Dlx2, Dlx1, and Dlx5, then Dlx6 (Porteus et al., 1994; Liu et al., 1997; Eisenstat et al., 1999; Panganiban and Rubenstein, 2002). In the embryonic GE, Dlx2 and Dlx1 are mainly found in neuroepithelial cells of the VZ, while Dlx5 is mostly expressed in cells of the SVZ, in cells of the rostral migratory stream (RMS), and of the olfactory bulb (OB) (Bulfone et al., 1993; Eisenstat et al., 1999; Levi et al., 2003). Whether this functional and histological organization of Dlxexpressing cells is retained in the neonatal SVZ is not entirely known, nor we have evidence of a function of Dlx genes during perinatal and postnatal neurogenesis in the SVZ-RMS-OB system.

The analysis of the brain phenotypes of mice with targeted disruption of individual Dlx genes ($Dlx1^{-/-}$, $Dlx2^{-/-}$, and $Dlx5^{-/-}$) or combinations of them ($Dlx1^{-/-}/Dlx2^{-/-}$) reveals partially overlapping functions of Dlx genes in the development of the basal forebrain. Dlx genes play fundamental roles in the control of migration of NPCs along the RMS toward the OBs, in the tangential migration from the GE into the dorsal cortex, and the differentiation of immature precursors into GABAergic neurons (Anderson et al., 1997b, 2001; Marin and Rubenstein, 2001; Panganiban and Rubenstein, 2002; Stuhmer et al., 2002; Levi et al., 2003). The brain of $Dlx1^{-/-}/Dlx2^{-/-}$ mice has been highly informative: in addition to a block in tangential migration from the GE to the cortex, these mice show a block in neurogenesis in the basal telencephalon and a block in the differentiation of GABAergic and dopaminergic interneurons in the OB and other brain areas (Marin et al., 2000; Marin and Rubenstein, 2001; Anderson et al., 1997a,b; Bulfone et al., 1998). Notably, both $Dlx2^{-/-}$ and $Dlx5^{-/-}$ mice show a reduction in the number of GABAergic and dopaminergic neurons in the OB (Qiu et al., 1995; Bulfone et al., 1998; Long et al., 2003; Levi et al., 2003).

In Dlx5-null mice (Acampora et al., 1999; Depew et al., 1999), a unique olfactory phenotype is observed, with defects affecting both the olfactory epithelium (OE) and the OBs. In these mice, we (Levi et al., 2003) and others (Long et al., 2003) have observed a profound disorganization of the OB cell layers and a severe impairment of dopaminergic and GABAergic interneuron differentiation. We could also demonstrate that the reduction of differentiated interneurons is a direct consequence of the mutation in a cell-autonomous fashion rather than the result of the lack of axonal connectivity between the OE and the OB (Levi et al., 2003). OB interneurons derive mainly from precursors that reside in VZ-SVZ of the forebrain, although neural stem cells (NSCs) have been isolated from all the rostral extension regions of the olfactory areas, including the OBs (Gritti et al., 2002). At later stages of development and in postnatal life, proliferating cells in the SVZ migrate rostrally along the RMS and home specifically to the OBs (Weiss et al., 1996; Lois and Alvarez-Buylla, 1994; Alvarez-Buylla et al., 2002; Alvarez-Buylla and Garcia-Verdugo, 2002). Based on these observations, we raise the hypothesis that the impaired OB interneuronal differentiation seen in Dlx5-null brains reflects an

intrinsic defect of differentiation of *Dlx5*-deficient NPCs. This would necessarily imply a function of *Dlx5* in the regulation of the intrinsic differentiation capacity of NPCs of the olfactory neurogenic system. Embryonic neurogenesis is severely impaired in the double *Dlx1–Dlx2*-null brains (Anderson et al., 1997a,b); this effect is associated to the loss of *Dlx5* expression via the *Dlx*-dependent sub-pallium-specific enhancer element in the intergenic *Dlx5–Dlx6* region (Zerucha et al., 2000). The disruption of *Dlx5*, in turn, leads to a less dramatic reduction of the progenitor cell compartment (Long et al., 2003).

The SVZ–OB system represents an ideal and well-characterized model to unravel mechanisms regulating the genesis, migration, and differentiation of NPCs in the postnatal mammalian brain (Peretto et al., 1999). However, efforts to define the role of *Dlx5* in the biology of NPCs have been hampered by the facts that (a) *Dlx* genes are expressed in CNS territories simultaneously involved in neurogenesis, migration, and differentiation, making in vivo histological analyses difficult, and (b) *Dlx5*-null mice die soon after birth, making the analysis of postnatal neurogenesis impossible.

In this study, we directly investigate growth and differentiation of $Dlx5^{-/-}$ NSCs by employing an in vitro approach based on neurosphere assay (Stemple and Anderson, 1992; Reynold and Weiss, 1992; Gritti et al., 1996, 1999). NSCs obtained from Dlx5null brains displayed a normal proliferation rate but exhibited a markedly reduced neuronal differentiation. Therefore, Dlx5 controls key steps necessary for neuronal differentiation of NSCs after cell proliferation.

Results

Expression of Dlx5 in the SVZ of the mouse brain

The pattern of expression of Dlx genes in the embryonic mouse brain has been reported (Bulfone et al., 1993; Simeone et al., 1994; Merlo et al., 2000; Panganiban and Rubenstein, 2002), but still little is known on the expression of Dlx5 and Dlx6 in the newborn and adult brain.

To study the pattern of expression of Dlx5 and Dlx6, we used the knock-in reporter *lacZ* as well as in situ hybridization; we have already shown that the expression of the lacZ reporter reproduces faithfully that of Dlx5 in lacZ-Dlx5 knock-in (Acampora et al., 1999), and we confirmed this observation in this study (data not shown). In the newborn brain, very high levels of expression of Dlx5, but not of Dlx6 (Figs. 1A and C) are observed adjacent to the lateral and ventral border of the telencephalic ventricles, a region corresponding to a subependymal layer where the presence of NPCs has been clearly documented. To clarify whether Dlx5expressing cells coincide with the NPCs, we stained adjacent sections with anti-Emx2, a marker of NPCs in the SVZ (Gangemi et al., 2001), and anti- β -galactosidase antibodies. The anti-Emx2 antibody stains a population of cells more adjacent to the ventricle with respect to the location of $Dlx5/\beta$ -gal positive cells. Although we cannot exclude that a fraction of NPCs are Dlx5 positive, the large majority of these cells are in a territory not compatible with an NPC identity where usually more differentiated cells are found.

No qualitative difference was observed in the distribution of β galactosidase positive cells, indicating that the cell lineage normally expressing *Dlx5* is not lost in the mutant animal. Analysis of lacZ-Dlx5 and Dlx6 positive cells along the RMS of normal and $Dlx5^{-/-}$ brains (Figs. 1G–J) indicates that the cell lineage normally expressing Dlx5 and Dlx6 is still present in the absence of Dlx5 (see also Long et al., 2003).

Expression of Dlx5 in cultured NSCs

The distribution of Dlx5 in the SVZ and the defective differentiation and lamination of the OB in $Dlx5^{-/-}$ mice could suggest that Dlx5 plays a role in the differentiation of NPCs. First, we examined if Dlx5 is expressed in cultured NSCs obtained from the SVZ of wild-type, $Dlx5^{+/-}$, or $Dlx5^{-/-}$ newborn brains. NSCs were maintained in culture as neurospheres under non-differentiating conditions (Gritti et al., 1996). We could not detect any significant difference either in the number, in the appearance, or in the self-renewal capacity of neurospheres generated from Dlx5-null mice compared to those from normal (wild type or $Dlx5^{+/-}$) brains. Similarly, neurosphere formation was unaffected when cells were derived from E12.5 brains.

Neurospheres were then dissociated, the resulting cell suspensions were plated, and the adhering cells were stained for the expression of *lacZ* before or after induction of differentiation. Few hours after plating, 50-60% of the undifferentiated $Dlx5^{+/-}$ or $Dlx5^{-/-}$ cells were positive (Figs. 2A–C), indicating that the Dlx5promoter is active. When lacZ expression was analyzed 24 h after induction of differentiation, the situation changed radically. We never observed lacZ expression in cell characterized by an astrocyte-like morphology (independently of their genotype) (Figs. 2E and F, black arrows). Cells with a phenotype characterized by long bipolar processes reminiscent of early neurons were clearly visible in cultures of $Dlx5^{+/-}$ NSCs (indicated by arrowheads in Figs. 2D and E), these cells express the *lacZ* reporter in $Dlx5^{+/-}$ cultures. We did not observe any of these cells in cultures of $Dlx5^{-/-}$ cells after 1 day of differentiation. It must be pointed out, however, that at these early stages of differentiation in culture, none of the classical "neuronal" marker is yet expressed; these markers appear much later during differentiation. It is therefore difficult to call these cells "neurons" as they are not yet completely differentiated. This observation and the defects in OB formation observed in vivo in $Dlx5^{-/-}$ mice (Long et al., 2003; Levi et al., 2003) suggested that the lack of Dlx5 impairs the proliferation and/or the proper neuronal differentiation of NSCs.

Proliferation and differentiation of cultured NPCs

Cell proliferation was assayed by incorporation of tritiated thymidine on culture neurospheres obtained from normal and $Dlx5^{-/-}$ brains. The same experiment was carried out on cells derived from either newborn or E12.5 brains. We did not observe any significant difference in the rate of thymidine incorporation of NSCs from normal, $Dlx5^{+/-}$, and $Dlx5^{-/-}$ animals of the same gestational age (either E12.5 or P0), suggesting that Dlx5 does not play a major role in the control of NSCs proliferation (data not shown).

Single cell suspensions derived from neurospheres of normal, $Dlx5^{+/-}$, and $Dlx^{-/-}$ mutant mice at E12.5 and at birth were replated in differentiating medium for 7 days. Under these conditions, wild type NSCs differentiate and cause astrocytes, oligo-dendrocytes, and neurons in a reproducible distribution. To identify and quantify these three cell populations, differentiated cultures



Fig. 4. Efficiency of neuronal differentiation in cultures of Dlx mutant NPCs. Cells obtained from embryonic brain (E12.5, left) and neonatal brains (P0, right) are compared. In both cases, the percentage of Tuj1-positive cells over the total number of cells examined is shown for the three Dlx5 genotypes (indicated on the bottom). The means (six independent experiments per point) and standard deviations are reported. Cultures of neonatal $Dlx5^{-/-}$ NSCs generate a significantly (double asterisk = P < 0.002) lower number of neurons.

were stained with antibodies that recognize population-specific markers (galactocerebrosidase for oligodendrocytes; β-tubulin for neurons; glial fibrillary acidic protein for astrocytes) (Fig. 3). To estimate the percentage of each cell type present, a minimum of 4,000 cells were counted in each individual experiment. The pattern of differentiation of cultures derived from $Dlx5^{+/-}$ brains did not differ significantly from that of wild type cells at any gestational age. In all experiments, a very low percentage of oligodendrocytes (1-2%) was found. This makes a quantitative comparison rather difficult. In spite of this, we could not observe any significant disturbance of the differentiation rate of this cell type. The absence of *Dlx5* was associated with a significant and consistent reduction of the percentage of neurons formed in cultures of newborn $Dlx^{-/-}$ NSCs (Figs. 3 and 4). Fig. 4 reports as an histogram the percentage of neurons obtained from differentiating normal and mutant NSCs.

The reduced number of neurons observed in the absence of Dlx5 could be explained either by impaired ability of NSCs to differentiate or by the activation of a cell death program after the initiation of differentiation. If the latter explanation were true, one should expect a higher number of neurons at early stages of differentiation, which would subsequently decline with time. To rule out this possibility, we examined differentiation following short induction times. No obvious neuronal differentiation could be observed at Day 1 (Fig. 3E and data not shown), and the number of β-tubulin positive cells remained low when cells were examined at intermediate times (starting from Day 3 when the first cells displaying this differentiation marker begin to appear). Moreover, we also examined apoptotic cell death either by estimating the number of morphologically apoptotic nuclei or by staining the cells with an antibody that reacts with activated caspase-3 (data not shown). No increased apoptotic cell death could be observed in $Dlx5^{-/-}$ NSCs cultures compared to the control cells.

When the same in vitro differentiation experiments were performed with NSCs derived from $Dlx5^{+/-}$ or $Dlx^{-/-}$ E12.5 embryos, we did not observe any significant difference in the

number of differentiated β -tubulin-immunoreactive cells or of any other cell type in the absence of *Dlx5* (Figs. 3 and 4).

Dlx5 transfection restores normal differentiation of Dlx5^{-/-} NSCs

To confirm that the reduced capacity to differentiate into neurons of newborn $Dlx5^{-/-}$ NSCs was a consequence of the absence of the gene, we evaluated if forced expression of Dlx5could rescue the neuronal differentiation pathway. NSCs derived from $Dlx5^{-/-}$ mice at birth were transduced in vitro with the pLXIN/Dlx5 retroviral vector (see Experimental Methods) and selected for 7 days in G418-containing medium. As a control, $Dlx5^{-/-}$ NSCs were mock transduced with an empty pLXIN retroviral vector and similarly selected. The transduced cells were then assayed by RT-PCR for Dlx5 mRNA expression. As shown in Fig. 5, a PCR product corresponding to Dlx5 mRNA was only present in transduced NSCs.

Mock transduced cells, nontransduced cells, and cells transduced with Dlx5 were then plated in the differentiating medium along with control cells, as described above. The relative number of the three different cell lineages was assessed by immunostaining as indicated above. Transfection of exogenous Dlx5 in $Dlx5^{-/-}$



Fig. 5. Transduction with wild-type *Dlx5* cDNA restores neuronal differentiation in *Dlx5^{-/-}* NPCs. Top: RT-PCR for the detection of murine *Dlx5* and β -actin mRNAs in samples from wild-type (left), *Dlx5^{-/-}* NSCs transduced with empty vector (middle), and *Dlx5^{-/-}* NSCs transduced with *pLXIN/Dlx5*. Bottom: Efficiency of neuronal differentiation in vitro of wild-type (left), *Dlx5^{-/-}* NSCs transduced with *pLXIN/Dlx5*. The percentage of Tuj1-positive cells over the total number of cells examined is shown. The means (three independent experiments per point) and standard deviations are reported. Cultures of neuronal *Dlx5^{-/-}* NSCs transduced with empty vector generate a significantly (*P* < 0.02) lower number of neurons compared both to wild type and *pLXIN/Dlx5*-transduced mutant cultures. The difference in neurogenic potential between wild type NSCs and *pLXIN/Dlx5*-transduced mutant NSCs is not statistically significant.

NSCs restored the capacity of these cells to produce β -tubulin positive neurons (Fig. 5), indicating that the absence of this gene is directly associated with the reduced neuronogenic potential of NSCs.

Discussion

Mammalian brain development and patterning depend on the concerted proliferation, migration, differentiation, and death of NPCs (Gage, 2000; Van der Kooy and Weiss, 2000; Anderson, 2001) and in the establishment and selection of appropriate connectivity. All these processes are under the control of gene regulatory networks that are beginning to be unravelled thanks to the generation of targeted mutations in vivo. Indeed, targeted mutations of genes expressed in NPCs can profoundly subvert their migration and homing of to specific target territories (Marin et al., 2000; Marin and Rubenstein, 2001). In this study, we used cultures of NPCs to gain insight on the role of *Dlx5*, a member of the *Dlx* homeobox gene family on the neurogenic process. Our results indicate that *Dlx5* is essential for neuronal differentiation of perinatal, but not embryonic, NPCs.

Around E12.5 of mouse development migratory progenitors from the medial and lateral ganglionic eminence (MGE and LGE) express members of the Dlx gene family, including Dlx1 and Dlx2 (Anderson et al., 1997a,b) and Dlx5 (Porteus et al., 1994; Long et al., 2003; Levi et al., 2003), and contribute to the generation of GABAergic interneurons both in the cortex and in the OB. Dlx genes are expressed in a partially overlapping pattern: Dlx2 is expressed in cells throughout the VZ, while Dlx5 is expressed in cells of the SVZ and in committed cells of the mantle zone. Dlx2 expression begins before that of *Dlx1* and marks the earliest progenitors; instead expression of Dlx5 appears later in Dlx1and Dlx2-positive postmitotic neuronal progenitors. Dlx6 expression is minimal in the SVZ and appears strongly in the mantle zone of the GE (Eisenstat et al., 1999). Moreover, progenitors from the GE have been shown to have the potential to differentiate into neurons and glia in vitro (He et al., 2001), and Dlx2-positive migratory NPCs can differentiate into astrocytes and oligodendrocytes in the dorsal telencephalon in vivo (Marshall and Goldman, 2002). Hence, while Dlx2 is expressed in the most uncommitted NPCs in the GE (embryonic) and in the SVZ (neonatal), Dlx5 and Dlx6 are likely to be involved in later steps of NPCs maturation; however, there is no information on the postnatal function of Dlx5 in the differentiation of SVZ cells, as Dlx5-null mice die at birth. The culture assay employed here reveals that Dlx5 does not play any major role in the genesis of astrocytes and oligodendrocytes. This result is not entirely unexpected as only Dlx2-positive uncommitted progenitors from the GE have the potential to differentiate into neurons and glia (He et al., 2001; Marshall and Goldman, 2002) while Dlx5 marks more committed NPCs.

In the embryonic and neonatal mouse brain, Dlx5 is expressed in both the MGE and the LGE. There are significant differences in the fate of NPCs in these adjacent structures. The LGE hosts progenitors that either generate striatal projection neurons (Deacon et al., 1994; Olsson et al., 1995, 1997, 1998; Anderson et al., 1997a; Wichterle et al., 2001) or migrates along the RMS and contributes granule cells and interneurons to the OBs. The phenotype of mice with a combined loss-of-function mutation of Dlx1and Dlx2 is consistent with the presence of a common precursor for striatal projection neurons and OB interneurons in the LGE since differentiation defects are observed in both of these compartments. The MGE contributes to some precommitted interneuronal precursor populations that migrate tangentially to various areas of the dorsal telencephalon, hippocampus, and projection neurons in the basal ganglia (Anderson et al., 1997a, 2001; Ladvas et al., 1999; Sussel et al., 1999; Pleasure et al., 2000; Wichterle et al., 1999, 2001; Yun et al., 2002; reviewed in Marin and Rubenstein, 2001).

The most evident lesion observed in the $Dlx5^{-/-}$ brain is the overall size reduction of the OB (about 60-70% of its normal size) accompanied by an enlargement of its VZ-SVZ. This defect has been shown by examining the expression pattern of Dlx1, Dlx2, Dlx5^{lacZ}, and Dlx6 in the mutant OB (Long et al., 2003; Levi et al., 2003). This observation suggests an expansion of the NPCs and/or precommitted cells compartment linked to a reduction of the number of committed cells. In fact, a detailed analysis of OB differentiated interneurons indicates a severe reduction of both THand GAD67-positive cells. Our in vitro results indicate that Dlx5 does not have a major role in controlling NSCs proliferation. This is in line with in vivo studies on $Dlx5^{-/-}$ mice: BrdU incorporation analysis and in situ hybridization with Hes5 have not shown any significant difference in the rate of mitosis and in the size of the proliferative area of the VZ-SVZ in $Dlx5^{-/-}$ brains (Long et al., 2003; Levi et al., 2003). The fact that Dlx5 does not play any major role in control of NSCs cell cycle is also in line with the fact that expression of Dlx5 in the embryonic VZ-SVZ appears to be mostly confined to postmitotic precursors (Liu et al., 1997; Eisenstat et al., 1999; Stuhmer et al., 2002). Conversely, it is interesting to note that the absence of *Dlx5* does not increase the rate of cell death in the OB (Long et al., 2003; Merlo, unpublished observation). The question remains as to what is the cellular basis for the reduced size of the OB. Most likely, Dlx5 plays a significant role in the secondary neuronogenesis that occurs in the postnatal brain, while embryonal neuronogenesis is minimally or not affected. The results presented here, based on neurosphere assays, warrant a detailed in vivo analysis of mice bearing a conditional Dlx5 mutation when these will be available.

If proliferation and apoptosis are not affected, the simpler way to explain the severe reduction of the neurogenic capacity of perinatal $Dlx5^{-/-}$ NSCs is to assume that these cells have a preferential capacity to differentiate into glial cells and have lost, in great part, the capacity to differentiate into neurons. This is in agreement with two other findings. First, cultures of $Dlx5^{+/-}$ NSCs differentiate in neurons and glia, but Dlx5 is expressed only in differentiating neurons suggesting that this transcription factor is essential for the neuronal lineage. The second finding derives from our in vivo analyses of $Dlx5^{-/-}$ olfactory system: in the $Dlx5^{-}$ mice, the axons of OE receptor neurons are present but fail to produce functional connections with the OBs. This defect appears to be intrinsic to the OE (Levi et al., 2003). Conversely, in the OB, immature interneurons fail to a large extent to express the differentiation markers TH or GAD67 (Long et al., 2003; Levi et al., 2003). On the basis of in vivo grafting experiments, we could also rule out the possibility that the lack of GABAergic and dopaminergic interneurons in the OB is a mere consequence of the lack of innervation (Levi et al., 2003). This led to the hypothesis that the absence of Dlx5 directly causes an intrinsic block in the ability of precursor cells to differentiate into neurons.

Lineage tracing using Dlx5/6-reporter transgenic mice have indicated that the SVZ of the LGE contains two distinct populations of progenitor cells, both expressing Dlx genes: one that expresses the LIM-homeobox protein Islet1 (ISL1) and one that

is negative for this protein but positive for the ETS transcription factor Er81 (Stenman et al., 2003). These two cell populations have a clearly distinct fate: the $Dlx^+/ISL1^+$ population composes mainly striatal progenitors, while the $Dlx^+/Er81^+$ cells contribute mainly to granule cells and interneurons of the OBs. It is interesting to note that defects in the striatal formation have not been reported in $Dlx5^{-/-}$ mice. In the Dlx5 mutant brain, cells in the striatum are properly organized and express major differentiation markers (Merlo, unpublished observation). This observation suggests that in spite of a much wider distribution in the LGE and MGE, Dlx5 controls the fate of only a subset of NPCs: those implicated in the development of the olfactory system. As the contribution of neurons to the OB from the SVZ via RMS and migration begins mostly perinatally, this could explain why, in our setting, we observe no significant difference in neuronal differentiation from embryonic NSCs.

Experimental methods

Generation of Dlx5 null mice

Mice with targeted disruption of Dlx5 have been reported (Acampora et al., 1999). The targeted allele had the first and second exons of the gene replaced with the *lacZ* reporter. The modified allele, denominated $Dlx5^{lacZ}$, allows for easy detection of the Dlx5-expressing cell lineage. β -galactosidase expression in $Dlx5^{+/-}$ embryos recapitulates the known Dlx5 expression pattern (Simeone et al., 1994; Merlo et al., 2000). Genotypes were determined by PCR amplification of the wild-type and mutant alleles, as described (Acampora et al., 1999).

Brain histology and histochemistry

Embryos up to E14.5 were immersion fixed in 4% PAF in 0.1 M phosphate buffer (PB, pH 7.4) for 6 h, washed in PBS, and either paraffin embedded or cryprotected (20% sucrose). Animal E16.5 or older were perfused with 4% PAF, the brain was dissected, fixed for additional 2–3 h in 4% PAF, then paraffin embedded. For vibratome sectioning, brains were dissected following perfusion, postfixed for 30 min, and sectioned at 100 μ M. For the detection of Emx2 protein in histology sections, brains were perfused with NaCl 0.9%, dissected, snap frozen in isopenthane/liquid N₂ for 20–30 s, then transferred in methanol at -70° and stored for 30 days at -70° . The brains were then transferred in 100% ethanol and paraffin embedded.

β-gal was detected by X-gal staining of either whole-mount embryos or vibrating microtome sections by incubating the samples for 16–24 h at 32°, as described (Acampora et al., 1999). Stained sections were fixed in PAF 4% for 2–4 h before mounting and microscopic examination. Immunohistochemistry was carried out in Tris buffer saline using standard protocols. The following primary antibodies were used: rabbit anti-β-gal (1:5000; ICN Biomedical), rabbit anticalretinin (1:1000; Chemicon), rabbit antityrosine hydroxylase (TH; 1:2000; Chemicon), rabbit antiglutamic acid decarboxylase 67 kDa (GAD67; 1:2000: Chemicon), and rabbit anti-Emx1 and anti-Emx2 (1:500). Rabbit and goat antisera were revealed with goat antirabbit (EnVision+, Dako) and rabbit antigoat (Rockland) peroxidase-conjugated secondary antibodies, respectively. Mouse monoclonal antibodies were revealed with the ARK kit (Dako) according to the manufacturer's specifications. Peroxidase was developed with DAB (Dako).

Probes and in situ hybridization

In vitro transcribed (Promega) DIG-labeled *Dlx5* cRNA probe was obtained from the entire murine cDNA (*Dlx5*), while the *Dlx6* probe is obtained from a partial cDNA fragment of 260 bp comprising exons III and IV. These probes specifically recognize the correct mRNA on Northern blot; in situ hybridization of the *Dlx5* probe on *Dlx5* null embryo yielded negligible background. Hybridization was carried out on 100-µm thick vibratome sections of newborn brains perfused with PAF 4% according to the published procedures (Wilkinson, 1992). Prehybridization and hybridization were done in 50% formamide, 5× SSC pH 4.5, 1% SDS, 50 µg/ml heparin, 50 µg/ml of yeast tRNA, and 1 µg/ml of DIG-labeled probe at 70°C. Signal was detected with APconjugated anti-DIG Fab fragments (1:5000; Roche-Boehringer) followed by color development with NBT/BCIP. Three brains of each genotype were analyzed.

Primary cultures of NSCs

Primary cultures of NSCs were established as described (Gangemi et al., 2001; Gritti et al., 1996, 1999). Briefly, brains of PO normal, $Dlx5^{+/-}$, and $Dlx5^{-/-}$ mice were microdissected to obtain the rostral periventricular region after coronal sections; brains of E12.5 mice were microdissected to obtain the whole telencephalic vesicle. The tissue was dissociated with 0.1% trypsin in phosphate-buffered saline (PBS) for 10 min at 37°C in the presence of 10 mM glucose. After centrifugation, cells were carefully dissociated and resuspended in serum-free NSA medium (Euroclone) supplemented with N2 (Gibco/BRL), 2 mM glutamine, and 20 ng/ ml EGF and 10 ng/ml bFGF (Peprotech). Neurospheres were mechanically dissociated every 3 days and replated in tissue culture flasks. To assess self-renewal, primary floating neurosphere colonies were subcloned by mechanically dissociating a single neurosphere colony in 0.2 ml of serum-free medium containing FGF2, heparin, and EGF and plated in uncoated 96well (0.2 ml/well) plates (Nunclon). Stem-cell self-renewal was assessed by identifying new neurospheres after a further 7 days in vitro.

For the examination of differentiation properties, dissociated neurospheres were plated at 3×10^4 cells/well in 24-well plates (Costar) on matrigel-coated glass coverslips (Becton Dickinson). After adhesion, cells were cultured for 5 days in serum-free medium containing bFGF and then in medium containing 1% serum for 3–7 days.

Immunocytochemistry on cultured NSCs

Cells were fixed with 4% PAF in PBS for 15 min at room temperature (RT), then incubated with the appropriate primary antibody for 1 h, washed twice with PBS, and incubated with the secondary antibody for 45 min. The following primary antibodies were used: monoclonal anti-galactocerebrosidase (Boehringer Mannheim) 1:10; monoclonal anti β -tubulin (Sigma) 1:100; rabbit anti cow glial fibrillary acidic protein (Dako) 1:300, and rabbit anticaspase-3 1:1000 (BD Pharmingen). Secondary anti-

bodies were: goat antimouse IgG (H + L) FITC and goat antirabbit TRITC (Jackson Immunoresearch) used at 1:200 and 1:250, respectively. For β -gal staining, cells were fixed in glutaraldehyde 0.5% for 15 min, rinsed with PBS, and stained as above.

Estimation of NSCs proliferation in culture

The number of cells in S-phase of the cell cycle was determined by thymidine incorporation. Ten thousand cells/well were plated on flat bottom 96-well plates in quadruplicate and grown for 24, 48, and 72 h. ³H-metyl-thymidine, 1 μ Ci/well (Amersham) was added 12 h before harvesting. Cells were lysed at the desired time, blotted on filter paper, and following addition of 5 ml of scintillation fluid, ³H emission was measured in a β -counter.

Retroviral vector construction and infection

A bicistronic retroviral vector (pLXIN2, Clontech) was constructed with the complete *Dlx5* cDNA and the *neo* gene under the control of the viral LTR (pLXIN/*Dlx5*). The same bicistronic vector with the *neo* gene alone was used as control. Retroviral plasmids were used to transfect the Phoenix packaging cell line. The retroviral titer was evaluated on 3T3 cells and ranged from $5 \times$ 105 to 1×106 colony forming unit (CFUs). The efficiency of NSC infection ranged from 40% to 50%. The infection was repeated twice for 8 h, then NP cells were selected for 7 days with G418. Expression of exogenous *Dlx5* mRNA in the transduced cells was tested by RT-PCR. Total RNA was prepared from normal and *Dlx5^{-/-}* newborn mice and from pLXIN/*Dlx5*-infected NSCs using a commercial kit (RNeasy, Qiagen). PCR amplification of *Dlx5* was performed for 35 cycles with the following 5' and 3' oligonucleotides:

5' AGAGTCCCAAGCATCCGATCC 3' 5' CCAGCACAACACTGTAGTCCC 3'

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