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## ***Dlx* transcription factors promote migration through repression of axon and dendrite growth**

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### **Abstract**

In the mouse telencephalon, *Dlx* homeobox transcription factors are essential for the tangential migration of subpallial-derived GABAergic interneurons to neocortex. However, the mechanisms underlying this process are poorly understood. Here, we demonstrate that *Dlx1&2* have a central role in restraining neurite growth of subpallial-derived immature interneurons at a stage when they migrate tangentially to cortex. In *Dlx1<sup>-/-</sup>;Dlx2<sup>-/-</sup>* mutants, neurite length is increased and cells fail to migrate. In *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mutants, while the tangential migration of immature interneurons appears normal, they develop dendritic and axonal processes with increased length and decreased branching, and have deficits in their neocortical laminar positions. Thus, *Dlx1&2* are required for coordinating programs of neurite maturation and migration. In this regard, we provide genetic evidence that in immature interneurons *Dlx1&2* repression of the p21-activated kinase PAK3, a downstream effector of the Rho-family of GTPases, is critical in restraining neurite growth and promoting tangential migration.

### **Keywords**

neuronal differentiation; cortical development; cytoskeleton; medial ganglionic eminence

## **INTRODUCTION**

The cerebral cortex consists of two main neuronal classes, pyramidal neurons (projection neurons) and interneurons (local-circuit neurons). They differ in their embryonic origin, migration properties, cell morphology, and neurotransmitter production. Pyramidal neurons are generated in the proliferative zones of the pallium from progenitors expressing the transcription factors *Emx1*, *Neurogenin1* and *2*, *Pax6*, and *Tlx1* and *2*, and migrate radially into the cortical plate (Guillemot *et al.*, 2006). Substantial progress has been made in identifying the mechanisms that control radial migration and differentiation of pyramidal cells (Kriegstein and Noctor, 2004; Bielas *et al.*, 2004; Guillemot *et al.*, 2006). However, relatively less is known about the cellular and molecular mechanisms that control the

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migration and differentiation of cortical GABAergic interneurons. Cortical interneurons are born in the proliferative zones of the subpallium (ventral telencephalon) from progenitors expressing transcription factors including *Nkx2.1*, *Mash1*, *Dlx1* and *2*, and *Lhx6* (Campbell, 2003; Marin and Rubenstein, 2003; Guillemot, 2005). Postmitotic immature interneurons exit the subpallial subventricular zone (SVZ) and take tangential migratory routes to enter the cortex. Within the cortex, they change their mode of migration from tangential to radial to invade the cortical plate and acquire their laminar positions, where they differentiate into a variety of interneuron cell types (Marin and Rubenstein, 2003; Metin *et al.*, 2006; Wonders and Anderson, 2006; Ayala *et al.*, 2007). Thus, postmitotic subpallial-derived interneurons must delay their maturation while they are migrating to their final cortical positions. The mechanisms that coordinate their migration and neuronal maturation are unknown.

*Dlx* homeobox genes have major roles in regulating GABAergic differentiation in the forebrain (Panganiban and Rubenstein, 2002). Analysis of *Dlx* loss-of-function mouse mutants has demonstrated that these genes regulate the production of GABAergic neurons from progenitors located in the SVZ (Anderson *et al.*, 1997a,b; Marin *et al.*, 2000; Anderson *et al.*, 2001; Yun *et al.*, 2002). In *Dlx1<sup>-/-</sup>;Dlx2<sup>-/-</sup>* double mutants (*Dlx1/2<sup>-/-</sup>*), tangential migration of GABAergic cortical interneurons from the subpallial SVZ to neocortex and hippocampus is nearly abolished. As a result, interneuron precursors accumulate in the SVZ of the medial and caudal ganglionic eminences (MGE, CGE) (Anderson *et al.*, 1997a). These findings raised the question of whether *Dlx* genes simply regulate cell motility or have further functions in neuronal differentiation. Expression data is consistent with additional functions of *Dlx* genes in neuronal differentiation: cortical interneurons express *Dlx1&2* genes during all developmental stages and into adulthood, with robust expression of *Dlx1&2* in SVZ, high expression of *Dlx1&2* in migrating cells, and moderate expression of *Dlx1* and low expression of *Dlx2* in post-migratory cells (Liu *et al.*, 1997; Yun *et al.*, 2002; Cobos *et al.*, 2005, 2006). Moreover, recent analysis of *Dlx1* single knockouts showed that *Dlx1* cell autonomously regulates dendrite maturation of at least a subset of cortical interneurons (Cobos *et al.*, 2005).

Here, we investigate *Dlx1&2* functions in regulating migration and neuronal differentiation of cortical interneurons. Using *Dlx1/2<sup>-/-</sup>* double mutants, we demonstrate that *Dlx* genes regulate the growth of neuronal processes of immature presumptive GABAergic interneurons. Mutant interneurons exhibited longer neurites. In *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* compound mutants tangential migration takes place, but immature neocortical interneurons show defects in the acquisition of their final laminar positions, and have dendritic and axonal processes with increased length and decreased branching. To dissect the molecular pathways underlying these defects, we used microarray analysis comparing gene expression of *Dlx1/2<sup>-/-</sup>* and wild-type MGE-derived cells. We focused on PAK3, a member of the p21-activated serine/threonine kinases (PAKs) family. PAK kinases are major downstream effectors of the Rho-family GTPases Rac1 and Cdc42 (Bokoc, 2003; Hofmann *et al.*, 2004; Kumar *et al.*, 2006). Rho GTPases integrate different extracellular and intracellular signals to orchestrate the changes in the cytoskeleton that are ultimately responsible for cell motility, neurite outgrowth, and axon and dendrite guidance (Luo, 2000; Govek *et al.*, 2005). We show that in wild-type embryos, PAK3 expression is low in migrating interneurons, and is up-regulated once they have arrived to cortex. We provide genetic evidence that PAK3

repression by *Dlx1&2* in MGE-derived immature interneurons is critical in restraining the growth of their neurites and promoting their tangential migration.

## RESULTS

### Interneuron precursors from *Dlx1/2*<sup>-/-</sup> double mutants have longer neuronal processes

Precursors of mouse cortical GABAergic neurons are generated in the proliferative zones (VZ/SVZ) of the subpallium from where they migrate tangentially to cortex. Lack of *Dlx1/2* function produces a cell-autonomous block in this migration that results in accumulation of interneuron precursors in the subpallial SVZ (Anderson *et al.*, 1997a; 2001; Marin *et al.*, 2000). The *Dlx1/2*<sup>-/-</sup> SVZ contains both proliferating cells and cells that express markers of differentiating neurons, such as microtubule associated protein-2 (MAP2). These cells extend neurites when dissociated and grown *in vitro* (Anderson *et al.*, 1997b). These observations show that *Dlx1/2* function is required for initiating tangential migration of interneurons, but is not required for neurogenesis or for initiating neurite outgrowth. To characterize the ability of interneuron precursors lacking *Dlx1/2* to extend and branch their neurites, we analyzed the morphology of individual MGE-derived neurons using an *in vitro* cell transplantation assay (Xu *et al.*, 2004). Donor cells were dissociated from the MGE and plated at low density over high-density feeder cells prepared from P0 wild-type cortices. The experiment was performed using E15.5 MGE, an age when it is primarily producing cells that will become cortical interneurons. Green fluorescent protein (GFP) expression was used to visualize the morphology of donor cells using the *Lhx6*-GFP BAC transgene (<http://www.gensat.org>). This transgene is a good reporter for cortical interneurons in our experimental paradigm because: i) *Lhx6* is expressed by a large number of GABAergic neurons in embryonic and postnatal cortex (Cobos *et al.*, 2006; Liodis *et al.*, 2007); ii) nearly all *Lhx6*-positive neurons migrating throughout the embryonic cerebral cortex are *Dlx*-positive (Figure S1 in Supplemental data); and iii) expression levels of *Lhx6* mRNA in the telencephalon are not greatly changed in *Dlx1/2* mutants (Cobos *et al.*, 2005).

First, we analyzed neurite outgrowth in GFP-expressing interneurons between 1 and 3 days *in vitro* (DIV), from cultures prepared with low numbers of E15.5 donor cells (1%). We obtained confocal images of randomly selected GFP-expressing cells, and measured in each cell the number of neurites, total neurite length (TNL), average neurite length (ANL), and length of longest neurite (LN). Our analysis showed that, by 3 DIV, neurite length (TNL, ANL and LN) had significantly increased in mutant cells as compared to control cells (TNL: ~45%; ANL: ~35%; LN: ~65%), whereas the number of neurites was similar in mutants and controls (Figure 1A–D). We performed a similar analysis using E12.5 MGE donor cells, when cortical interneurons are beginning to migrate from MGE, and found results that were similar to those obtained with E15.5 MGE donors (Figure S2 in Supplementary data). Thus, our *in vitro* analysis showed that MGE-derived neurons from *Dlx1/2*<sup>-/-</sup> mutants develop longer neurites.

Next, we analyzed E15.5 cultures grown *in vitro* for 5 days. At this stage we were able to distinguish reliably axons and dendrites in both wild-type and mutant neurons, based on the more segregated expression of Tau1 into axons and MAP2 into nascent dendrites (Dehmelt and Halpain, 2005). Analysis of dendrite morphogenesis showed increased (~30%) average

dendrite length in mutant cells compared to controls, and similar number of dendritic branches in both genotypes. Analysis of axon morphogenesis showed increased (~75%) average axon length and reduced (~40%) number of axonal branches in mutant cells (Figure 1E–H). Thus, *Dlx1/2*<sup>-/-</sup> immature interneurons developed longer dendrites, and axons that were longer and had fewer branches.

### Interneuron precursors from *Dlx1/2*<sup>-/-</sup> undergo apoptosis

Previous studies have shown that the *Dlx* genes are required for cell survival (De Melo *et al.*, 2004; Cobos *et al.*, 2005). In our *in vitro* cell transplantation assay using MGE-derived interneuron precursors, there was a dramatic reduction in numbers of GFP-expressing *Dlx1/2*<sup>-/-</sup> cells after 5DIV; those remaining frequently had shrunken cell bodies with retracting processes and pyknotic nuclei. Quantification of numbers of GFP<sup>+</sup> cells revealed that the number of *Dlx1/2*<sup>-/-</sup> mutant cells decreased to 80% by 5 DIV, and were further decreased to 7% by 10 DIV (Figure S3 in Supplemental data). To directly assess apoptosis, we used TUNEL staining and anti-active caspase-3 immunocytochemistry. Whereas double TUNEL<sup>+</sup>/GFP<sup>+</sup> and double caspase3<sup>+</sup>/GFP<sup>+</sup> cells were rarely observed in control cultures, they were increased ~10-fold in mutants at the time when there was a reduction in GFP<sup>+</sup> cells. Furthermore, TUNEL assay of sections from *Dlx1/2*<sup>-/-</sup> neonatal brains showed a robust increase in the number of TUNEL<sup>+</sup> cells in the basal ganglia (SVZ and mantle) (Figure S3). Thus, our results support a requirement of *Dlx1/2* for the survival of MGE-derived GABAergic neurons.

### Interneurons from *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> mutants tangentially migrate to cortex but show defects in dendritic and axonal growth

Development of the forebrain, branchial arches and limbs are sensitive to *Dlx* dosage (Depew *et al.*, 2005). Because postnatal *Dlx1*<sup>-/-</sup> mice have mild defects in dendrite morphogenesis in a subset of cortical interneurons (Cobos *et al.*, 2005), we tested whether *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> compound mutants exhibited a phenotype intermediate to the single and double *Dlx* mutants in interneuron differentiation.

*Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> compound mutant pups died shortly after birth, probably because they have cleft palate. To first verify that *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> embryos are hypomorphic relative to *Dlx1/2*<sup>-/-</sup>, we studied key phenotypic features of the double null mutants, i.e., disruption of development of the striatal SVZ, reduced production of striatal projection neurons, and reduced migration of interneurons to olfactory bulb and to cerebral cortex (Anderson *et al.*, 1997a,b; Marin *et al.*, 2000; Yun *et al.*, 2002). Consistent with a disruption of striatal SVZ differentiation through altered Notch signaling (Yun *et al.*, 2002), E13.5 compound heterozygous embryos showed moderate increases in mRNA expression of the Notch ligand *Delta-like1 (Dll1)*, the transcription factor *Hes5*, and the bHLH transcription factor *Mash1*. As a result, the striatal mantle of E17.5 embryos was smaller (Figure S4 in Supplemental data). Consistent with defects in migration of interneurons to olfactory bulb, we detected reduced numbers of cells expressing several interneuron markers (i.e., *GAD67*, *ER81*, *Arx*, and *Reelin*) in the granular and periglomerular layers. Finally, we found that cortical interneuron precursors were able to migrate to cerebral cortex, based on expression of *GAD67* and other interneuron markers (Figures S4 and S5 in Supplemental data). Thus, the

*Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* compound mutants exhibit a hypomorphic phenotype relative to *Dlx1/2<sup>-/-</sup>* double mutants, but maintain tangential migration.

To compare the effects of the *Dlx1<sup>-/-</sup>*, *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>*, and *Dlx1/2<sup>-/-</sup>* mutations on interneuron morphology, we used our *in vitro* cell transplantation assay (Figure 1A). E15.5 MGE donors from all genotypes were analyzed (all were *Lhx6-GFP<sup>+</sup>*). This analysis showed that *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* cells, by 3 DIV, had increased neurite length (TNL, ANL and LN) as compared to control cells (TNL: ~25%; ANL: ~25%; LN: ~40%), and similar number of neurites (Figure 1D). By 5 DIV, the average length of axons was increased (~50%) (Figure 1G,H). These defects were similar but milder than in *Dlx1/2<sup>-/-</sup>* cells. By contrast, *Dlx1<sup>-/-</sup>* cells did not show significant differences in dendrite or axon morphogenesis (Figure 1D,G,H).

We next prepared dissociated neuronal cultures from P0 cortices (*Lhx6-GFP<sup>+</sup>*) of *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mutants and littermate controls (Figure 2A). At 1–3 DIV, *GFP<sup>+</sup>* cells from *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* cortices showed increased (~20%) average neurite length and increased (~30%) length of their longest neurite, as compared with controls (data not shown). At 5 DIV, the *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mutant cells had reduced numbers of both dendritic and axonal branches (~25%) (Figure 2B–E). Thus, *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* cells showed defects in neurite growth (i.e., increased length and decreased branching) that were similar but milder than *Dlx1/2<sup>-/-</sup>* cells. Together, these results show that *Dlx* genes restrain neurite growth in immature interneurons, and additionally control their axonal and dendritic growth at later stages of maturation in the neocortex.

### Interneurons from *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mutants show defects in neocortical layer acquisition

At perinatal stages, neocortical interneurons radially migrate “inward” from the marginal zone (MZ) to the cortical plate, and “outward” from the VZ/SVZ and intermediate zone (IZ) to the cortical plate, to adopt their laminar identities (Nadarajah *et al.*, 2002; Tanaka *et al.*, 2003; Hevner *et al.*, 2004). We tested whether *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mutation affected the ability of neocortical interneurons to migrate to their laminar positions. First, we analyzed the expression of interneuron markers in P0 cortices (Figure 3A–D). We observed that, although the total numbers of *GAD67<sup>+</sup>* neurons in neocortex was normal, there were increased numbers of *GAD67<sup>+</sup>* neurons in the marginal and subventricular zones (MZ, SVZ; Figure 3 B–D, and Supplemental Figure 5). Next, we used the *Lhx6-GFP* BAC allele to visualize the interneurons by GFP immunofluorescence. At P0, the numbers of *GFP<sup>+</sup>* neurons in both MZ and VZ/SVZ were increased in the mutant brains (Figure 3E, F). In superficial neocortical layers of control brains, interneurons were radially-oriented, consistent with an inward migration of *Lhx6<sup>+</sup>* interneurons from superficial to middle/deeper layers. By contrast, *GFP<sup>+</sup>* cells in superficial neocortical layers of compound mutants lacked this radial orientation (Figure 3G). Furthermore, organotypic cultures prepared from P0 telencephalic slices of *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>;Lhx6-GFP* mice that were maintained up to 7 DIV, showed defects in the laminar distribution of *GFP<sup>+</sup>* cells (Figure 3H–K). At 7 DIV, ectopic *GFP<sup>+</sup>* cells were frequently observed in layer I, and there was a 2-fold increase in *GFP<sup>+</sup>* cells in superficial layers (II–III), a ~40% decrease in middle layers (IV–V), and a ~60% increase in deep layers (VI). Thus, *Dlx1&2* regulate the acquisition of laminar positions of neocortical

interneurons. Together, these results show that *Dlx* genes promote both tangential and radial migration of neocortical interneurons, while they restrain their neurite growth.

### Microarray expression analysis from *Dlx1/2*<sup>-/-</sup> interneuron precursors reveals molecular pathways affecting neuronal differentiation and migration

To identify molecular pathways that regulate interneuron differentiation, which are deregulated in *Dlx1/2*<sup>-/-</sup> mutants, we carried out microarray analysis comparing gene expression of cells derived from the MGE of *Dlx1/2*<sup>-/-</sup> versus *Dlx1/2*<sup>+/+</sup> embryos. For that, we used dissociated cells that were obtained from the VZ/SVZ of the MGE at E15.5 and grown *in vitro* for 3 days, a stage of differentiation in which we observed abnormal, longer neurites, but did not detect significant neuronal death. The data can be found at the NIH Neuroscience Microarray Consortium website (<http://arrayconsortium.tgen.org>). We concentrated on a set of genes that are known to regulate neuronal differentiation and cell migration, and verified that their expression is indeed altered *in vivo* based on *in situ* hybridization and/or immunocytochemistry (Figure S6 and Table I in Supplemental data). We identified signaling molecules that are relevant for cell communication and cell adhesion, and for the regulation of the cytoskeleton, and that were deregulated in *Dlx1/2* mutants. Altered functions of these molecules often result in phenotypes affecting cell morphology and migration (Luo, 2000; Guan and Rao, 2003; Govek *et al.*, 2005).

Notably, a number of genes encoding molecules that regulate the cytoskeleton were up-regulated in *Dlx1/2*<sup>-/-</sup> mutants. These include *Mtap2* (MAP2), *Mapt* (Tau1), *GAP43* (growth associated protein 43), and *PAK3* (p21-activated kinase 3) (Figure 4). The increased mRNA expression resulted in increased protein levels in neurites, as determined using immunocytochemical detection of MAP2 (2a + 2b), Tau1, GAP43 and PAK3 in primary cell cultures derived from E15.5 MGE of *Dlx1/2*<sup>-/-</sup> and control littermates. Immunoblots from mutant cultures lysates confirmed the increase in protein expression (MAP2: ~50%; Tau1: ~30%; GAP43: 3-fold; PAK3: 3-fold) (Figure 4A–G). We next examined MAP2, Tau1, GAP43 and PAK3 expression in sections from *Dlx1/2*<sup>-/-</sup> and littermate control embryos at E17.5. Whereas in controls their expression was low/undetectable in the SVZ of the MGE, in the mutants they were prematurely expressed in the SVZ (Figure 4H–M). These results are consistent with our phenotypic analysis showing increased neurite length of *Dlx1/2* mutant interneuron precursors. We focused our next studies on the expression and function of PAK3 in interneurons.

### *Dlx1/2* genes control growth of neurites and cell migration in part through negative regulation of PAK3 expression

PAK serine/threonine kinases are critical for coordinating the dynamics of the actin and microtubule cytoskeletons during cell motility and cell growth and/or transformation (Bokoc, 2003; Hofmann *et al.*, 2004; Kumar *et al.*, 2006). We tested the hypothesis that PAK3 overexpression in *Dlx1/2*<sup>-/-</sup> neurons contributed to the excessive neurite growth and block in migration in MGE-derived interneurons.

First, we analyzed the developmental expression of PAK3 in the wild-type mouse telencephalon (Figure 5A–C). At early and mid-gestational stages (E11.5–E15.5), *PAK3*

mRNA expression was nearly undetectable in the MGE and cells migrating out of the MGE (Figure 5A, B). At P0 and postnatal stages, MGE-derived cells, including projection neurons of the globus pallidus, striatal interneurons, and cortical and hippocampal interneurons, showed high expression of *PAK3* mRNA (Figure 5C, D, and Supplemental Figure 7). *PAK3* was also expressed by neocortical and hippocampal pyramidal neurons, as described in previous studies (Allen *et al.*, 1998). In *Dlx1/2<sup>-/-</sup>* mutant brains, *PAK3* expression was robust in the SVZ of the MGE (Figure 5A'-C'). The *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mutant MGE showed a slight elevation in *PAK3* expression (Supplemental Figure 8). Immunocytochemistry in cell cultures prepared from wild-type E15.5 MGE showed a diffuse *PAK3* distribution throughout the soma and neuronal processes (Figure 5E-G). Notably, *PAK3* expression in wild-type cultures was up-regulated as neurons differentiated, as assessed by immunocytochemistry (3-7 DIV; Figure 5E-G) and immunoblots from culture lysates (Figure 5H).

To test whether *PAK3* was necessary for neurite growth in MGE-derived neurons, we reduced its expression ~8-fold by transfecting dissociated MGE cells with *PAK3* siRNA oligos [see Figure S9 in Supplementary data for the siRNA efficiency in *PAK3* down-regulation, and Boda *et al.*, (2004) for the specificity of *PAK3* down-regulation by *PAK3* siRNA]. Dissociated cells from E15.5 wild-type MGE (VZ/SVZ) were transfected with *PAK3* siRNA (or non-silencing siRNA for controls), and plated over cortical feeders prepared from wild-type E18.5/P0 brains (Figure 6). Co-electroporation of a GFP-expressing DNA plasmid served as a reporter for visualizing the morphology of transfected cells. Morphometric analysis of randomly selected GFP-expressing cells after 3 DIV showed significant decreases in total neurite length, average neurite length and longest neurite length in *PAK3* siRNA-treated neurons (TNL: ~15%; ANL: ~15%; LN: ~25%), whereas the number of neurites was unchanged (Figure 6 A-C, H). Next, to test whether *PAK3* was mediating the overgrowth of neurites in *Dlx1/2<sup>-/-</sup>* neurons, we performed the same experiments in mutant cells. Our results showed that after 3 DIV, *PAK3* siRNA-treated neurons showed a significant decrease in average neurite length and length of longest neurite when compared to control cells (ANL: ~15%; LN: ~25%), and similar number of neurites (Figure 6 D,E, H).

To test whether *PAK3* overexpression could increase the length of neurites in wild-type MGE-derived neurons, we transfected dissociated cells with an expression vector coding the full-length sequence of *PAK3*; this resulted in ~4-fold increased *PAK3* expression (Figure S9). Analysis of neurite growth from cells co-transfected with *PAK3* and *GFP* after 3 DIV did not show significant differences in either length or number of neurites (Figure 6F-H). Thus, our results revealed that *PAK3* contributes to the lengthening of neuronal processes in MGE-derived cells, both in wild-type and *Dlx1/2* mutants, and its overexpression is not sufficient alone to promote neurite growth.

PAK proteins regulate cell motility (Bokoch *et al.*, 2003). Thus, we asked whether *PAK3* overexpression in the *Dlx1/2<sup>-/-</sup>* MGE contributed to the defect in interneuron migration. To test this possibility, we used a slice tissue culture assay (Figure 7). Brain slices were prepared from E14.5 wild-type embryos, focally co-electroporated with *PAK3* and *GFP* expression vectors in the MGE, and allowed to develop in culture for 3 days (Figure 7A-D).

Quantification of GFP<sup>+</sup> cells in the cortex showed a ~70% reduction in cell migration after 3 DIV in *PAK3* electroporations, compared to control electroporations (Figure 7E). These results suggest that MGE-derived interneurons have to maintain PAK3 expression at low levels while they tangentially migrate to the cortex.

To test whether reducing PAK3 was sufficient to rescue the block in migration in *Dlx1/2*<sup>-/-</sup>, we focally co-electroporated *PAK3* siRNA and a *GFP* expression vector in MGE of *Dlx1/2*<sup>-/-</sup> slices. Electroporations with control siRNA showed very few GFP<sup>+</sup> cells in the cortex; these cells were located in the cortical subventricular zone adjacent to the subpallial/pallial boundary (Figure 7J). By contrast, *PAK3* siRNA resulted in a ~4-fold increase in the number of cells that tangentially migrated into the mantle zone of the dorsolateral cortex (control siRNA: ~7 cells, *PAK3* siRNA ~28 cells; versus ~250 cells in wild-type slices, Figure 7J,K,P).

Finally, we tested whether reducing MAP2 in the MGE of *Dlx1/2*<sup>-/-</sup> could also contribute to rescue the migration defect [see Figure S9 for the siRNA efficiency in MAP2 down-regulation]. MAP2 is expressed primarily in post-migratory neurons where it exerts important functions in stabilizing microtubules during dendrite growth (Fujimori *et al.*, 2002; Dehmelt and Halpain, 2005). In *Dlx1/2*<sup>-/-</sup> mutant brains, MAP2 was prematurely expressed in the SVZ of the MGE (Figure 4J; Anderson *et al.*, 1997b). We found that electroporating *MAP2* siRNA into the mutant MGE resulted in a ~4-fold increase in the number of tangentially migrating cells reaching the cortex (~28 cells; Figure 7L,P). Moreover, co-electroporation of *PAK3* and *MAP2* siRNAs further increased (~8-fold) the number of GFP<sup>+</sup> cells that tangentially migrated into the dorsolateral cortex (~47 cells; Figure 7M–P). This represented a ~12% rescue of the tangential migration defect in *Dlx1/2*<sup>-/-</sup> mutants, when compared to the migration in wild-type slices (~326 cells; Figure 7N). Thus, these results indicate that reducing PAK3 (and MAP2) contributes, at least partially, to rescue the tangential migration defect in *Dlx1/2*<sup>-/-</sup> mutants.

## DISCUSSION

Precursors of mouse neocortical GABAergic interneurons originate in the subpallial telencephalon, from where they tangentially migrate to reach the cortical plate, and then radially migrate to acquire their laminar positions. Within neocortex, they develop their dendritic and axonal arborizations. Previous studies have shown that *Dlx* transcription factors are required for the tangential migration of subpallial-derived GABAergic interneurons (Anderson *et al.*, 1997a; Panganiban and Rubenstein, 2002). In the present study, we demonstrate a central role of *Dlx1&2* genes in inhibiting neurite growth in immature interneurons at a stage while they migrate tangentially to cortex. Using *Dlx1*<sup>-/-</sup>; *Dlx2*<sup>+/-</sup> mutants, we demonstrate that developing interneurons also require *Dlx* genes for migrating to their neocortical laminar positions, and for regulating the growth and branching of their dendrites and axons in neocortex. We provide evidence that premature and excessive expression of several proteins that regulate the cytoskeleton (i.e., PAK3, MAP2) in subpallial-derived *Dlx1/2*<sup>-/-</sup> neurons contributes to the excessive neurite growth and the block in tangential migration.



## Evidence that *Dlx* genes regulate neuronal differentiation and migration of cortical GABAergic interneurons

*Dlx1* and *2* genes are co-expressed by progenitor cells of the subpallial subventricular zone, where they have redundant functions in controlling the development of GABAergic neurons (Liu *et al.*, 1997; Anderson *et al.*, 1997b, Yun *et al.*, 2002). In *Dlx1/2*<sup>-/-</sup> embryos, interneuron precursors fail to migrate out of the SVZ, where they partially differentiate (Anderson *et al.*, 1997a,b). We provide three lines of evidence that *Dlx* genes have a central role in controlling neuronal differentiation that may contribute to their migration defects. First, we show that primary cultures from *Dlx1/2*<sup>-/-</sup> MGE produce neurons with increased neurite length. As neurons further differentiate, they show increased dendritic and axonal length, and decreased axonal branching. Second, we confirmed this phenotype by analyzing neocortical interneurons from *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> mutants, which maintain tangential migration but exhibit abnormalities in the growth and branching of their neuronal processes. Third, there was precocious and robust expression of several proteins that regulate the cytoskeleton (i.e., MAP2, Tau1, GAP43, and PAK3) both in the MGE of *Dlx1/2*<sup>-/-</sup> embryos and in MGE cultures (see also Anderson *et al.* 1997b). *In vivo*, these proteins are found primarily in post-migratory cells, where they exert important functions in controlling the growth of axons and dendrites (Figure 4–6, and Aigner *et al.*, 1995; Fujimori *et al.*, 2002; Dehmelt and Halpain, 2005). Because loss of *Dlx1&2* produces neurons with increased complexity (i.e., longer neurites) and up-regulation of genes that are normally expressed by more mature, post-migratory interneurons, we hypothesize that *Dlx* genes have a central role in repressing molecular pathways in migrating interneurons that promote their later differentiation steps. Thus, in the absence of *Dlx* function, immature interneurons are unable to maintain the undifferentiated state that is necessary for their migration (see model in Figure 8).

An alternative interpretation for this phenotype could be a switch in cell fate. In this context, it could be possible that *Dlx* loss-of-function favored the differentiation of specific neuronal subtypes characterized by increased neurite length and decreased neurite branching. This seems unlikely, because changes in cell fate have not been observed in the *Dlx1/2*<sup>-/-</sup> double mutant MGE (Anderson *et al.*, 1997b; Marin *et al.*, 2000; Yun *et al.*, 2002; De Melo *et al.*, 2005; Cobos *et al.*, 2005). Furthermore, the MGE of the *Dlx1/2*<sup>-/-</sup> embryos maintains expression of several interneuron markers including somatostatin and neuropeptide Y (Cobos *et al.*, 2005). In addition, analysis of interneuron markers in the neocortex of *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> neonates and in primary cultures from *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> neocortex do not show obvious changes in GABAergic interneuron cell fate (Cobos and Rubenstein, unpublished data).

We have previously shown that postnatal *Dlx1*<sup>-/-</sup> single mutant mice have decreased branching of dendrites in subsets of cortical interneurons (e.g. bitufted somatostatin<sup>+</sup> interneurons; Cobos *et al.*, 2005). Here we report progressively more severe morphological defects in *Dlx1/2*<sup>-/-</sup> > *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> > *Dlx1*<sup>-/-</sup> GABAergic interneurons. *Dlx1*<sup>+/-</sup>;*Dlx2*<sup>-/-</sup> compound mutants have defects in forebrain development that are similar to those observed in the *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> mutants (Cobos and Rubenstein, unpublished data). These findings suggest redundant functions of *Dlx1* and *Dlx2* in GABAergic differentiation, and are consistent with previous studies showing that *Dlx* dosage is critical in development of neural

and non-neural structures (Anderson et al., 1997b; Robledo *et al.*, 2002; Depew *et al.*, 2005). It does remain possible that *Dlx1* and *Dlx2* also have distinct functions in GABAergic neurons.

In addition to the role of *Dlx* genes in controlling neuronal morphogenesis in GABAergic interneurons, we present the first evidence that *Dlx* affect the acquisition of their normal laminar positions in neocortex. In *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>* mice, excess *Lhx6<sup>+</sup>* interneurons accumulate in deep and superficial layers of neocortex, and fail to sufficiently populate middle (IV–V) layers. Neocortical interneurons have been shown to migrate radially to adopt their laminar identities (Nadarajah *et al.*, 2002; Tanaka *et al.*, 2003; Hevner *et al.*, 2004; Pla *et al.*, 2006). Future studies will be aimed at elucidating the molecular mechanisms regulating radial migration of GABAergic interneurons by *Dlx* genes.

Finally, our results show that *Dlx* function is required for the survival of GABAergic neurons developing *in vivo* and *in vitro*. Previous studies have reported a requirement of *Dlx1&2* for cell survival in the retina (De Melo *et al.*, 2004), and of *Dlx1* in mature interneurons of the neocortex and hippocampus (Cobos *et al.*, 2005). The mechanisms underlying cell death in *Dlx1/2<sup>-/-</sup>* are unknown, although we propose that it has a cell autonomous basis because mutant MGE cells show apoptosis when transplanted onto wild type cortical cells (Supplemental Figure 3). Whether *Dlx* genes regulate the expression of trophic factor receptors and/or pre- and pro-apoptotic genes remains to be determined.

### ***Dlx* genes coordinate neuronal differentiation and migration through regulation of cytoskeletal proteins: role of *PAK3***

We show that immature MGE-derived *Dlx1/2<sup>-/-</sup>* interneurons exhibit premature and excessive expression of several cytoskeletal proteins, including MAP2, Tau1, GAP43 and PAK3. We focused on the analysis of PAK3 because of the central role of PAK kinases in cytoskeletal reorganization and cell behavior. PAK kinases are major downstream effectors of the Rho-family of GTPases (RhoA, Rac1, Cdc42), a family of key signaling proteins that integrate different extracellular and intracellular signals to regulate the actin cytoskeleton (Govek *et al.*, 2005; Rossman *et al.*, 2005). PAKs usually interact with and are activated by Rac1 and Cdc42, although they can also be activated by a variety of Rho GTPase-independent mechanisms (Bokoc, 2003). In *Drosophila*, PAK kinases have essential roles in axon guidance (Hing *et al.*, 1999; Fan *et al.*, 2003). In the mammalian brain, they play crucial roles in regulating neurite outgrowth, cell migration, spine morphogenesis, and synapse formation (Bokoc, 2003; Hofmann *et al.*, 2004; Boda *et al.*, 2006). We found that in MGE primary cultures PAK3 expression is up-regulated as neurons differentiate. *In vivo*, *PAK3* expression is up-regulated in cortical interneurons once they have migrated into the cortex, a stage when they develop their dendritic and axonal arborizations. By reducing PAK3 levels using RNA interference, we show that PAK3 promotes the lengthening of neuronal processes. These results are in agreement with previous studies showing that PAK proteins regulate the cytoskeleton during neurite growth and remodeling. For example, PAK1 is implicated in neurite outgrowth (Rashid *et al.*, 2001; Daniels *et al.*, 1998), and GEFT, a guanine nucleotide exchange factor that activates the Rac1/Cdc42-PAK signaling pathway, promotes dendrite growth in primary hippocampal neurons (Bryan *et al.*, 2004).

On the other hand, PAK3 overexpression in MGE neurons was insufficient to increase the lengthening of neuronal processes. This is in line with previous studies in cell lines showing that wild-type PAK and cytoplasmically expressed PAK do not cause efficient neurite outgrowth and/or elongation, while membrane-targeted forms of PAK are able to increase neurite extension. Local recruitment and activation of PAK to the membrane, together with Paxillin and GIT1, form a signaling complex that appear to be necessary for mediating polarized actin polymerization and subsequent neurite extension (Daniels *et al.*, 1998; Obermeier *et al.*, 1998; Nayal *et al.*, 2006).

Major *in vivo* functions of PAK3 in mammalian neurons involve dendrite spine morphogenesis and synapse formation and plasticity (Allen *et al.*, 1998; Boda *et al.*, 2004; Zhang *et al.*, 2005; Meng *et al.*, 2005). Here we provide evidence that PAK3 promotes neurite growth in cortical GABAergic interneurons. Notably, we found that in *Dlx1/2<sup>-/-</sup>* mutants, *PAK3* is prematurely expressed in the MGE, and that down-regulating PAK3 in the mutant MGE is sufficient to rescue the excessive growth of neurites in the mutants. These findings support our hypothesis that *Dlx1&2* have a major role in immature interneurons for repressing molecular pathways that promote axon and dendrite maturation.

PAK proteins also regulate cell motility (Bokoc, 2003). In fibroblasts and epithelial cells, PAK (PAK1, PAK3) is a key effector for adhesion turnover and protrusions dynamics. In these cells, PAK is targeted with Paxillin and GIT1 near their leading edge to increase adhesion turnover, protrusion and motility (Zhao *et al.*, 2000; Nayal *et al.*, 2006). Although the functions of PAK in the cytoskeletal structure are well established, its mechanism in regulating neuronal migration is less understood. In the mammalian cortex, PAK1 co-localizes with p35/Cdk5 and Rac1 at the leading edges of axonal growth cones. p35/Cdk5 is essential for the radial migration of immature pyramidal neurons and is required for cortical lamination (Chae *et al.*, 1997; Nikolic *et al.*, 1998). p35/Cdk5 hyperphosphorylate and subsequently down-regulate PAK1, which is crucial for the proper regulation of the cytoskeleton during neurite growth and migration (Nikolic *et al.*, 1998; Rashid *et al.*, 2001). Thus, these studies indicate that in mouse neocortex PAK1 activity has to be inhibited for the proper regulation of cytoskeletal dynamics during neuronal migration. In agreement with this concept, we found that PAK3 expression was low/absent in wild-type MGE-derived migratory interneurons, and that PAK3 overexpression in the MGE was sufficient to arrest the tangential migration of interneurons to neocortex. Moreover, down-regulating PAK3 with RNA interference in the MGE of *Dlx1/2<sup>-/-</sup>* tissue culture slices was sufficient to increase the numbers of neurons migrating tangentially to neocortex. Tangential migration in *Dlx1/2<sup>-/-</sup>* tissue culture slices was further increased using RNA interference against *MAP2* together with *PAK3*. Although the rescue of migration was incomplete, these results demonstrate that *Dlx* genes promote tangential migration at least in part through negative regulation of neurite growth. Deficits in additional signaling pathways in *Dlx1/2<sup>-/-</sup>* embryos may well also contribute to the migration failure. For instance, changes in expression of the Slit receptor Robo1, the Semaphorin receptor Neuropilin2, or the Neuregulin1 receptor ErbB4 (Supplemental Figure 6), which have been implicated in cortical interneuron migration (Marin *et al.*, 2001; Flames *et al.*, 2004; Andrews *et al.*, 2006), may also explain the tangential migration defect of *Dlx1/2<sup>-/-</sup>* mutants.

## METHODS

### Mice

The mouse mutant strains with double null alleles of *Dlx1* and *Dlx2* (*Dlx1/2*) and single null allele of *Dlx1* (Qiu *et al.*, 1997) were used. *Dlx1*<sup>-/-</sup>; *Dlx2*<sup>+/-</sup> compound mutant mice were generated by mating *Dlx1*<sup>+/-</sup> with *Dlx1/2*<sup>+/-</sup> animals. The transgenic line *Lhx6*-GFP BAC (NINDS GENSAT project) was bred to the *Dlx1*<sup>+/-</sup> and *Dlx1/2*<sup>+/-</sup> lines to generate *Dlx1*<sup>+/-</sup>; *Lhx6*-GFP and *Dlx1/2*<sup>+/-</sup>; *Lhx6*-GFP animals, respectively. Mating of these mice to either *Dlx1* or *Dlx1/2* mice were used to generate *Dlx1*<sup>-/-</sup>; *Lhx6*-GFP, *Dlx1*<sup>-/-</sup>; *Dlx2*<sup>+/-</sup>; *Lhx6*-GFP, and *Dlx1/2*<sup>-/-</sup>; *Lhx6*-GFP embryos. Littermate mice (WT or, when not possible, heterozygous) were used as controls for all experiments. Currently, no phenotype has been identified in the brain of *Dlx1/2*<sup>+/-</sup> mice. Mouse colonies were maintained at the University of California, San Francisco, in accordance with National Institutes of Health and UCSF guidelines.

### Histology

Pregnant females and neonatal pups were deeply anesthetized in a CO<sub>2</sub> chamber and sacrificed by cervical dislocation or decapitation. Mouse pups (P5–P7) were anesthetized with Avertin (0.2cc/10g body weight), perfused intracardially with 4% paraformaldehyde (PFA) in phosphate-buffered solution (PB 0.1 M, pH 7.4), and their brains removed and post-fixed overnight in the same fixative. E13.5–P0 brains were dissected and fixed by immersion in 4% PFA in PB. The tissue was cryoprotected by immersion in 30% sucrose, embedded in OCT (Tissue-Tek), and cryostat sectioned (10–20 μm).

Immunohistochemistry on cryostat sections was performed according to standard methods. The antibodies used were as follows: mouse anti-Tuj1 (1:1000, Covance), mouse anti-MAP2 (2a + 2b; 1:2000, Sigma), mouse anti-Tau1 (1:1000, Chemicon), rabbit anti-GAP43 (1:4000, Chemicon), goat anti-PAK3 (1:100, Santa Cruz), rabbit anti-pan-Dll antibody (1:500, G. Panganiban), rabbit anti-GFP (1:2000, Molecular Probes), and mouse anti-GFP (1:500, Molecular Probes). Fluorescent secondary antibodies (goat anti-rabbit, goat anti-mouse, or donkey anti-goat), conjugated with either Alexa-488 or Alexa-594 (1:300; Molecular Probes), were used.

*In situ* RNA hybridization on cryostat sections was performed according to standard methods. The probes used and their sources were as follows: *GAD67* (B. Condie), *Lhx6* (V. Pachnis), *Dll1* and *Hes5* (G. Weinmaster), *Mash1* (F. Guillemot), *ErbB4* (C. Lai), *Robo1*, *Nrp2* and *Sema3A* (M. Tessier-Lavigne), and *Mef2c* (E. Olson). Some probes were made from EST clones (Invitrogen): *Elmo1* (BI694830), *CD47* (BG964433), *DSCAM* (CO043786), *PAK3* (AW049432), *IGSF4a* (BF021469), and *Rapgef5* (BE310387). Our expression data was in agreement with public electronic databases [BGEM (<http://www.stjudebgem.org>), NINDS GENSAT project (<http://www.gensat.org>)] and published data (Supplemental Figure 6).

### Primary cell cultures

*Dlx1/2<sup>-/-</sup>* and *Dlx1/2<sup>+/-</sup>* control brains were used for primary MGE cultures (E15.5), and for transplantation of MGE donor cells (E15.5) over cortical feeders following the method of Xu *et al.* (2004). The brains were removed in ice-cold Hanks buffer (HBSS; Invitrogen), and the proliferative zones (VZ/SVZ) from the anterior part of the MGE were isolated (Cobos *et al.* 2005). The cells were dissociated mechanically in Neurobasal/B27 medium (NB/B27, Invitrogen) containing 1 U/ml DNase I (Roche). Then, they were pelleted, resuspended in DMEM (Invitrogen) with 10% fetal bovine serum (FBS, HyClone), and plated at a density of  $2.5 \times 10^5$  cells per  $\text{cm}^2$ , in either 16-well, 8-well or 2-well chamber slides (0.4  $\text{cm}^2$ , 0.8  $\text{cm}^2$ , and 4  $\text{cm}^2$ , respectively; Lab-Tek, Nalgene). The slides were previously coated with poly-lysine (10  $\mu\text{g/ml}$ ; Sigma) followed by laminin (5  $\mu\text{g/ml}$ ; Invitrogen). Primary cortical cultures were prepared from E18.5 or P0 brains. The neocortex was dissected in ice-cold HBSS, macerated using fine forceps, trypsinized (0.05% trypsin, 37°C, 20 min), and further triturated using a fire-polished glass pipette. For transplantations of MGE donor cells over neocortical feeders, we isolated *Dlx1<sup>-/-</sup>*, *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>*, *Dlx1/2<sup>-/-</sup>*, and control cells (all *Lhx6-GFP<sup>+</sup>*), and plated them on E18.5/P0 wild-type feeders prepared 1 day earlier. One thousand donor cells were added per well (8-well slides; 0.8  $\text{cm}^2$ ) in experiments designed to image and analyze cell morphology; ten thousand donor cells were used in experiments designed to quantify cell survival.

### Image analysis and quantification of neurite growth

Quantification of neurite growth was performed in GFP-expressing immature interneurons from primary cultures prepared with either *Dlx1<sup>-/-</sup>*, *Dlx1<sup>-/-</sup>;Dlx2<sup>+/-</sup>*, or *Dlx1/2<sup>-/-</sup>* mutants and littermate controls (all *Lhx6-GFP<sup>+</sup>*). Transplantation of donor cells at low density, and dilution of GFP cortices with WT, non-GFP cortices, as described above, allowed identification of individual GFP<sup>+</sup> cells so that their processes could be clearly distinguished from those of other GFP<sup>+</sup> cells. For sampling of cultured neurons, we used an unbiased, non-stereological method that gave every cell an equal chance of being sampled. We defined a systematic series of fields of view in the culture area, and randomly chose 1–3 cells in each field for imaging. The experimenter was blinded to genotype during sampling, image analysis, data collection and statistical analysis. We sampled 20–40 interneurons for each time point and each genotype, from at least 6 different wells obtained from 3 independent experiments. Images were acquired on a confocal microscope (Radiance 2000, BioRad) with a 20× or 40× objective. The confocal settings were chosen so that the GFP fluorescence was within the brightness range required to clearly visualize the entire cell, and were maintained for all experiments of the same category. Series of z-stack images (3–10 optical series, depending on the age) were collected encompassing all neuronal processes of each cell. Digitized images were imported to Image J (National Institute Health software; Neuron J plugin) for tracing and quantifying neurites. Total number of branches (filopodia longer than 5  $\mu\text{m}$  were considered branch tips), total neurite length, average neurite length, and length of longest neurite were determined for each cell. Statistical analyses were performed with SPSS 10.0. Differences between means were assessed by ANOVA, followed by Tukey-Kramer *post hoc* test. Significance level was taken as  $p < 0.05$ .

## DNA plasmids and siRNA

The expression vector coding the full-length sequence of the mouse *PAK3* was generated subcloning *PAK3* (clone ID 30060082, MGC full-length) into pCAGGS, a chicken  $\beta$ -actin promoter expression vector (Stuhmer *et al.*, 2002). An expression vector encoding enhanced green fluorescent protein (*GFP*) cloned into pCAGGS (Stuhmer *et al.*, 2002) was used for co-transfection experiments. siRNA targeting the mouse *PAK3* and *MAP2* genes were used. siRNA was obtained from IDT (duplexed RNAi oligonucleotides). The target sequence for *PAK3* previously published by Boda *et al.* (2004) (5' TAGCAGCACATCAGTCTGAATA), and for *MAP2* used by Fontaine-Lenoir *et al.* (2006) (5' TTCGCTGAGCCTTTAGACA), resulted in a consistent reduction of ~90% of *PAK3* and *MAP2* protein levels, respectively ( $n = 5$  each; as measured by western blot analysis). The target sequence used as non-silencing control was 5' AATTCTCCGAACGTGTCACGT (Boda *et al.*, 2004).

## Transient transfection of primary neurons

For transfections of MGE cells with either siRNA or DNA plasmids we utilized Nucleofector technology (Amaxa biosystems). The VZ/SVZ of the MGE from *Dlx1/2<sup>-/-</sup>* or control embryos, aged E15.5, was dissected and their cells dissociated as described above. We conducted several control experiments to determine the optimal volume of sample (number of cells) and substrate (siRNA and/or DNA plasmid) being transfected for maximum gene transfer efficiency and cell viability. Based on these results, we used  $3 \times 10^6$  cells (pooled from 3–5 embryos) for each transfection, and 0.2 nmol of siRNA or 2  $\mu$ g of DNA, which resulted in > 90% efficiency in gene delivery. Transfections were performed according to the manufacturer guidelines (Amaxa biosystems), using Program O-05 and the primary mouse neuron kit. Identical conditions were used for transfections of DNA, RNA, and co-transfections with DNA and RNA together. After transfections, the cells were resuspended in DMEM/10% FBS and plated at high density. For analysis of cell morphology, MGE cells were co-transfected with either *PAK3* siRNA or the *PAK3* expressing vector, and the *GFP* expressing vector, and plated over wild-type neocortical feeders prepared 1 day previously.

## Slice tissue cultures and electroporations

Preparation of slice tissue cultures from embryonic mouse brains followed the methods of Anderson *et al.* (1997a). To achieve focal electroporations of MGE cells, we pressure injected, through a glass micropipette, *PAK3* siRNA (200  $\mu$ M), *MAP2* siRNA (200  $\mu$ M) or the *PAK3* expressing vector (1  $\mu$ g/ $\mu$ l), and the corresponding controls in the contralateral brain hemisphere. In all cases we co-electroporated the *GFP*-expression vector to assess the efficiency of the electroporations, and to quantify the migration of the targeted cells to the cortex. Electroporations followed the methods of Stuhmer *et al.* (2002). Five square wave pulses of 100 volts and 5 ms duration were applied using a T820 Electro Square Porator (BTX). Cell viability was assessed using 25  $\mu$ g/ml propidium iodine (Molecular Probes) added to the medium. Each slice was examined for GFP expression under an epifluorescent dissecting microscope and photographed at 1, 2 and 3 days *in vitro* (DIV). After 3–4 DIV, the slices were fixed in 4% PFA in PB, cryoprotected in 30% sucrose, embedded in OCT compound, and re-sectioned on a cryostat (20–75  $\mu$ m) for immunohistochemistry.

## Microarrays

RNA was extracted from E15.5 MGE cells (*Dlx1/2<sup>-/-</sup>* and *Dlx1/2<sup>+/?</sup>* littermates) that were dissociated and grown *in vitro* for 3 days. Genotypes were determined based on the presence of cleft palate in mutants and PCR. Total RNA was isolated using Absolutely RNA Miniprep kit (Stratagene). RNA from at least 6 embryos, each genotype, was pooled to average genetic and experimental variations. 6–20 µg of RNA, each genotype, were used. cDNA reverse transcription, amplification, labeling and hybridization to Affymetrix GeneChip 430 2.0 arrays was performed by the NIH Neuroscience Microarray Consortium.

## Western blots

For western blot analysis, cells from primary MGE cultures were lysed in RIPA buffer (50 mM Tris HCl pH7.5, 150 mM NaCl, 0.5% DOC, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors (protease inhibitors cocktail, Roche). Protein samples (10 µg each) were resolved on polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were pre-incubated in 5% nonfat dry milk in TBT (Tris-buffered solution and 0.1% Tween-20), and incubated O/N with the primary antibodies in the same solution. Membranes were probed with HRP-couple anti-mouse, -rabbit or -goat antibodies (1:5000, Bio-Rad) and developed using ECL reagent (GE Healthcare). Each experiment was done at least three times. Quantification of optical density was done using NIH Image software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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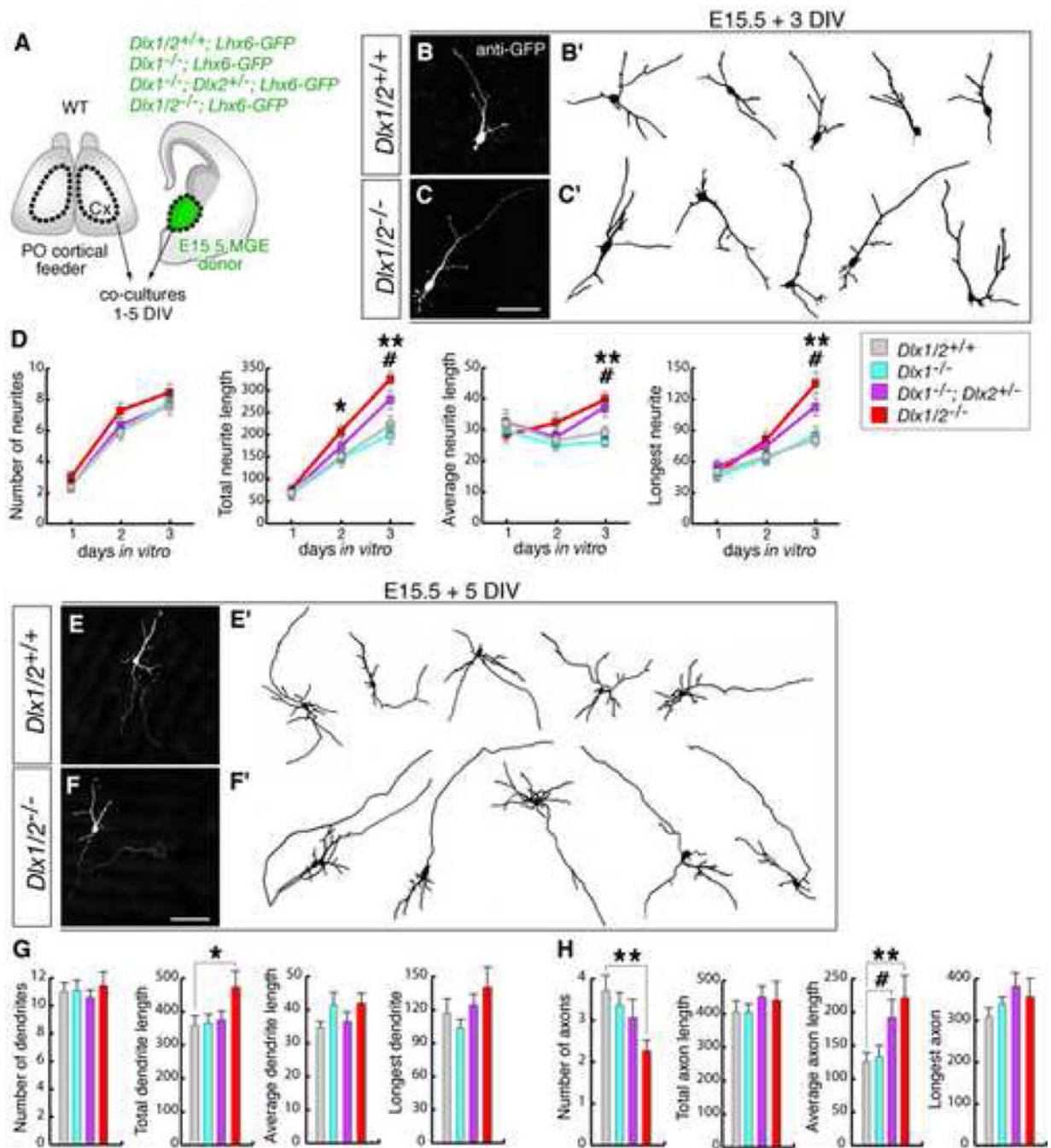
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**Figure 1. Interneuron precursors from *Dlx* mutant MGE cultures develop longer neuronal processes**

(A) Experimental assay used to characterize the morphology of interneuron precursors derived from progenitors of the MGE from *Dlx1*<sup>-/-</sup>, *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup>, and *Dlx1/2*<sup>-/-</sup> mutants.

(B, C) Examples of *Dlx1/2*<sup>-/-</sup> mutant and control neurons from E15.5 MGE cultures stained with anti-GFP antibody after 3 DIV. (B', C') are drawings of representative neurons.

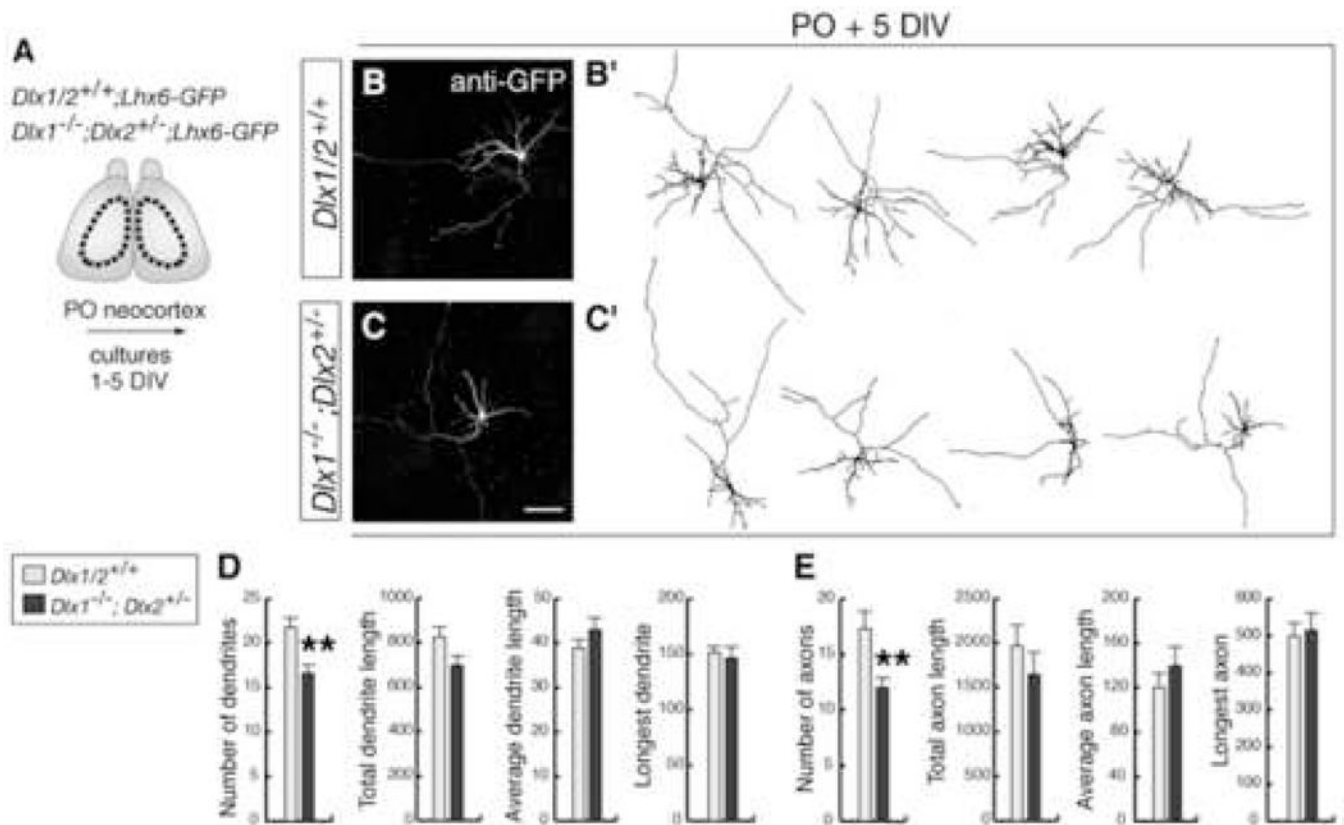
(D) Quantification of number of neurites, total neurite length (TNL), average neurite length (ANL), and length of longest neurite (LN) from control (gray lines), and *Dlx* mutants (color

lines) after 1–3 DIV. Tukey-Kramer test revealed significant increases of TNL, ANL and LN in *Dlx1/2*<sup>-/-</sup> and in *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> mutant cells as compared to control cells by 3 DIV [*Dlx1/2*<sup>-/-</sup>: (in  $\mu\text{m}$ ) TNL:  $325 \pm 12.8$ , ANF:  $39.8 \pm 2.1$ , LN:  $134.2 \pm 10$ ; *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup>: TNL:  $280 \pm 20.2$ , ANF:  $37.8 \pm 2.8$ , LN:  $112.9 \pm 8.2$ ; control: TNL:  $228.5 \pm 19.3$ , ANF:  $30.9 \pm 1.9$ , LN:  $80.3 \pm 3.9$ ;  $n = 25$ , each genotype]. \*  $p < 0.05$ , \*\*  $p < 0.01$  (*Dlx1/2*<sup>-/-</sup> versus controls); #  $p < 0.05$  (*Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> versus controls).

(E, F) Examples of *Dlx1/2*<sup>-/-</sup> mutant and control neurons after 5 DIV. (E', F') are drawings of representative neurons.

(G, H) Quantification of axonal and dendritic morphology in control (gray bars) and *Dlx* mutants (color bars) after 5 DIV. (Average dendrite length: *Dlx1/2*<sup>-/-</sup>:  $42 \pm 3.1 \mu\text{m}$ ; control:  $33.3 \pm 2.4 \mu\text{m}$ ; average axon length: *Dlx1/2*<sup>-/-</sup>:  $221.1 \pm 33.7 \mu\text{m}$ ; *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup>:  $190.7 \pm 27.8 \mu\text{m}$ ; control:  $122.2 \pm 14.3 \mu\text{m}$ ; number of axonal branches: *Dlx1/2*<sup>-/-</sup>:  $2.27 \pm 0.26$ ; control:  $3.88 \pm 0.38$ ;  $n = 25$  each genotype). \*  $p < 0.05$ , \*\*  $p < 0.01$  (*Dlx1/2*<sup>-/-</sup> versus controls); #  $p < 0.05$  (*Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> versus controls; Tukey-Kramer test).

Scale bars = 50  $\mu\text{m}$  (B,C) and 100  $\mu\text{m}$  (E,F).



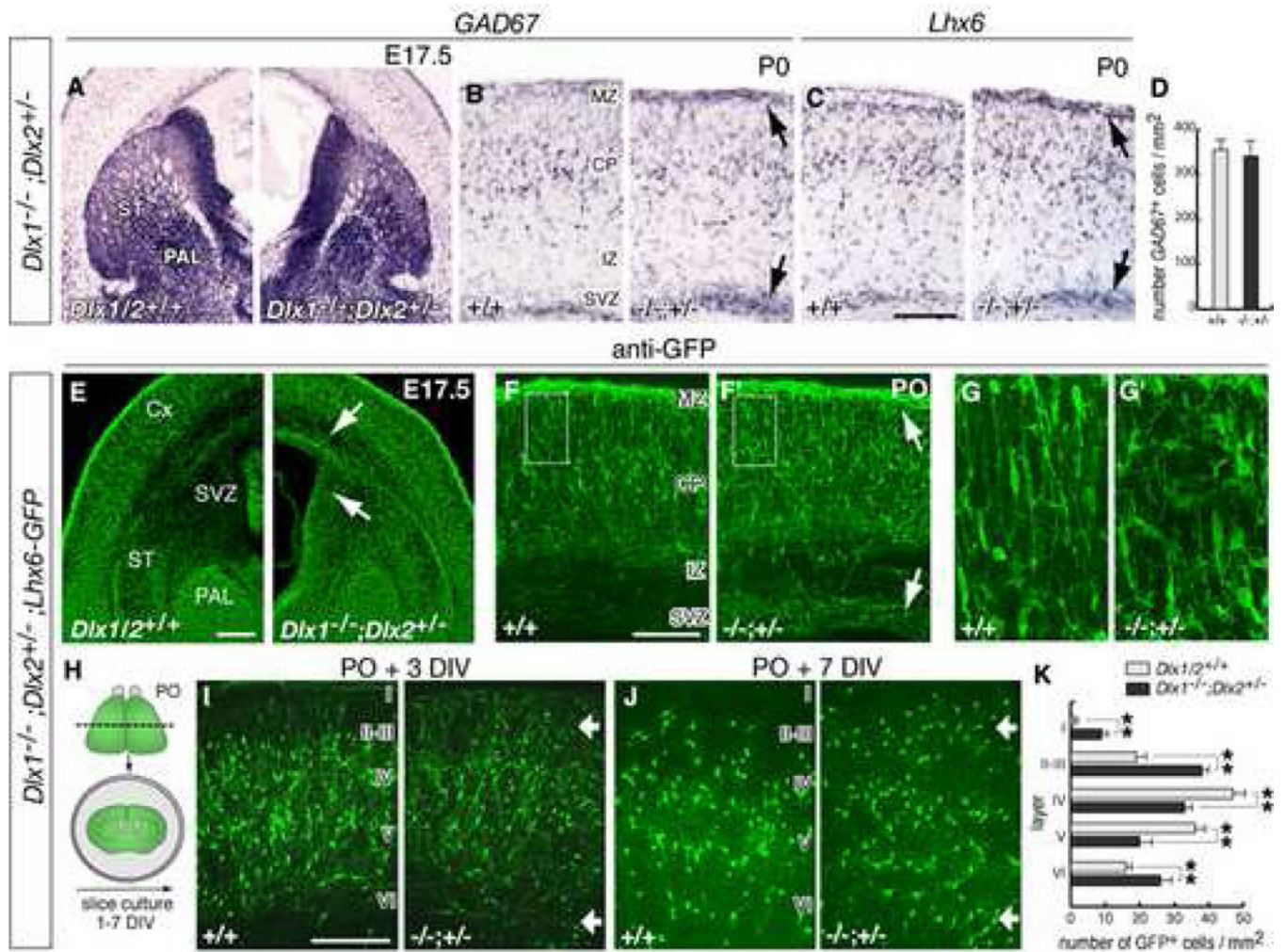
**Figure 2. Interneurons from neocortex of *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> compound mutants develop neuronal processes with fewer branches**

(A) Experimental assay used to characterize the morphology of interneurons.

(B, C) Examples of interneurons isolated from P0 neocortex and immunostained with anti-GFP after 5 DIV. (B', C') are drawings of representative neurons.

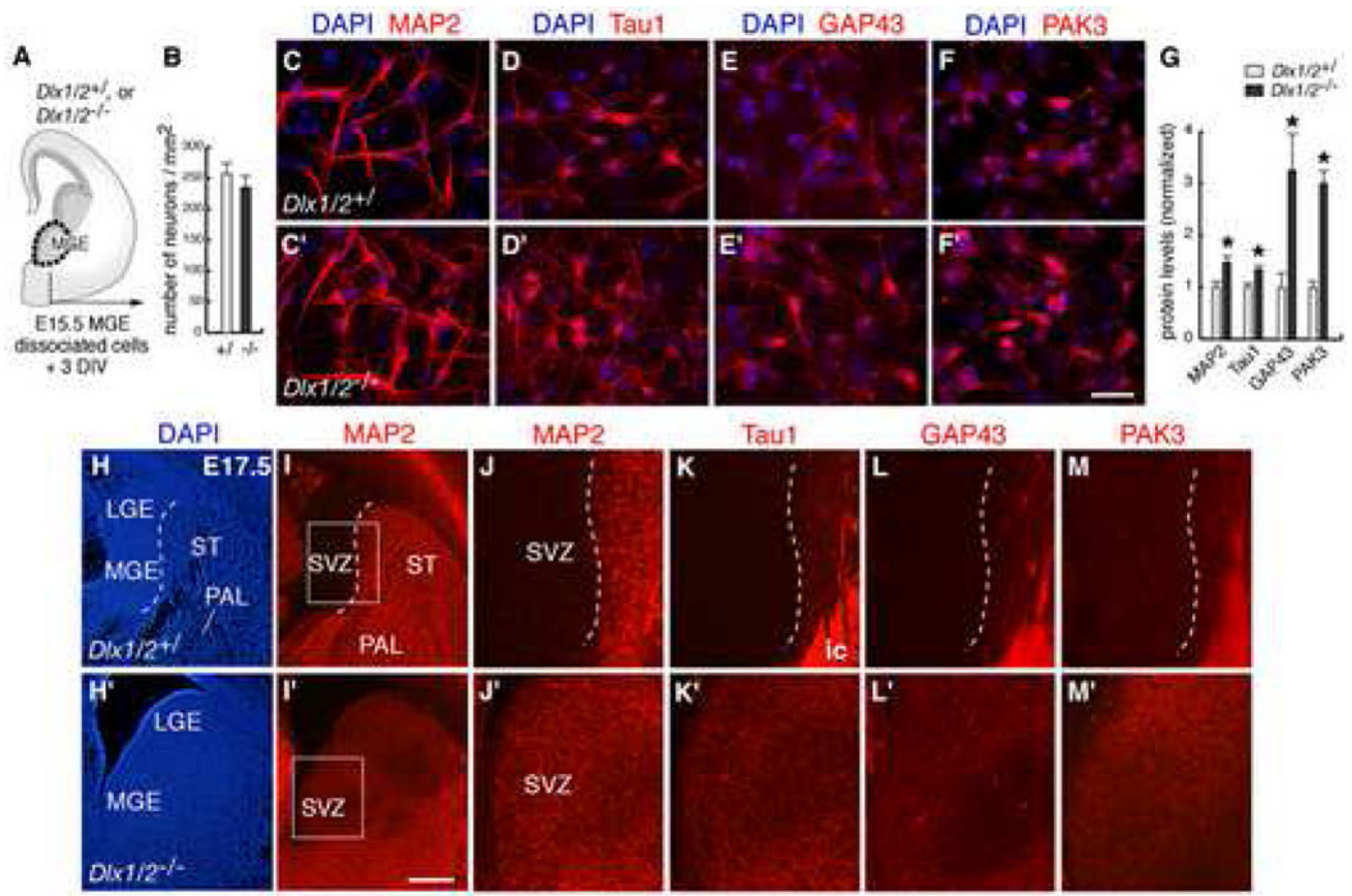
(D, E) Quantification of axonal and dendritic morphology from control (gray bars) and *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> (black bars) interneurons after 5 DIV. Student's *t*-test revealed significantly decreases in the numbers of dendritic and axonal branches in mutant cells (dendrites: mutant:  $16.6 \pm 1$ ; control:  $21.8 \pm 1.2$ ,  $p < 0.01$ ; axons: mutant:  $12 \pm 0.8$ ; control:  $17.3 \pm 1.6$ ,  $p < 0.01$ ;  $n = 30$ ). \*\*  $p < 0.01$ .

Scale bar = 100  $\mu\text{m}$  (B,C).



(I–J) Coronal sections through the neocortex from organotypic cultures prepared from P0 brains and maintained 3 and 7 DIV. Roman numbers approximate the position of cortical layers. Arrows indicate accumulation of GFP<sup>+</sup> neurons in superficial (I–III) and deep (VI) layers of mutant brains.

(K) Quantification of the distribution of GFP<sup>+</sup> cells in neocortical layers of control and *Dlx1*<sup>-/-</sup>; *Dlx2*<sup>+/-</sup> organotypic cultures after 7 DIV (controls: I:  $0.15 \pm 0.13$ ; II–III:  $19.1 \pm 2.3$ ; IV:  $47.3 \pm 4.9$ ; V:  $36.6 \pm 3.7$ ; VI:  $16.1 \pm 2.1$ ; mutants: I:  $9.3 \pm 1.9$ ,  $p < 0.01$ ; II–III:  $38.5 \pm 2.5$ ,  $p < 0.01$ ; IV:  $33.4 \pm 2.8$ ,  $p < 0.01$ ; V:  $19.9 \pm 4.0$ ,  $p < 0.01$ ; VI:  $26.2 \pm 4.8$ ,  $p < 0.01$ ;  $n = 7$ , each genotype). \*\*  $p < 0.01$  (Student's *t* test). Scale bars = 500  $\mu\text{m}$  (A,E) and 250  $\mu\text{m}$  (B,C,F,G and I,J).



**Figure 4. MGE-derived neurons from *Dlx1/2*<sup>-/-</sup> show increased expression of several proteins that regulate the cytoskeleton**

(A) *In vitro* experimental assay used to analyze neuronal differentiation of *Dlx1/2*<sup>-/-</sup> MGE.

(B) Quantification of numbers of neurons from control and mutant MGE cultures after 3 DIV.

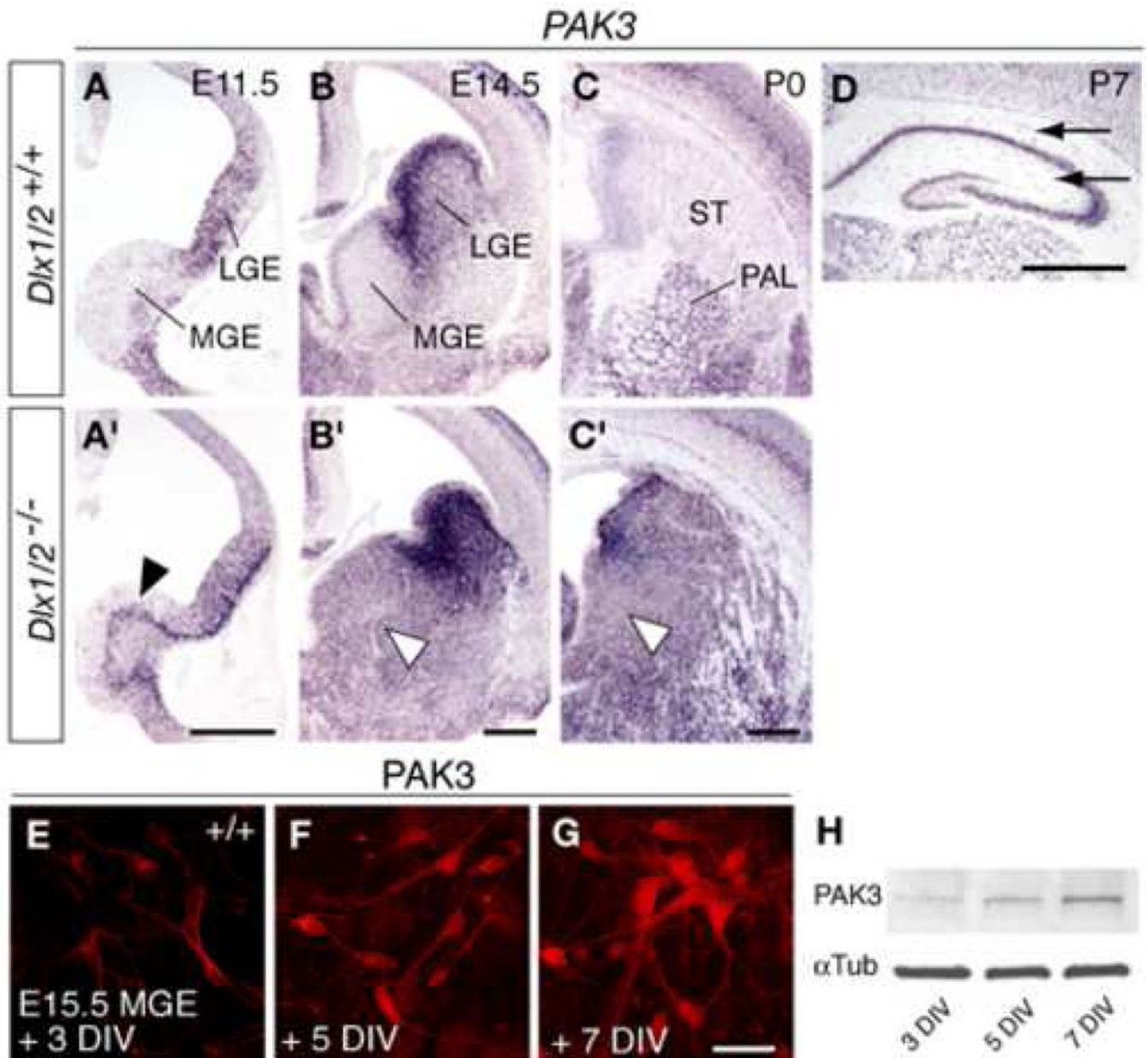
(C–F) Immunofluorescent staining for MAP2 (2a + 2b), Tau1, GAP43, and PAK3 in mutant (C'–F') and control (C–F) MGE cultures after 3 DIV.

(G) Quantification of protein levels from immunoblots of control and mutant MGE cultures after 3 DIV. \*  $p < 0.05$  (Student's *t* test).

(H–M) Immunofluorescent staining for MAP2, Tau1, GAP43 and PAK3 in histological sections from E17.5 *Dlx1/2*<sup>-/-</sup> (H'–M') and controls (H–M), showing increased levels of these proteins in the SVZ of the MGE. (J–M) are higher magnifications of the areas boxed in (I). Dashed lines approximate the boundary between SVZ and mantle (ST: striatum; PAL: globus pallidum). Strong expression of Tau1, GAP43 and PAK3 in (K–M) labels fibers of the internal capsule (ic).

Scale bars = 50  $\mu$ m (C–F) and 500  $\mu$ m (H,I).





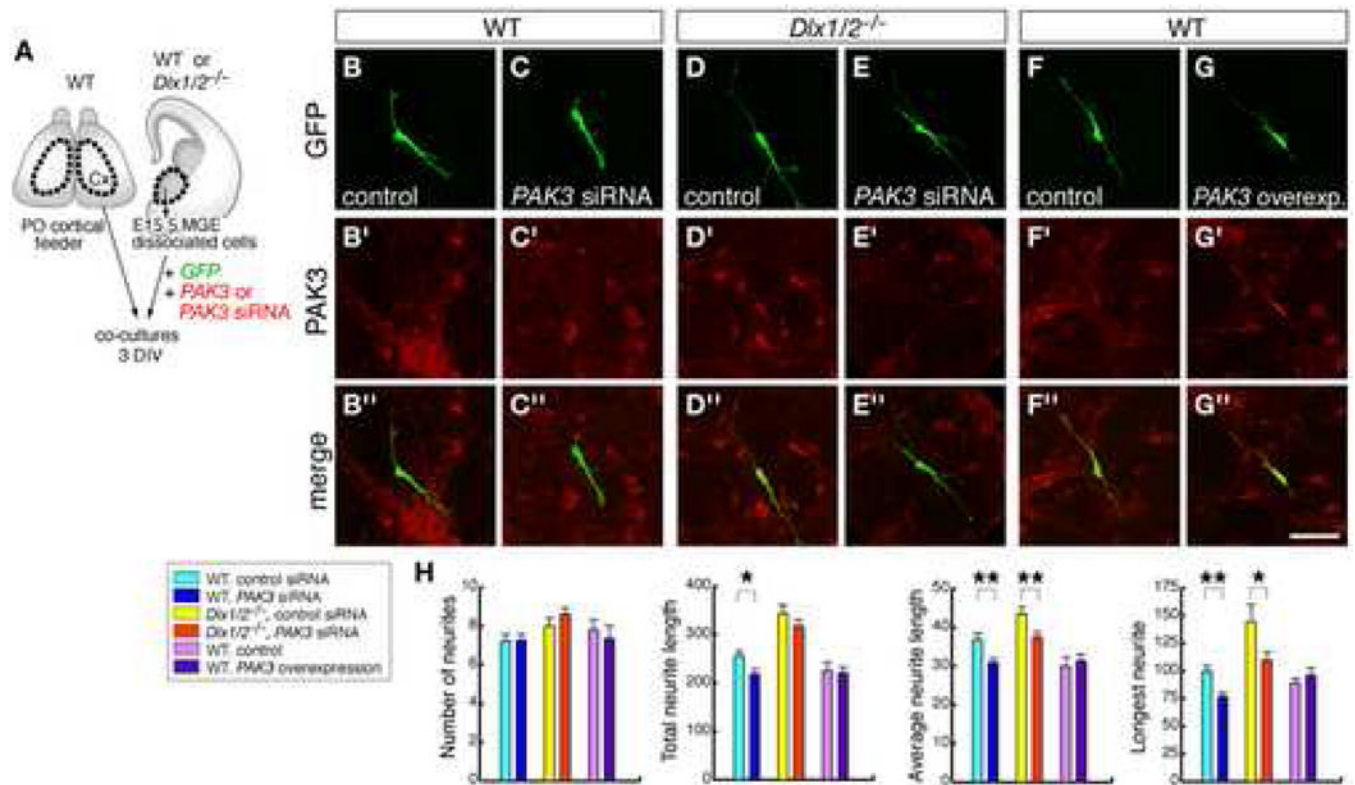
**Figure 5. Analysis of PAK3 expression in the mouse telencephalon**

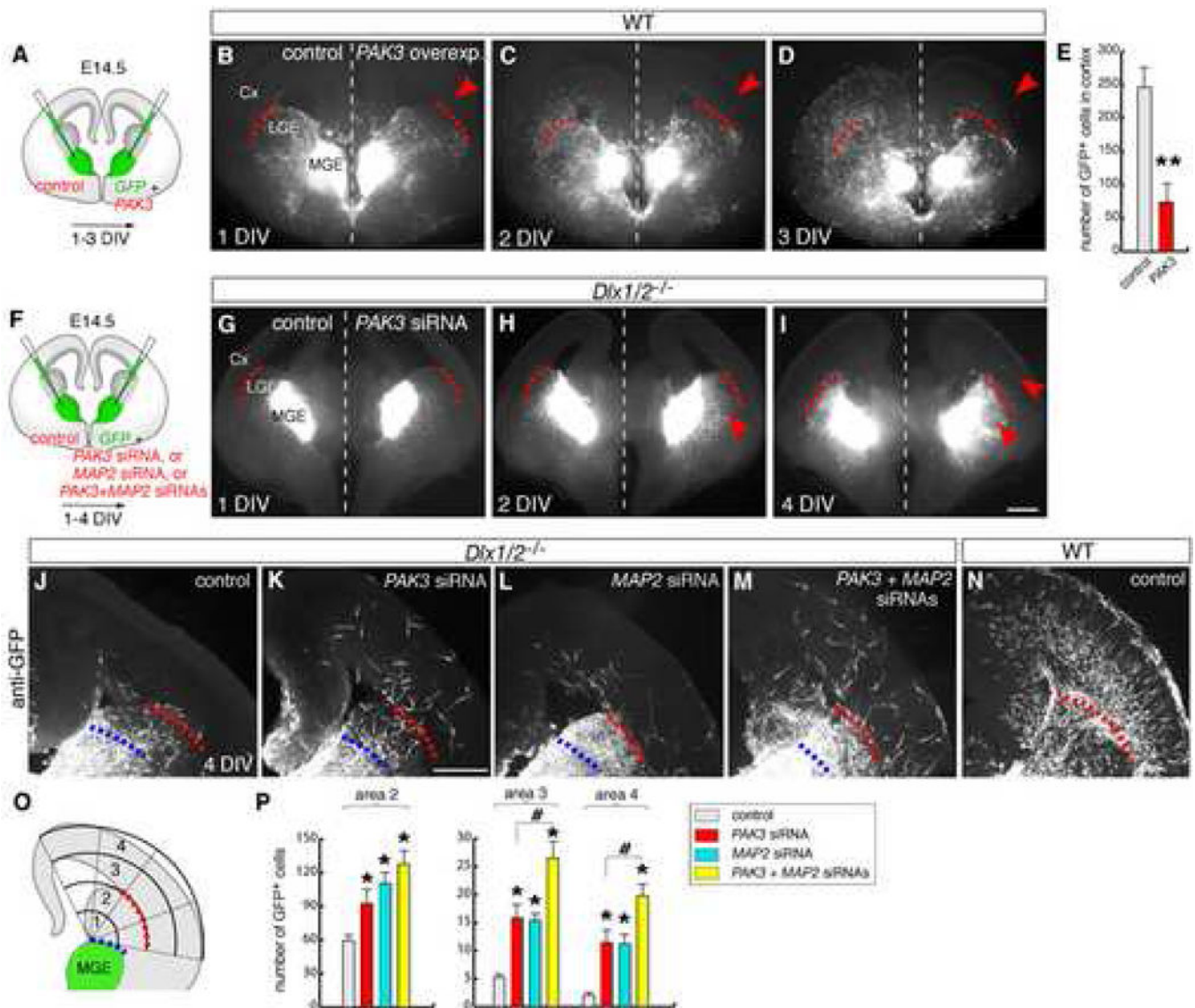
(A–D) RNA *in situ* hybridization for *PAK3* in coronal sections through the telencephalon of wild-type (A–D) and *Dlx1/2*<sup>-/-</sup> mutant (A'–C') embryos at E11.5, E14.5, and P0. Note the absence of detectable *PAK3* expression in the MGE SVZ of wild-type embryos, and increased expression in the MGE SVZ of *Dlx1/2*<sup>-/-</sup> mutants (arrowheads in A'–C'). (D) *PAK3* expression in wild-type postnatal (P7) hippocampus. Black arrows point to *PAK3* expression in hippocampal layers that contain interneurons.

(E–G) Immunofluorescent staining for PAK3 protein in cultures from wild-type E15.5 MGE after 3, 5 and 7 DIV.

(H) Immunoblot analysis of PAK3 from cell lysates of MGE cultures after 3, 5 and 7 DIV.

Scale bars = 500  $\mu$ m (A,B,C,D) and 50  $\mu$ m (E–G).



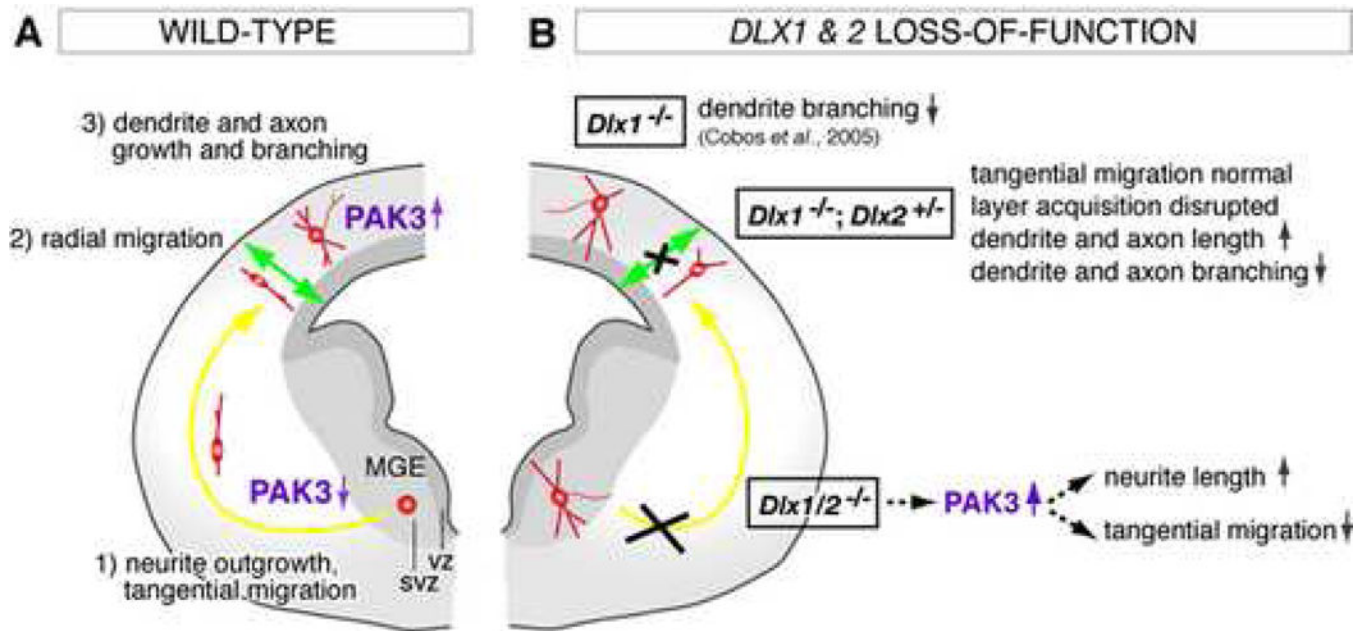


(G–I) *In vivo* labeling of GFP neurons from one *Dlx1/2<sup>-/-</sup>* embryo after co-electroporation with *PAK3* siRNA and *GFP*-expressing vector (right hemisphere), and siRNA control and *GFP*-expressing vector (left hemisphere). Arrowheads in (H, I) indicate increased migration to dorsal LGE and cortex.

(J–N) Anti-GFP immunohistochemistry in representative *Dlx1/2<sup>-/-</sup>* slices that were electroporated with siRNAs: control (J), *PAK3* (K), *MAP2* (L), or *PAK3+MAP2* (M) siRNAs. The slices were fixed and re-sectioned (75  $\mu$ m). Note increased migration of GFP<sup>+</sup> cells to cortex after *PAK3* and *MAP2* siRNA electroporations. Migration in one representative wild-type slice that was electroporated with siRNA control is shown in (N).

(O, P) Quantification of numbers of GFP<sup>+</sup> cells migrated to cortex in *Dlx1/2<sup>-/-</sup>* slices after 4 DIV. A grid with concentric circles was superimposed to each microphotograph, with its center placed at the dorsal limit of the electroporated area (typically between MGE and LGE; blue dash lines). The number of GFP<sup>+</sup> cells in areas 2 (dorsal LGE), 3 (ventrolateral cortex) and 4 (dorsal cortex) were counted [control: 59.3  $\pm$  4.9 (area 2), 5.2  $\pm$  0.7 (area 3), 2  $\pm$  0.5 (area 4); *PAK3*: 92.4  $\pm$  12.3 (area 2), 15.8  $\pm$  2.5 (area 3), 11.4  $\pm$  2.2 (area 4); *MAP2*: 110.0  $\pm$  9.2 (area 2), 15.4  $\pm$  1.2 (area 3), 11.2  $\pm$  1.7 (area 4); *PAK3+MAP2*: 127.5  $\pm$  12.3 (area 2), 26.5  $\pm$  3 (area 3), 19.7  $\pm$  2.1 (area 4); *n* = 15 slices for each type). \* *p* < 0.05 versus control; # *p* < 0.05 versus *PAK3* siRNA group (Tukey-Kramer test).

Scale bars =  $\mu$ m (B–D, G–I, and J–N).



**Figure 8. Model for *Dlx1&2* functions in the migration and neuronal differentiation of GABAergic interneurons**

(A) Most mouse precursors for cortical GABAergic interneurons (red) are generated in the medial ganglionic eminence (MGE) in the ventral telencephalon. Their differentiation program involves the outgrowth of neurites and tangential migration (yellow arrow) towards the cortical plate (Marin and Rubenstein, 2003; Metin *et al.*, 2006; Wonders and Anderson, 2006). Within the cortical plate, they change their mode of migration from tangential to radial (green arrow) to acquire their laminar positions (Nadarajah *et al.*, 2002; Tanaka *et al.*, 2003; Hevner *et al.*, 2004; Pla *et al.*, 2006). In neocortex, they grow and pattern their dendritic and axonal arbors, at the time that they up-regulate expression of the PAK3 kinase. Reducing PAK3 levels using siRNA against *PAK3* inhibits the lengthening of neuronal processes.

(B) *Dlx* dosage has a profound effect on the migration and differentiation of GABAergic interneurons. In *Dlx1/2*<sup>-/-</sup> double mutants, interneuron precursors fail to migrate tangentially; instead they partially differentiate within the subventricular zone (SVZ) of the MGE (Anderson *et al.*, 1997a). They show increased neurite length. In *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> compound mutants, interneurons are able to migrate tangentially to neocortex, but show defects in the acquisition of their laminar positions, and exhibit dendritic and axonal processes with increased length and decreased branching. In *Dlx1*<sup>-/-</sup> single mutants, a subset of neocortical interneurons show subtle defects in dendritic morphogenesis; these mice survive to adulthood and develop epilepsy (Cobos *et al.*, 2005). Complete loss of *Dlx1&2* results in premature expression of several proteins that regulate the cytoskeleton (i.e., MAP2, Tau1, GAP43 and PAK3) in the SVZ of the MGE. Overexpression of PAK3 in the MGE is sufficient to block tangential migration. Reducing PAK3 and MAP2 levels in the MGE of *Dlx1/2*<sup>-/-</sup> mutants partially rescues the tangential migration defect.