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## Ldb1 is essential for development of Nkx2.1 lineage derived GABAergic and cholinergic neurons in the telencephalon

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### Abstract

The progenitor zones of the embryonic mouse ventral telencephalon give rise to GABAergic and cholinergic neurons. We have shown previously that two LIM-homeodomain (LIM-HD) transcription factors, *Lhx6* and *Lhx8*, that are downstream of *Nkx2.1*, are critical for the development of telencephalic GABAergic and cholinergic neurons. Here we investigate the role of *Ldb1*, a nuclear protein that binds directly to all LIM-HD factors, in the development of these ventral telencephalon derived neurons. We show that *Ldb1* is expressed in the *Nkx2.1* cell lineage during embryonic development and in mature neurons. Conditional deletion of *Ldb1* causes defects in the expression of a series of genes in the ventral telencephalon and severe impairment in the tangential migration of cortical interneurons from the ventral telencephalon. Similar to the phenotypes observed in *Lhx6* or *Lhx8* mutant mice, the *Ldb1* conditional mutants show a reduction in the number of both GABAergic and cholinergic neurons in the telencephalon. Furthermore, our analysis reveals defects in the development of the parvalbumin-positive neurons in the globus pallidus and striatum of the *Ldb1* mutants. These results provide evidence that *Ldb1* plays an essential role as a transcription co-regulator of *Lhx6* and *Lhx8* in the control of mammalian telencephalon development.

### Keywords

Differentiation; Forebrain development; Interneuron; Mouse; Tangential migration

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## Introduction

Functioning of the mammalian telencephalon is dependent on the formation of complex circuitry by different types of neurons. Many of these neurons are derived from distinct progenitor domains in the ventral telencephalon during embryonic development. Largely through mouse mutant analysis, the molecular genetic program controlling the generation of these neurons has begun to be unveiled.

Nkx2.1 is a transcription factor expressed in the medial ganglionic eminence (MGE) and preoptic area (POA) of the developing telencephalon. Loss-of-function studies have revealed that Nkx2.1 is essential for both patterning of the MGE/POA and the specification/differentiation of a variety of MGE/POA-derived neurons, including GABAergic interneurons, GABAergic projection neurons, and cholinergic neurons (Sussel et al., 1999, Marin et al., 2000, Butt et al., 2008, Du et al., 2008, Flandin et al., 2011).

The function of Nkx2.1 in the developing telencephalon is mediated by controlling the expression of two closely related LIM-homeodomain (LIM-HD) genes, *Lhx6* and *Lhx8*. Deletion of *Nkx2.1* impairs the *Lhx6* and *Lhx8* expression in the ventral telencephalon (Sussel et al., 1999, Marin et al., 2000). Exogenous expression of *Lhx6* restores the ability for the generation of cortical interneurons in *Nkx2.1*<sup>-/-</sup> MGE cells. Moreover, RNAi knockdown of *Lhx6* blocks the rescue of the interneuron phenotype in the *Nkx2.1*<sup>-/-</sup> cells by exogenous *Nkx2.1* expression (Du et al., 2008). Nkx2.1 has also been shown to bind directly to a promoter region of the *Lhx6* and to activate reporter gene expression (Du et al., 2008).

Roles of *Lhx6* and *Lhx8* in telencephalon development have been extensively analyzed. Deletion of *Lhx6* severely impairs tangential migration and specification of telencephalic GABAergic interneurons (Liodis et al., 2007, Zhao et al., 2008, Neves et al., 2012). Inactivation of *Lhx8* causes defects in telencephalic cholinergic neuron development (Zhao et al., 2003, Mori et al., 2004, Fragkouli et al., 2005, Fragkouli et al., 2009, Lopes et al., 2012). Analysis of double mutants further revealed that *Lhx6/Lhx8* are required to directly control *Sonic hedgehog* (*Shh*) gene expression in the MGE and to regulate the development of the globus pallidus (Flandin et al., 2011).

It has been suggested that LIM-HD proteins regulate gene transcription by forming multi-protein complexes through binding to other nuclear factors (Hobert and Westphal, 2000). Ldb1 (LIM-domain-binding protein 1) is one of such factors (Agulnick et al., 1996, Jurata et al., 1996, Bach et al., 1997). Previous mouse mutant studies have shown that Ldb1 plays important roles in controlling multiple aspects of embryogenesis ranging from early embryo patterning to limb, pancreas, and spinal cord development (Thaler et al., 2002, Mukhopadhyay et al., 2003, Tzchori et al., 2009, Hunter et al., 2013). To determine the role of Ldb1 in development of the Nkx2.1-lineage derived neurons in the telencephalon, we generated a *Ldb1* conditional mutant by crossing a *Ldb1* floxed mouse line (Zhao et al., 2007) with a BAC-transgenic line expressing the Cre recombinase under control of enhancer elements of the *Nkx2.1* (Xu et al., 2008). Our studies show that Ldb1 is essential for the development of multiple types of neurons in the telencephalon.

## Materials and Methods

### Animals

Animals were maintained and handled by following the National Institutes of Health guidelines and procedures approved by the animal care and use committee of the National Institute of Child Health and Human Development.

To specifically inactivate *Ldb1* function in the cells of the *Nkx2.1*-lineage, mice carrying one null allele of the *Ldb1* gene (*Ldb1*<sup>+/-</sup>) (Mukhopadhyay et al., 2003) were crossed to a BAC-transgenic mouse line that expresses the Cre recombinase under control of regulatory elements of the *Nkx2.1* gene (*Nkx2.1-Cre*<sup>+</sup>) (Xu et al., 2008). Offspring double heterozygous for the *Ldb1* and the *Nkx2.1-Cre* alleles (*Ldb1*<sup>+/-</sup>; *Nkx2.1-Cre*<sup>+</sup>) were mated with homozygous *Ldb1* floxed (*Ldb1*<sup>fl/fl</sup>) mice (Zhao et al., 2007) to generate *Ldb1/Nkx2.1-Cre* conditional mutant (*Ldb1*<sup>fl/-</sup>; *Nkx2.1-Cre*<sup>+</sup>) and control (*Ldb1*<sup>fl/+</sup>; *Nkx2.1-Cre*<sup>+</sup>, *Ldb1*<sup>fl/+</sup>; *Nkx2.1-Cre*<sup>-</sup>, or *Ldb1*<sup>fl/-</sup>; *Nkx2.1-Cre*<sup>-</sup>) animals for analysis. For the experiments that required genetic marking of the *Nkx2.1*-lineage cells, the *Ldb1*<sup>+/-</sup>; *Nkx2.1-Cre*<sup>+</sup> mice were crossed to the *Rosa26-Yellow Fluorescent Protein (YFP)* reporter (*R26R-YFP*) line (*R26*<sup>+/LoxP-stop-LoxP-YFP) (Srinivas et al., 2001) for two generations to generate offspring that were heterozygous for the *Ldb1*-null and *Nkx2.1-Cre* alleles, and homozygous for the *YFP* reporter allele (*Ldb1*<sup>+/-</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>LoxP-stop-LoxP-YFP/LoxP-stop-LoxP-YFP</sup>). These mice were mated with homozygous *Ldb1* floxed (*Ldb1*<sup>fl/fl</sup>) mice to produce *Ldb1* conditional mutant (*Ldb1*<sup>fl/-</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+/LoxP-stop-LoxP-YFP</sup>) and control (*Ldb1*<sup>fl/+</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+/LoxP-stop-LoxP-YFP</sup>) animals for analysis. The *Ldb1*-null, *Ldb1*-floxed, and the *Nkx2.1-Cre*<sup>+</sup> alleles were maintained in a C57/BL6 background, whereas the *R26-YFP* reporter allele was in a CD-1 background. Genotyping of the various alleles was performed by PCR as previously described in the reference for each of these alleles.</sup>

### Tissue preparation

To obtain embryos of specific developmental stages, female mice were checked daily for the presence of vaginal plug after mating. Noon of the day when a plug was detected was considered embryonic day (E) 0.5. Embryo heads (E12.5 to E14.5) or brains (E18.5) were fixed by immersion in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) overnight at 4°C. After wash in phosphate-buffered saline (PBS; pH 7.4), the tissue was dehydrated through a series of ethanol solutions with ascending concentrations and embedded in paraffin, or cryo-protected in sucrose/PBS (pH 7.4) (15%, and then 30%) solutions, embedded in Tissue-Tek OCT compound, and frozen on dry ice.

Postnatal mice were fixed by transcardial perfusion with 4% PFA/0.1 M PB (pH 7.4). The brains were dissected and post-fixed in PFA overnight at 4°C. The brains were washed in PBS (pH 7.4), cryo-protected in sucrose (15%, and then 30%)/PBS solutions, embedded in OCT compound, and frozen on dry ice.

### Immunohistochemistry

The primary antibodies and their dilutions used are as follows: rabbit anti-Ldb1 (a gift from Drs. Liqi Li and Paul Love, NICHD, Li et al., 2011, 1:2000), chick anti-Green Fluorescent Protein (GFP) (Aves Labs, GFP-1020, also reactive to YFP, 1:500), goat anti-Choline Acetyltransferase (ChAT) (Millipore, AB144, 1:250), rat anti-Somatostatin (SOM) (Millipore, MAB354, 1:150), mouse anti-Parvalbumin (PV) (Sigma, P3088, 1:2000).

Paraffin (5 μm thick) or frozen (16 μm) sections of the embryo heads (E12.5 and E14.5) or brains (E18.5) were cut and mounted onto silanized (KD Medical) or Superfrost Plus (Fisher) microscope slides. For immunofluorescent staining of frozen sections using antibodies from a non-mouse species, the sections were blocked in PBS containing 2% bovine serum albumin (BSA, Vector Laboratory) and 5% normal serum from the same species where the secondary antibody was derived. The sections were incubated overnight at 4°C in the primary antibodies diluted with PBS containing 2% of the normal serum. After washes in PBS, sections were incubated with secondary antibodies labeled with Alexa-488 or Alexa-568 (Invitrogen). The sections were washed in PBS and coverslipped with a ProLong Gold antifade reagent (Invitrogen). Staining using mouse primary antibodies was

carried out with a M.O.M kit (Vector Laboratory) by following the manufacturer's instruction. For immunofluorescent staining of paraffin sections, the sections were deparaffinized in xylene, and rehydrated through a series of ethanol solutions with descending concentrations. Sections were treated with an antigen retrieval solution (Buffer A, Electron Microscopy Sciences) using a PickCell 2100 retriever (Electron Microscopy Sciences), and then processed by following the same procedure used for the staining of frozen sections. For immunoperoxidase staining, paraffin sections were processed in procedures similar to those used for immunofluorescent staining with an additional 15-minute incubation in a hydrogen peroxide solution (0.3% in methanol) before rehydration through the ethanol. After primary antibody incubation, staining was detected by using an ABC elite kit (Vector Laboratory) and AEC solution (Millipore) as a peroxidase substrate.

Frozen sections of postnatal mouse brains were cut at a thickness of 40  $\mu\text{m}$  on a cryostat and kept free-floating at 4°C in PBS. Staining of the sections was carried out in 6-well plates by procedures similar to those described above except for lengthening the primary antibody incubation to 40 hours and for adding Triton X-100 to PBS (at a concentration of 0.4%) for washing and primary antibody incubation.

### Cell counting

To count immunostained neurons, comparable coronal sections (40  $\mu\text{m}$  thick) of the striatum (anterior to the crossover of the anterior commissure), septum, nucleus Basalis, the anterior hippocampus, and the cortex (anterior to the crossover of the anterior commissure) from both control and *Ldb1/Nkx2.1-Cre* mutants were photographed under a 4x objective on a Nikon (E1000) microscope. Labeled cells within the entire regions of the striatum, septum, nucleus Basalis, and hippocampus shown on the photos were counted manually. Labeled cells in the cortex were counted only in a medial area between the midline and 1 mm lateral to the midline.

### In situ hybridization

*In situ* RNA hybridization experiments were performed using digoxigenin labeled riboprobes on 20  $\mu\text{m}$  frozen sections as previously described (Flandin et al., 2011). All riboprobes used were previously described (Flandin et al., 2011).

### Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the kit from Pierce. Briefly, each reaction (20 $\mu\text{l}$ ) consisted of 2  $\mu\text{g}$  nuclear extract and 1 fmole/ $\mu\text{l}$  of biotinylated probes, in binding buffer consisting of: 10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 5% glycerol, 1 mM EDTA, 50 ng/ $\mu\text{l}$  poly (dI-dC) (Sigma) and 50 ng/ $\mu\text{l}$  BSA (New England Biolabs).

LHX6, LHX8 and LDB1 proteins were generated by Fugene6 transfection of HEK 293 cells. After 48 hours, nuclear extracts were prepared using the Pierce nuclear extract kit. Biotinylated DNA probes: Probe A corresponded to the 26–64 bp of the SBE3 *Shh* enhancer and included LHX site A (Flandin et al., 2011); mutated probe A had the same nucleotide sequence as the wild type probe A, but the LHX site core sequence (TAATCA) was changed to TTTTTT.

## Results

### Expression of Ldb1 in Nkx2.1-lineage cells derived from the ventral telencephalon

To determine whether Ldb1 protein is expressed in the cells derived from the Nkx2.1-lineage in the developing telencephalon, we genetically marked these cells in the mouse embryos that carried both the *Nkx2.1-Cre* transgene and the *R26R-YFP* reporter allele; cells

expressing *Nkx2.1-Cre* become YFP<sup>+</sup> (Xu et al., 2008). Immunostaining revealed that at E12.5 most cells in the MGE and the POA expressed YFP (Figs. 1B, C, and data not shown). Scattered YFP<sup>+</sup> cells were also detected within the LGE (Fig. 1D); many of these cells are likely to be MGE-derived striatal interneurons (Marin et al., 2000; Nóbrega-Pereira et al., 2008). Two streams of YFP<sup>+</sup> cells were found in the cortical interneuron tangential migrating routes (Fig. 1D; Marin and Rubenstein 2001).

Ldb1 protein was detected extensively in the developing telencephalon, including in many *Nkx2.1*-lineage (YFP<sup>+</sup>) cells in the MGE and LGE (Figs. 1A, C, D). By E18.5, a large number of migrating YFP<sup>+</sup> cells had reached the cortex and hippocampus, where they continued to express Ldb1 (Figs. 1G, H). In addition, Ldb1 was detected in *Nkx2.1*-lineage (YFP<sup>+</sup>) cells in the ventral telencephalon, including in the septum (Fig. 1E), the striatum (not shown), and the globus pallidus (Fig. 1F).

Since the *Nkx2.1*-lineage cells contribute to a variety of differentiated neuronal cell types in different regions of the telencephalon, we examined whether Ldb1 protein was expressed in these neurons in postnatal (P18) animals. Double immunofluorescent staining of Ldb1 and ChAT showed that Ldb1 was expressed in cholinergic interneurons in the striatum (Fig. 2A), and in cholinergic projection neurons in the septum, nucleus Basalis, and the magnocellular preoptic nucleus (Figs. 2B, C, D).

Double staining of Ldb1/PV and Ldb1/SOM revealed that Ldb1 was also expressed in PV<sup>+</sup> (Figs. 2E, F, G, H, M) and SOM<sup>+</sup> (Figs. 2I, J, K, L) subtypes of GABAergic interneurons, respectively, throughout the telencephalon, including the striatum, septum, globus pallidus, neocortex and hippocampus. Thus, Ldb1 was expressed in the *Nkx2.1*-lineage cells in the telencephalon early in embryonic development through P18.

#### Deletion of *Ldb1* in the ventral telencephalon of the *Ldb1/Nkx2.1-Cre* conditional mutant

Homozygous *Ldb1*-null mutants die around E9.5 with severe developmental defects in the head and heart (Mukhopadhyay et al., 2003). To determine the function of Ldb1 in the development of the *Nkx2.1*-lineage derived cells in the telencephalon, we generated a conditional mutant (*Ldb1*<sup>f/f</sup>; *Nkx2.1-Cre*<sup>+</sup>) with a specific deletion of the *Ldb1* in these cells by crossing the *Ldb1* floxed mice (*Ldb1*<sup>f/f</sup>, Zhao et al., 2007) with mice that carry one *Ldb1* null allele and an *Nkx2.1-Cre* transgene (*Ldb1*<sup>+/-</sup>; *Nkx2.1-Cre*<sup>+</sup>). Consistent with the result that Ldb1 was expressed in *Nkx2.1*-lineage cells (Fig. 1), immunostaining of Ldb1 in both E12.5 and E14.5 embryos confirmed that Ldb1 was missing in most of the cells in the MGE and the underlying POA of the *Ldb1/Nkx2.1-Cre* mutants (Figs. 3A–H). However, at both E12.5 and E14.5, some Ldb1<sup>+</sup> cells were still present in the mutants, especially in the dorsal and the rostral regions of the MGE (Figs. 3B, D, F, H). The presence of these Ldb1<sup>+</sup> cells was probably due to a previously reported lack of expression of the Cre recombinase in the dorsal and rostral regions of the MGE of the *Nkx2.1-Cre* transgenic mouse (Xu et al., 2008).

#### Ldb1 is required for *Shh* expression and *in vitro* binding of Lhx6 and Lhx8 to the *Shh* enhancer in MGE neurons

*Lhx6*<sup>-/-</sup>/*Lhx8*<sup>-/-</sup> double mutants fail to express *Shh* in early born neurons of the MGE (Flandin et al., 2011). Thus, we began our molecular analysis of the conditional *Ldb1* mutant by studying *Shh* RNA expression in the ventral telencephalon. At E12.5, while *Shh* expression in the ventricular zone (VZ) of the POA and ventral MGE is preserved, *Shh* expression in MGE mantle zone (MZ) is greatly reduced, except in the rostral MGE (Figs. 4A–C, A'–C').

Previously, we demonstrated that Lhx6 and Lhx8 can compensate for each other in the regulation of *Shh* expression in the MGE MZ, and that both proteins can bind to the *Shh* enhancer (SBE3 domain) in electrophoretic mobility shift assays (Flandin et al., 2011). In those assays, we included Ldb1 protein. Here we tested whether Ldb1 was required for the binding of Lhx6 and Lhx8 to the SBE3 *Shh* enhancer. We found that when Ldb1 was not added to nuclear extracts, Lhx6 and Lhx8 failed to bind to the SBE3 *Shh* enhancer (Fig. 5). Furthermore, mutating the core Lhx6/Lhx8 binding site of SBE3 *Shh* enhancer resulted in a strong reduction of the amount of the complex. Thus, this provides *in vitro* biochemical evidence that Ldb1 is required to enable Lhx6 and Lhx8 to bind the SBE3 enhancer. Lack of binding may underlie the reduced *Shh* expression in the MGE MZ of the *Ldb1* conditional mutant (Figs. 4A–C, 4A'–C').

### Molecular defects in the MGE and MGE-derived pallidal neurons of the *Ldb1/Nkx2.1-Cre* mutant

We continued our study of the conditional *Ldb1* mutant by *in situ* hybridization analysis of expression of a series of genes in the VZ, SVZ, and MZ (including the GP) of the developing MGE. At E12.5, similar to *Shh*, the expression of the *Gbx2* homeobox gene in the MZ of the MGE was reduced in the mutant (Figs. 4D–F, 4D'–F'). At E14.5 we found gene expression defects in the basal ganglia (Fig. 6) and in cortical interneurons (Fig. 7). There were no definitive VZ defects, although *Nkx2.1* expression may be increased in both the VZ and SVZ (Figs. 6A–C, 6A'–C'). There was reduced SVZ expression of *Cux2*, *Lmo3*, *Mafb* and *NPY* (Figs. 6 and 7), whereas SVZ expression of *Arx*, *Dlx1*, *Er81*, *ErbB4*, *Lhx6*, *Lhx8* and *Sox6* appeared normal (Figs. 6 and 7). In the MZ, while there were increased *Nkx2.1*<sup>+</sup> scattered cells, there were much fewer *Arx*, *Gbx1*, *Lhx6*, *Lhx8*, and *Somatostatin* expressing cells (Figs. 6 and 7). The GP formed, based on *Nkx2.1* expression (Figs. 6A–C, 6A'–C'); however it was smaller and less compact, based on expression of *Dlx1*, *Er81*, *Gbx1*, *Lhx6*, *Lhx8*, *Lmo3* and *Sox6* (Figs. 6 and 7) (black arrow). Likewise the ventral pallidal and pallidal-septal cells expressed less *Gbx1*, *Lhx6* and *Lhx8* (Fig. 6).

### Molecular defects in the MGE and MGE-derived cortical and striatal interneurons of the *Ldb1/Nkx2.1-Cre* mutant

Due to the severe defects in the differentiation of MGE subpallial derivatives, we analyzed development of MGE-derived cortical interneurons at E14.5, which at that stage are tangentially migrating through the cortex (Figs. 6 and 7). Expression of *Arx*, *Cux2*, *Cxcr4*, *ErbB4*, *NPY* and *Somatostatin* was greatly reduced in both the superficial and deep cortical migration streams; expression of *Dlx1*, *Mafb* and *Lhx6* were preferentially reduced in the superficial stream (Figs. 6 and 7). Both neocortical and paleocortical interneurons showed reduced expression of *Arx*, *Cux2*, *Cxcr4*, *Dlx1*, *ErbB4*, *Mafb*, *Lhx6*, *NPY* and *Somatostatin* (black and white arrowheads, respectively).

Striatal interneuron marker expression was also reduced in the conditional *Ldb1* mutant. Striatal expression of *Lhx8*, *Er81*, *ErbB4*, *NPY* and *Somatostatin* was not detectable (Figs. 6 and 7). Striatal expression of *Nkx2.1* and *Lhx6* was clearly reduced, but persistent in some cells (Fig. 6).

### Defects in the distribution of the Nkx2.1-lineage cells in the developing telencephalon of the *Ldb1/Nkx2.1-Cre* mutant

To further examine whether deletion of *Ldb1* affected the migration and distribution of the cells derived from the Nkx2.1-lineage, we crossed our mice to the *R26R-YFP* reporter line to generate control (*Ldb1*<sup>f/+</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+LoxP-stop-LoxP-YFP</sup>) and *Ldb1* mutant mice that also carried the *YFP* reporter allele (*Ldb1*<sup>f/-</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+LoxP-stop-LoxP-YFP</sup>). In these

mice the *YFP* reporter gene was activated specifically in the *Nkx2.1*-lineage cells. Immunostaining for YFP at E14.5 revealed fewer migrating YFP<sup>+</sup> cells that reached the dorsal part of the cortex in the mutant than those in the control (Figs. 8A, B), consistent with the *in situ* hybridization results (Fig. 6). The YFP<sup>+</sup> cells in the GP were reduced in number and were less organized in the mutant compared to the control (Figs. 8C, D), again consistent with the gene expression data (Fig. 6). At E18.5, fewer YFP<sup>+</sup> cells were present in the marginal zone and the cortical plate of the neocortex in the mutant compared to the control (arrowheads, Figs. 8E, E', F, F'). In contrast, more YFP<sup>+</sup> cells were present in the subventricular zone of the cortex in the mutant than those in the control (arrows, Figs. 8E, E', F, F'). YFP<sup>+</sup> cells were detected in the hippocampus of both control and *Ldb1* mutant embryos. However, unlike in the control, more of the cells were scattered in the stratum oriens (arrows, Figs. 8G, H) whereas fewer of them were seen in a compact layer adjacent to the pyramidal cell layer (arrowheads, Figs. 8G, H) in the mutant.

In the ventral telencephalon the mutant had an ectopic cluster of YFP<sup>+</sup> cells in the dorsal-lateral corner of the MGE, suggestive of a failure of migration from the progenitor zone (arrows, Figs. 8I, J). The mutant also showed a large reduction of YFP<sup>+</sup> cells in the GP, whose internal organization appeared disrupted (Figs. 8M, N). The striatum had a moderate reduction of YFP<sup>+</sup> cells (Figs. 8K, L). Together, these results indicate that the migration and distribution of the cells derived from the *Nkx2.1*-lineage were impaired in the *Ldb1/Nkx2.1-Cre* mutant.

Next we examined the YFP<sup>+</sup> cells in the postnatal *Ldb1/Nkx2.1-Cre* mutant. At around two weeks after birth (P12 to P16), the number of YFP<sup>+</sup> cells was reduced in the various telencephalic structures in the mutant. These included the neocortex, hippocampus, striatum, and globus pallidus (Figs. 9A–H).

### **Reduction in the number of cholinergic neurons in the telencephalon of the postnatal *Ldb1/Nkx2.1-Cre* mutant**

Previous studies have shown that *Nkx2.1*, and its downstream gene *Lhx8*, are required for the proper development of cholinergic neurons in the telencephalon (Sussel et al., 1999; Marin et al. 2000; Zhao et al. 2003; Lopes et al., 2012; Mori et al., 2004; Fragkouli et al., 2005). To determine whether *Ldb1* is required for the development of these neurons, we analyzed the *Ldb1/Nkx2.1-Cre* mutant by immunostaining of ChAT, an enzyme essential for acetylcholine synthesis. The number of cholinergic neurons was reduced in the striatum [average  $\pm$  standard deviation (sd): control  $190 \pm 24$ ; mutant  $21 \pm 5$ , nine sections from 3 animals for each group,  $P < 9 \times 10^{-9}$  (Figs. 10A, B, E, F)], septum [average  $\pm$  sd: control  $278 \pm 40$ ; mutant  $38 \pm 9$ , three sections from 3 animals for each group,  $P < 0.007$  (Fig. 10C, D)], and the nucleus Basalis [average  $\pm$  sd: control  $106 \pm 15$ ; mutant  $48 \pm 15$ , six sections from 3 animals for each group,  $P < 5 \times 10^{-5}$  (Fig. 10G, H)] in the *Ldb1* mutants at P16 and P18.

### **Reduction in the number of cortical and hippocampal GABAergic interneurons in the postnatal *Ldb1/Nkx2.1-Cre* mutant**

*Lhx6* is essential for the development of the PV<sup>+</sup> and SOM<sup>+</sup> subclasses of the GABAergic interneurons in the cortex and hippocampus (Liodis et al., 2007; Neves et al., 2012; Zhao et al., 2008). Thus, we examined these neurons in the *Ldb1/Nkx2.1-Cre* mutant. Immunostaining of PV and SOM revealed that the number of PV<sup>+</sup> and SOM<sup>+</sup> neurons in the cortex [PV<sup>+</sup> neurons, average  $\pm$  sd: control  $259 \pm 23$ ; mutant  $125 \pm 13$ , six sections from 3 animals for each group,  $P < 2 \times 10^{-6}$ ; SOM<sup>+</sup> neurons, average  $\pm$  sd: control  $138 \pm 31$ ; mutant  $83 \pm 21$ , four sections from 2 animals for each group,  $P < 0.04$ ] and hippocampus [PV<sup>+</sup> neurons, average  $\pm$  sd: control  $118 \pm 24$ ; mutant  $26 \pm 7$ , fourteen sections from 3 controls and twelve sections from 3 mutants,  $P < 8 \times 10^{-10}$ . SOM<sup>+</sup> neurons, average  $\pm$  sd: control  $60 \pm$



18; mutant  $28 \pm 8$ , four sections from 2 animals for each group,  $P < 0.03$ ] of the mutant was reduced compared to the control at P16 or P18 (Fig. 11). Thus, the reduction in number of the PV<sup>+</sup> and SOM<sup>+</sup> neurons in the mutant hippocampus was more pronounced than that in the cortex.

### Defects in development of the globus pallidus and striatal parvalbumin<sup>+</sup> neurons in the postnatal *Ldb1/Nkx2.1-Cre* mutant

Immunostaining of PV revealed additional defects in the basal telencephalon of the postnatal *Ldb1/Nkx2.1-Cre* mutant. Consistent with results from the molecular analysis of the defects in the E12.5 and E14.5 MGE and GP of the *Ldb1* mutant embryos (Figs. 6 and 7), the GP in the postnatal *Ldb1* mutant appeared irregular in shape and was smaller compared to the controls (Fig. 12 A, D). As reported previously (Zhao et al., 2003; 2008), the GP in *Lhx6* or *Lhx8* single mutants appeared normal (Figs. 12B, C). PV<sup>+</sup> interneurons in the striatum were not affected in the *Lhx8* mutant (Zhao et al., 2003). Although our previous study showed that the distribution of the PV<sup>+</sup> interneurons was altered in the *Lhx6* single mutant, the number of these neurons was not changed significantly (Zhao et al., 2008; Fig. 12F). However, a reduction in number of the PV<sup>+</sup> neurons in the striatum was observed in the *Ldb1/Nkx2.1-Cre* mutant [average  $\pm$  sd: control  $117 \pm 24$  (16 sections from 3 animals); mutant  $45 \pm 10$  (16 sections from 3 animals),  $P < 5 \times 10^{-10}$ ] (Fig. 12G). Thus the *Ldb1/Nkx2.1-Cre* mutant showed a broader phenotype in the ventral telencephalon than that was observed previously in either the *Lhx6* or *Lhx8* single mutant.

## Discussion

Previous studies have established that the LIM-HD factors *Lhx6* and *Lhx8* regulate the development of multiple types of neurons in the telencephalon (Zhao et al., 2003; 2008; Mori et al., 2004; Fragkouli et al., et al., 2005; 2009; Lodis et al., 2007; Lopes et al., 2012; Neves et al., 2012). However, the molecular mechanisms underlying how these transcription factors function have largely remained unclear. The nuclear factor *Ldb1* has been shown to directly bind to the LIM domains of LIM-HD factors (Aglunick et al., 1996). Disruption of *Ldb1* function in a number of developing systems has been shown to result in various defects similar to those observed after inactivation of certain LIM-HD factors (Aglunick et al., 1996; Mukhopadhyay et al., 2003; Zhao et al., 2007; Tzchori et al., 2009; Hunter et al., 2012). These studies indicate that *Ldb1* plays an essential role in mediating the function of the LIM-HD factors.

In the present study, we showed that *Ldb1* is expressed in telencephalic *Nkx2.1*-lineage cells, a major population that contributes to pallidal projection neurons, and striatal and pallial interneurons. To determine the function of *Ldb1* in the development of these neurons, we deleted the *Ldb1* in *Nkx2.1*-cell lineage by crossing the *Ldb1*-floxed mouse line with the *Nkx2.1-Cre* line. Inactivation of *Ldb1* resulted in striking reductions in the expression of genes that control MGE neuronal development, including those that are critically involved in the transcription regulation or cell signaling. As a result, the *Ldb1/Nkx2.1-Cre* conditional mutant showed severe defects in the generation and migration of cortical and sub-cortical GABAergic interneurons, pallidal GABAergic projection neurons, and telencephalic cholinergic neurons.

The phenotype of the *Ldb1/Nkx2.1-Cre* mutant is more severe than the *Lhx6* and *Lhx8* single mutants, or closely phenocopies the *Lhx6/Lhx8* double mutants. The developmental defects in GABAergic interneurons of the *Ldb1* conditional mutant are similar to those observed previously in the *Lhx6* mutant (Lodis et al., 2007; Zhao et al., 2008). Many defects in development of the cholinergic neurons are observed both in the *Ldb1* conditional mutant and in the *Lhx8* mutant (Zhao et al., 2003; Mori et al., 2004; Fragkouli et al., 2005). It is

possible that *Ldb1* mutant-dependent cholinergic defects also arise from its potential interaction with *Islet-1*, a LIM-HD transcription factor that like *Lhx8*, regulates telencephalic cholinergic neuronal development (Elshatory and Gan, 2008).

Both the *Ldb1* conditional mutant and the *Lhx6/Lhx8* double mutant share interneuron defects, but unlike the single mutants, also had an abnormal globus pallidus. Together these genetic results support that biochemical model that *Ldb1* is an essential co-factor for the *Lhx6* and *Lhx8* LIM-HD proteins in regulating the development of the *Nkx2.1*-lineage cells in the ventral telencephalon. Moreover, EMSA analysis showed that the binding of *Lhx6* or *Lhx8* to the *Shh* gene enhancer is dependent on the presence of *Ldb1*. This further supports the essential role of *Ldb1* as a co-factor with *Lhx6* and/or *Lhx8* in the transcriptional regulation of the development of the *Nkx2.1*-lineage derived neurons in the telencephalon.

Similar to the *Lhx6/Lhx8* double mutant, the *Ldb1/Nkx2.1-Cre* mutant showed a reduction of the *Shh* expression in the MGE mantle zone. We have reported previously that a conditional *Shh* deletion in the MGE mantle zone can also cause defects in the generation of cortical interneurons (Flandin et al., 2011). Thus the defects in cortical interneuron development observed here in the *Ldb1/Nkx2.1-Cre* mutant can result from, at least partially, from the impaired *Shh* expression in the MGE mantle zone.

Our analysis of the *Ldb1/Nkx2.1-Cre* conditional mutant has also revealed interesting new phenotypes that were not previously observed in the *Lhx6* and *Lhx8* single or double mutants. While the MGE-derived globus pallidus appears normal in *Lhx6* and *Lhx8* single mutants (Zhao et al., 2003; Zhao et al., 2008; and in this study, see Fig. 12), deletion of both *Lhx6* and *Lhx8* leads to severe molecular and morphological defects in the globus pallidus. This indicates a redundant function of *Lhx6* and *Lhx8* in the development of the globus pallidus (Flandin et al., 2011). Due to severe developmental defects in the craniofacial structures, the *Lhx6/Lhx8* double mutant dies neonatally, which precluded the postnatal analysis to further determine how this phenotype continues to manifest and how the later differentiated neuronal cell type such as the PV<sup>+</sup> neurons is affected in these mutants. In this study, we observed that the *Ldb1* mutant survives after birth. Thus, we were able to extend our analysis postnatally and found that PV<sup>+</sup> projection neurons in the globus pallidus and PV<sup>+</sup> striatal interneurons were greatly reduced.

The defects in the development of the GABAergic neurons in the cortex of the *Ldb1/Nkx2.1-Cre* mutant appeared less severe compared to the *Lhx6* mutant. This is probably in part due to an incomplete deletion of the *Ldb1* in the MGE, especially in the rostral-dorsal region of the MGE (Fig. 3), where not all of the *Nkx2.1*<sup>+</sup> cells express the Cre recombinase in the *Nkx2.1-Cre* BAC transgenic mice (Xu et al., 2008). In support of this idea, double immunofluorescent staining revealed that many of the remaining PV<sup>+</sup> cells in the cortex and striatum of the postnatal *Ldb1* mutants were *Ldb1*<sup>+</sup> (Supplemental Fig. S1). Double staining of ChAT and *Ldb1* also showed that the remaining ChAT<sup>+</sup> cells in the striatum of the *Ldb1* mutants were *Ldb1*<sup>+</sup> (Supplemental Fig. S2). However, double staining of PV/*Ldb1* or ChAT/*Ldb1* also revealed that the remaining PV<sup>+</sup> or ChAT<sup>+</sup> cells in the globus pallidus (Supplemental Fig. S1) or in the septum and nucleus Basalis (Supplemental Fig. S2) of the *Ldb1* mutants were *Ldb1*<sup>-</sup>. It is possible that the conditional *Ldb1* deletion might take place relatively later during the embryonic development in contrast to a germline gene deletion. Some of the neuron precursor cells might retain the *Ldb1* function and proceed with the development for a certain period before the gene was inactivated. Also, *Ldb2*, a gene closely related to *Ldb1* (Aglunick et al., 1996), may compensate for the loss of *Ldb1*, as shown recently in early development of the limb (Narkis et al., 2012).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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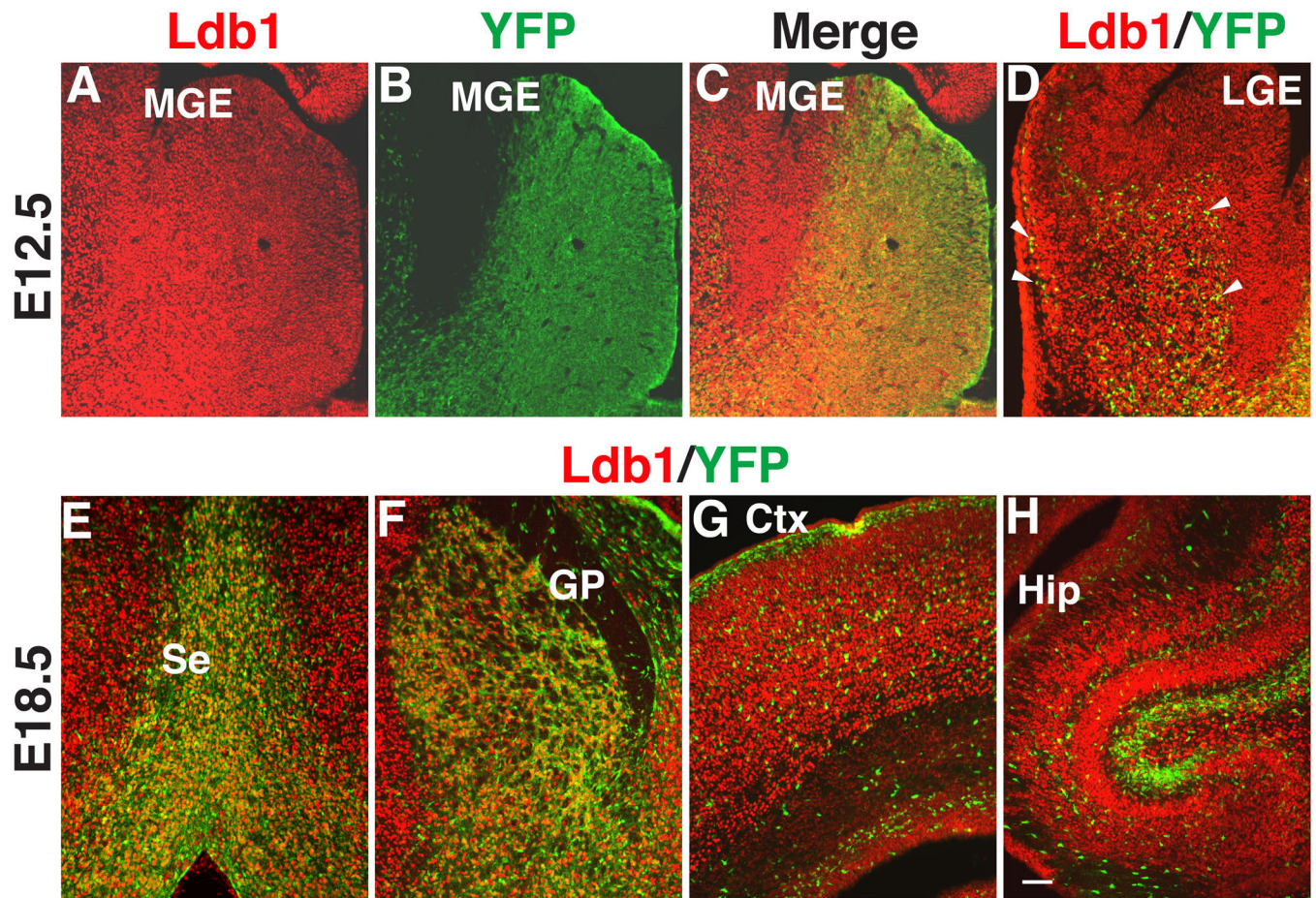
### Highlights

Ldb1 is expressed in the Nkx2.1 lineage in the developing ventral telencephalon.

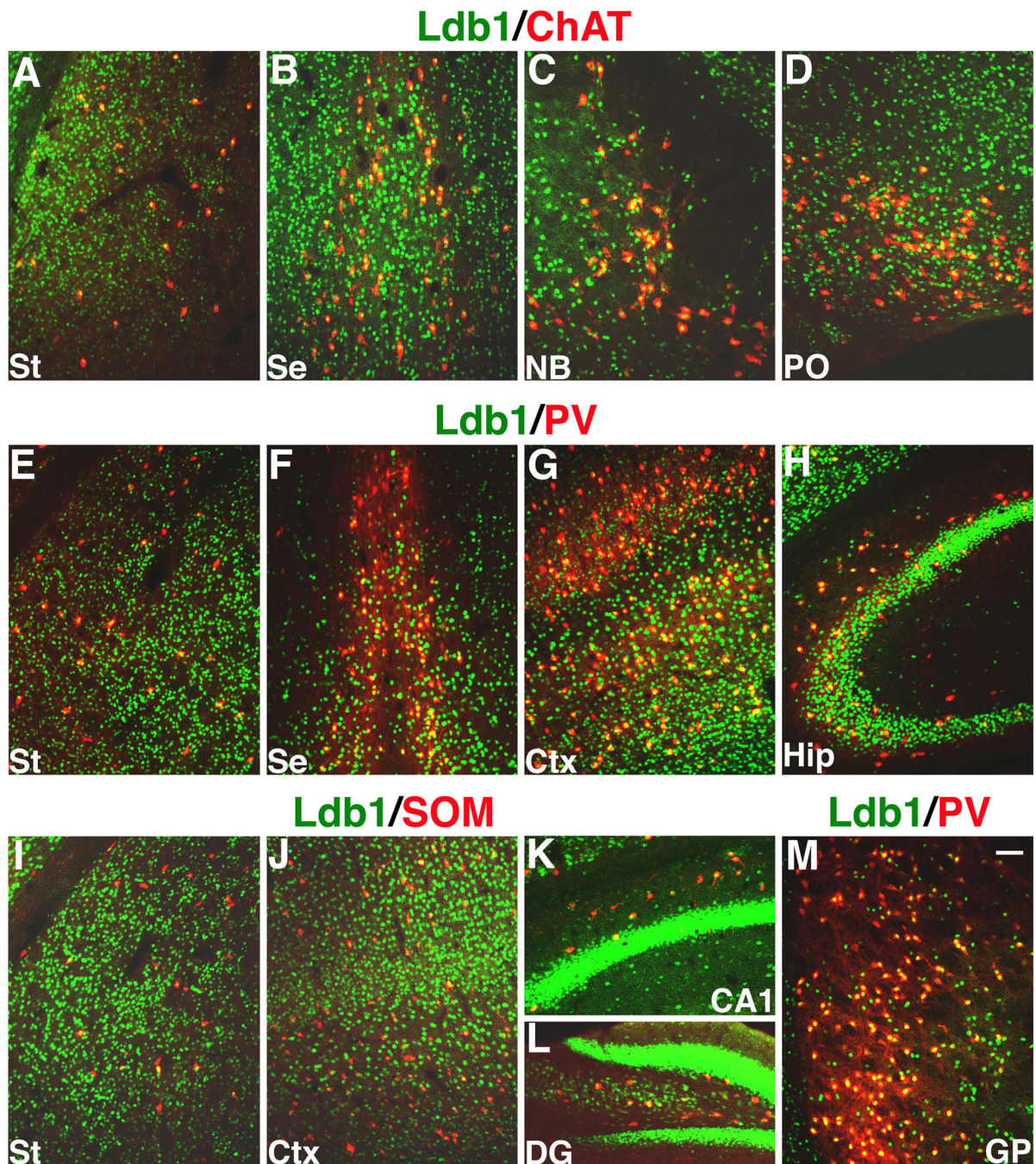
Ldb1 controls expression of a series of genes critical for telencephalon development.

Ldb1 regulates the tangential migration of ventral telencephalon derived neurons.

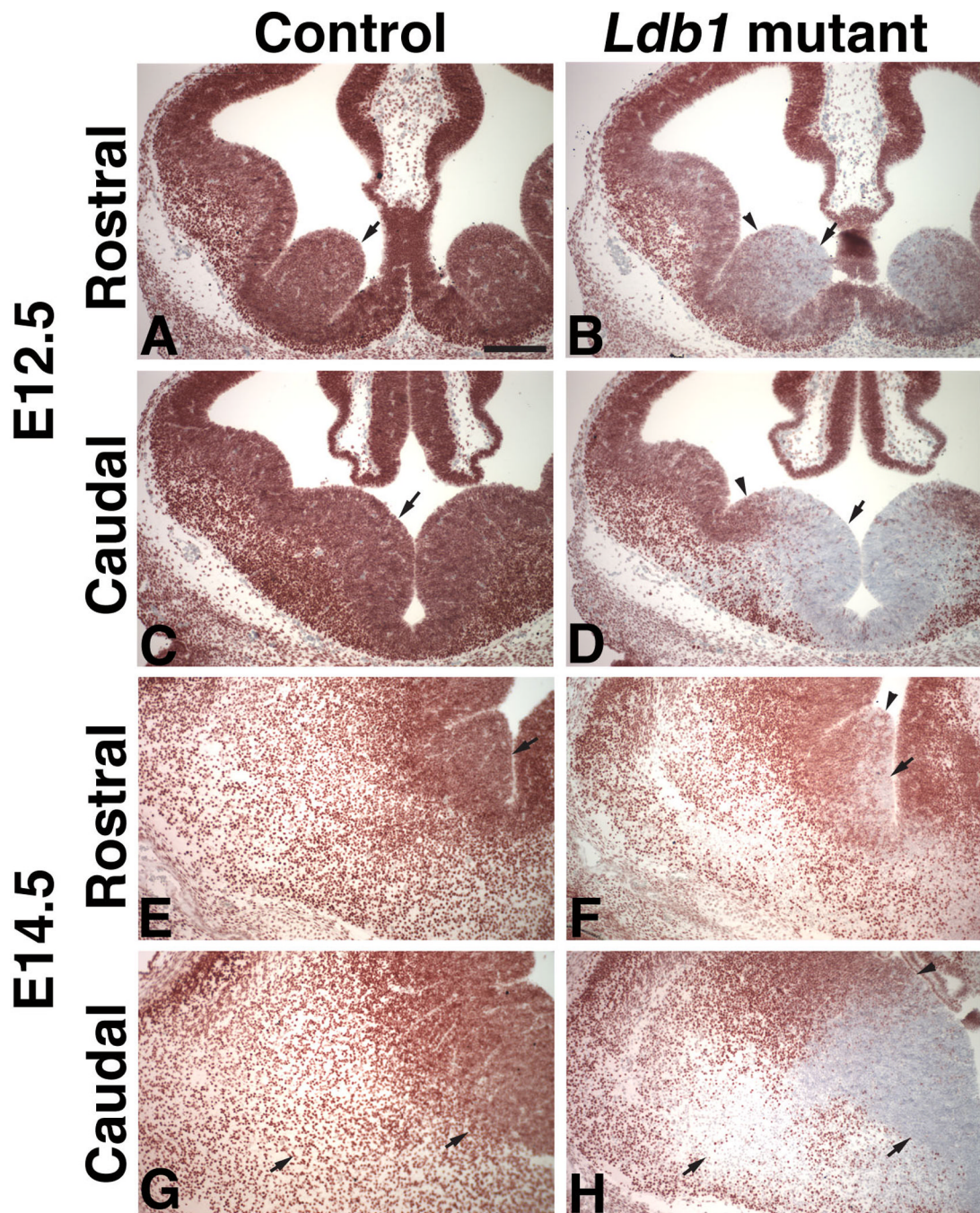
Ldb1 is essential for the generation of a variety of telencephalic neurons.

**Fig.1.**

Expression of *Ldb1* in *Nkx2.1*-lineage cells in the developing mouse telencephalon. Immunofluorescent staining of coronal sections from *Nkx2.1-Cre<sup>+</sup>; R26<sup>+/flox-stop-flox</sup>YFP* embryos shows that *Ldb1* is widely expressed in the ventral telencephalon at E12.5 (A, C), including in the *Nkx2.1*-lineage cells in the medial ganglionic eminence (MGE) labeled by staining of YFP (B, C). At this stage, *Ldb1* is also detected in *Nkx2.1*-lineage (YFP<sup>+</sup>) cells that have entered the lateral ganglionic eminence (LGE) or on their way migrate to the cortex (pointed by arrowheads in D). At E18.5, *Ldb1* is detected in *Nkx2.1*-lineage (YFP<sup>+</sup>) cells in various regions of the telencephalon including the septum (Se) (E), globus pallidus (GP) (F), cortex (Ctx) (G), and hippocampus (Hip) (H). Bar in H represents 100  $\mu$ m for all panels.

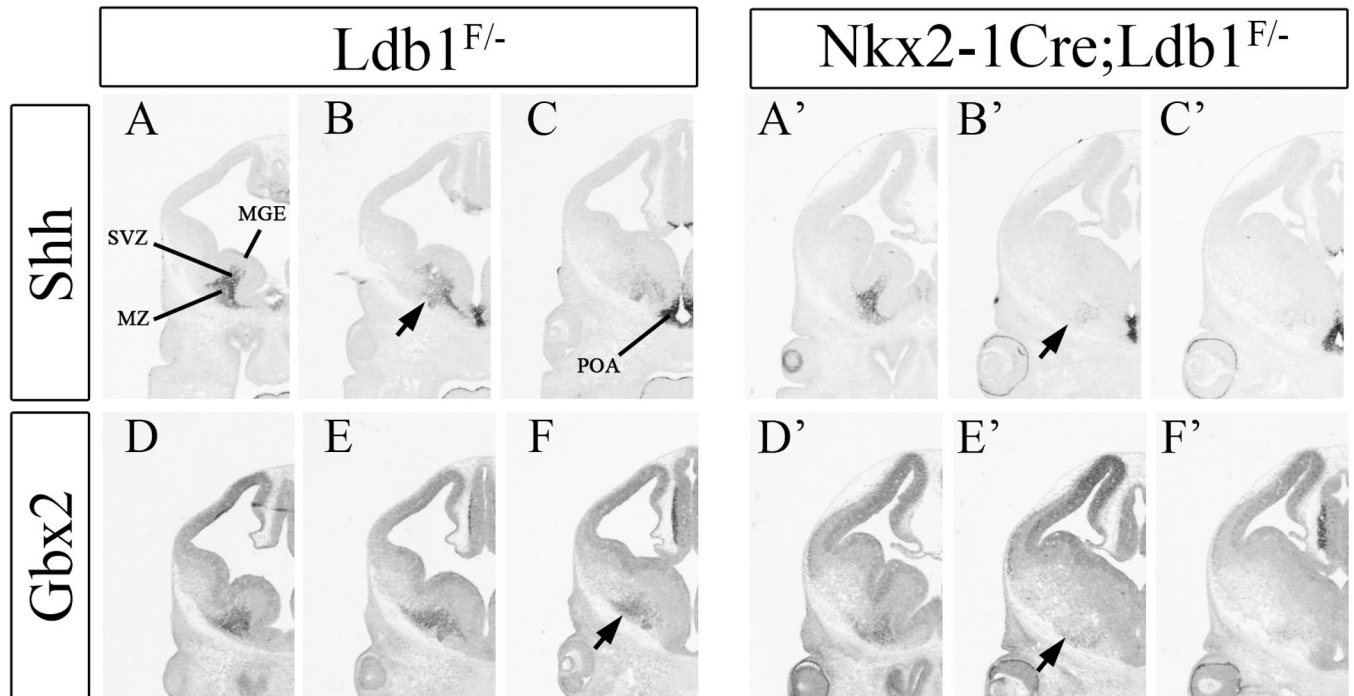


**Fig. 2.** Expression of Ldb1 in subtypes of neurons derived from the Nkx2.1-lineage in the postnatal (P18) mouse telencephalon. Immunofluorescent staining shows that Ldb1 is expressed in cholinergic (A–D), parvalbumin<sup>+</sup> (E–H, M), and somatostatin<sup>+</sup> (I–L) neurons in the various regions of the telencephalon including the striatum (St) (A, E, I), septum (Se) (B, F), globus pallidus (GP) (M), nucleus Basalis (NB) (C), preoptic area (PO) (D), cortex (Ctx) (G, J), hippocampus (Hip) (H, K), and dentate gyrus (DG) (L). Bar in M represent 100  $\mu$ m for all panels.

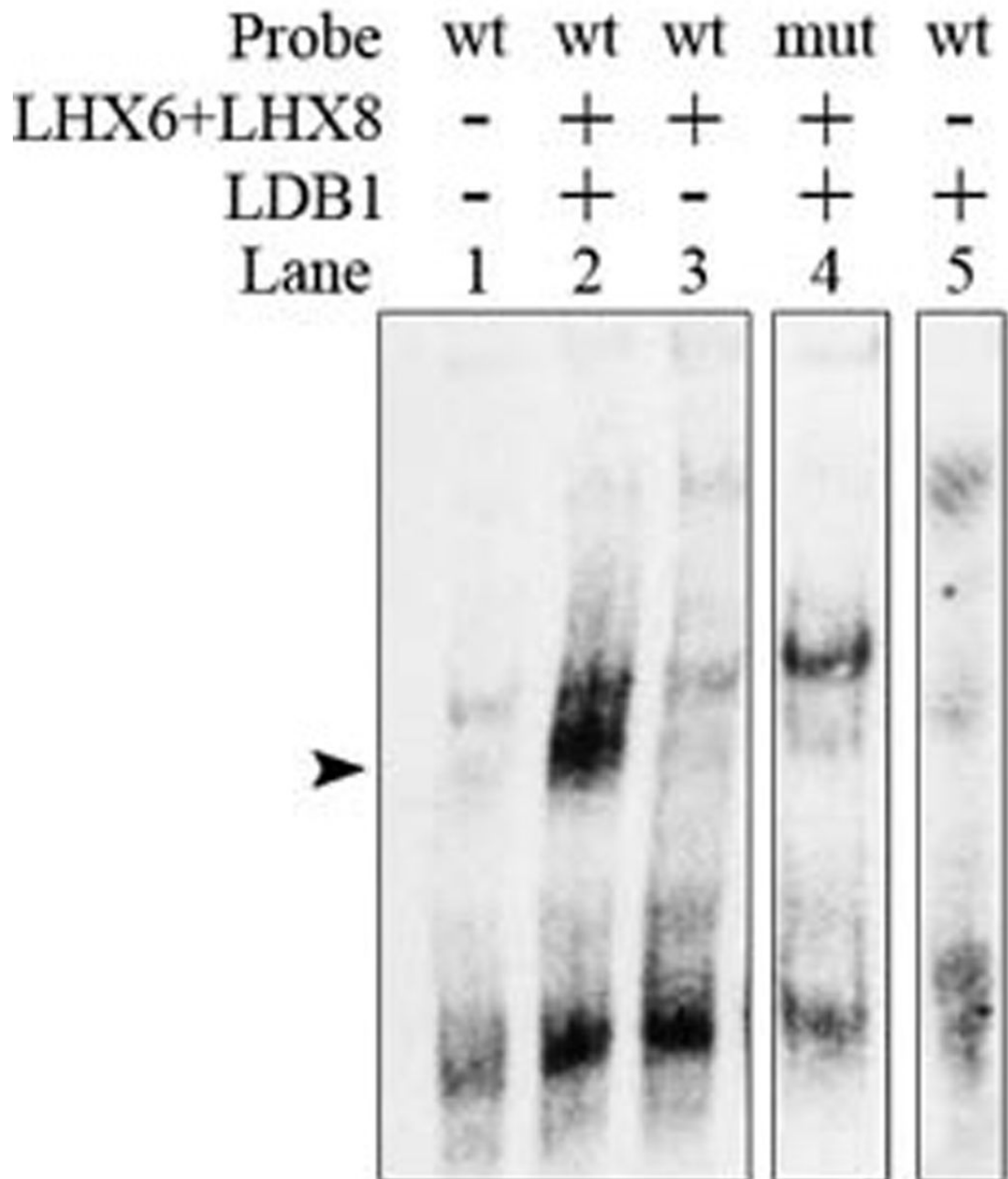


**Fig. 3.** Conditional deletion of *Ldb1* in the ventral telencephalon. Coronal sections through the telencephalon of E12.5 (A–D) and E14.5 (E–H) mouse embryos were immunostained with an antibody to *Ldb1*. A–B and E–F show sections from a rostral level. C–D and G–H show sections from a caudal level. Reduction of *Ldb1* staining was observed in the MGE of *Ldb1<sup>f/f</sup>; Nkx2.1-Cre<sup>+</sup>* conditional mutant (B, D, F, H) as compared to the control (A, C, E, G). Arrows in A–H point at regions of the MGE where *Ldb1* was detected in the control (A, C, E, G), but was missing in the mutant (B, D, F, H). Arrowheads in B, D, F, H point at the dorsal region of the MGE in the mutant where *Ldb1* was still detected. Bar in A represents 750  $\mu\text{m}$  for all panels.

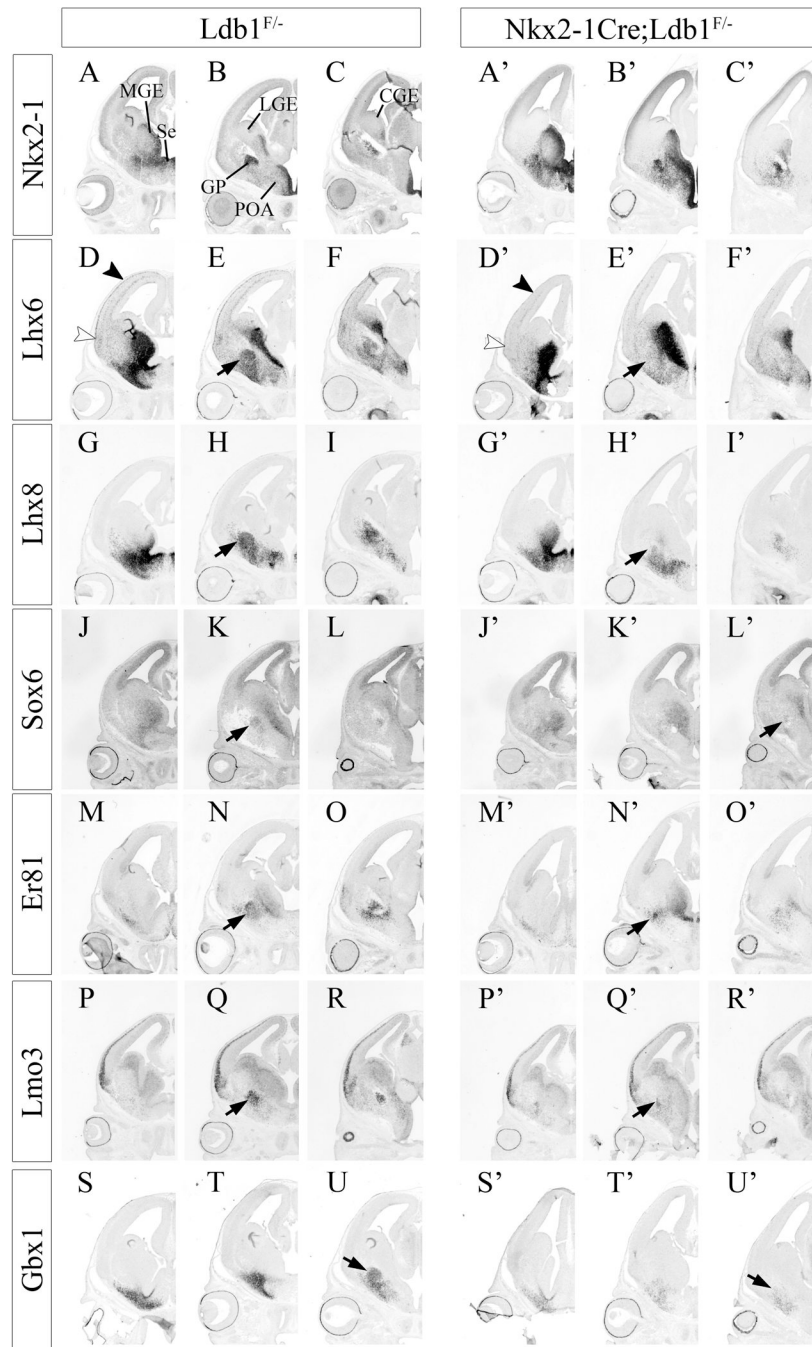




**Fig. 4.** Reduction of *Shh* (A–C, A'–C') and *Gbx2* (D–F, D'–F') expression in conditional *Ldb1* mutants, as determined by *in situ* hybridization at E12.5. Coronal telencephalic hemisections compare gene expression between control (*Ldb1*<sup>+/-</sup>; left) and mutant (*Nkx2-1-Cre*<sup>+</sup>; *Ldb1*<sup>-/-</sup>; right). Three planes of section are shown, with rostral at the left and caudal at the right. Arrows show reduced *Shh* and *Gbx2* expression in the mantle zone of the MGE. Abbreviations: MGE: medial ganglionic eminence, MZ: mantle zone, POA: preoptic area, SVZ: subventricular zone.

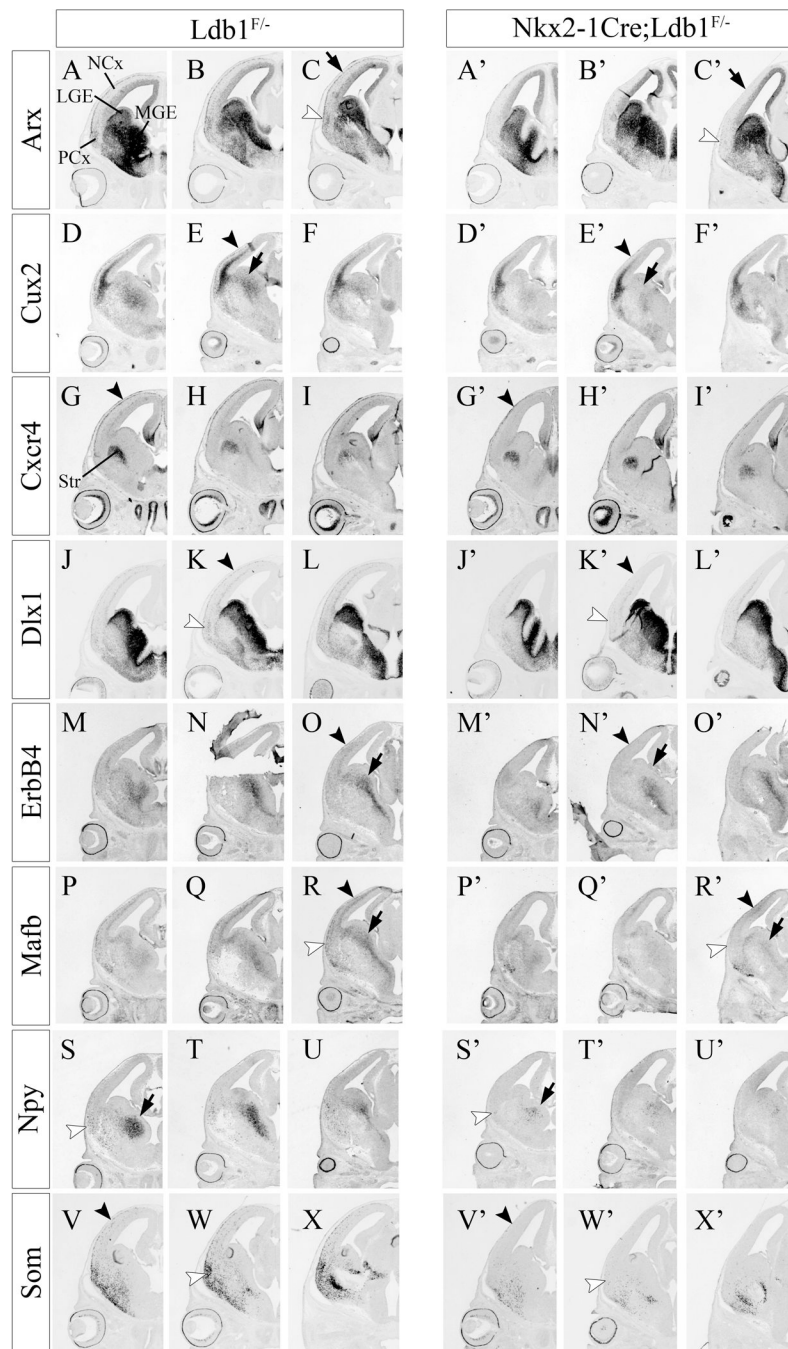


**Fig. 5.** Electrophoretic mobility shift assay showing that LHX6 and LHX8 require LDB1 to bind to the SBE3 *Shh* enhancer. In vitro DNA binding assay, using LHX6-, LHX8- and LDB1-containing nuclear extract, results in a strong probe (wt SBE3 *Shh* enhancer domain) - protein complex (arrowhead; lane 2), compared to control extract (lane 1). Addition of LHX6 and LHX8 extracts without LDB1<sup>+</sup> nuclear extra does not result in complex formation (lane 3). Mutating the LHX putative binding site of the probe (mut SBE3 *Shh* enhancer domain) also results in a strong reduction of the amount of complex (lane 4). LDB1 does not bind to the wt probe by itself (lane 5).



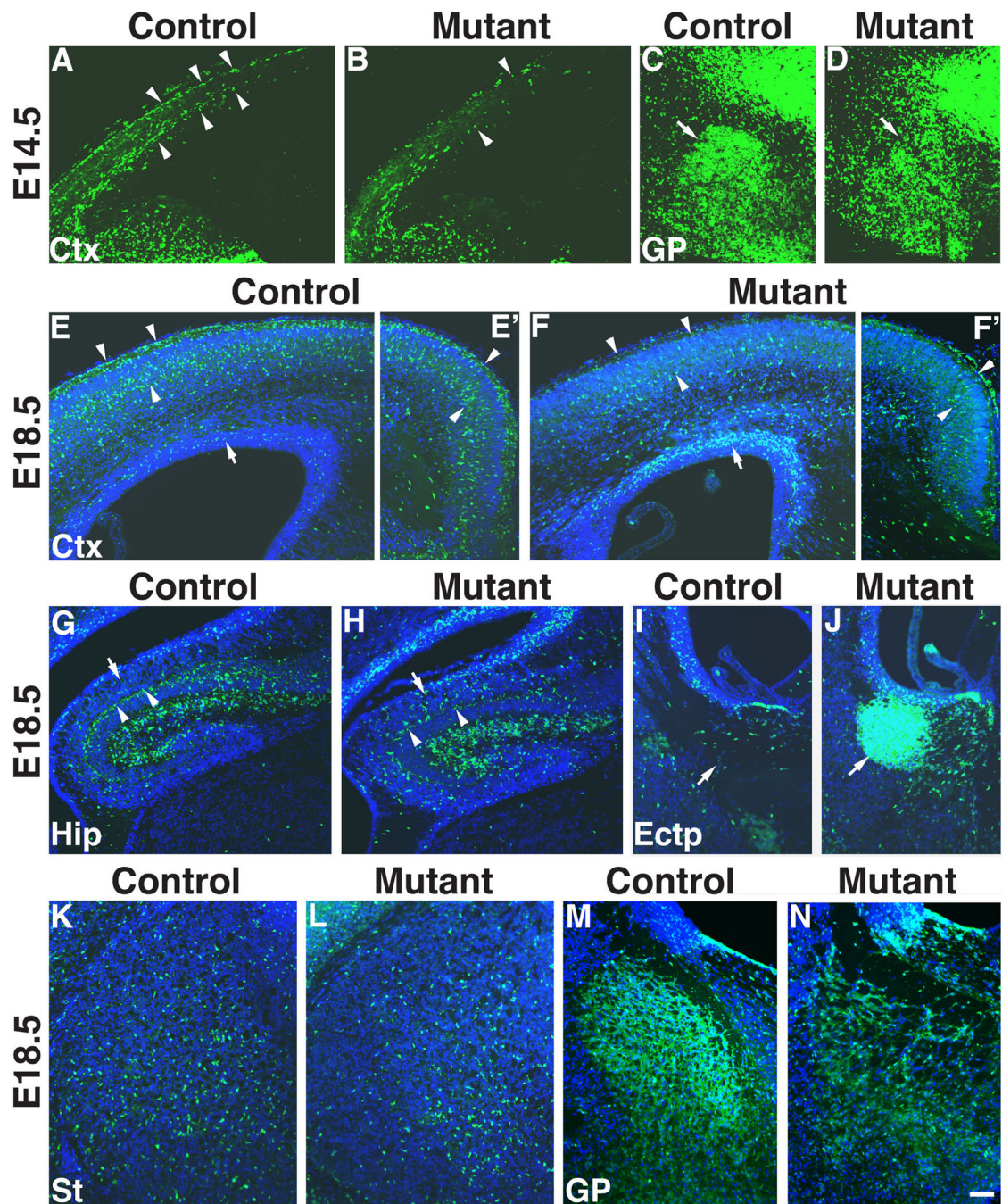
**Fig. 6.** Expression of genes that regulate and/or mark MGE development are reduced in conditional *Ldb1* mutants, as determined by *in situ* hybridization at E14.5. Coronal telencephalic hemisections compare gene expression between control (*Ldb1*<sup>+/-</sup>; left) and mutant (*Nkx2.1-Cre*<sup>+</sup>; *Ldb1*<sup>-/-</sup>; right). Three planes of section are shown, with rostral at the left and caudal at the right. *Nkx2.1* (A–C, A'–C'); *Lhx6* (D–F, D'–F'), black arrowhead shows reduced *Lhx6*<sup>+</sup> neocortical cortical interneurons in mutant; white arrowhead shows reduced *Lhx6*<sup>+</sup> paleocortical cortical interneurons in mutant; black arrow shows reduced GP in mutant; *Lhx8* (G–I, G'–I'), black arrow shows reduced GP in mutant; *Sox6* (J–L, J'–L'), black arrow

shows reduced GP in mutant; *Er81* (Etv1; M–O, M'–O'), black arrow shows reduced GP in mutant; *Lmo3* (P–R, P'–R'), black arrow shows reduced GP in mutant; *Gbx1* (S–U, S'–U'); black arrow shows reduced GP in mutant. Abbreviations: CGE: caudal ganglionic eminence, GP: globus pallidus, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, POA: preoptic area, Se: septum.



**Fig. 7.** Expression of genes that regulate and/or mark cortical interneuron development are reduced in conditional *Ldb1* mutants, as determined by *in situ* hybridization at E14.5. Coronal telencephalic hemisections compare gene expression between control (*Ldb1*<sup>+/+</sup>; left) and mutant (*Nkx2-1-Cre*<sup>+</sup>; *Ldb1*<sup>-/-</sup>; right). Three planes of section are shown, with rostral at the left and caudal at the right. *Arx* (A–C, A'–C'), black arrow and white arrowhead show reduced interneurons in the neocortex and paleocortex, respectively; *Cux2* (D–F, D'–F'), black arrowhead and arrow show reduced interneurons in the neocortex and striatum, respectively; *Cxcr4* (G–I, G'–I'), black arrowhead shows reduced interneurons in the

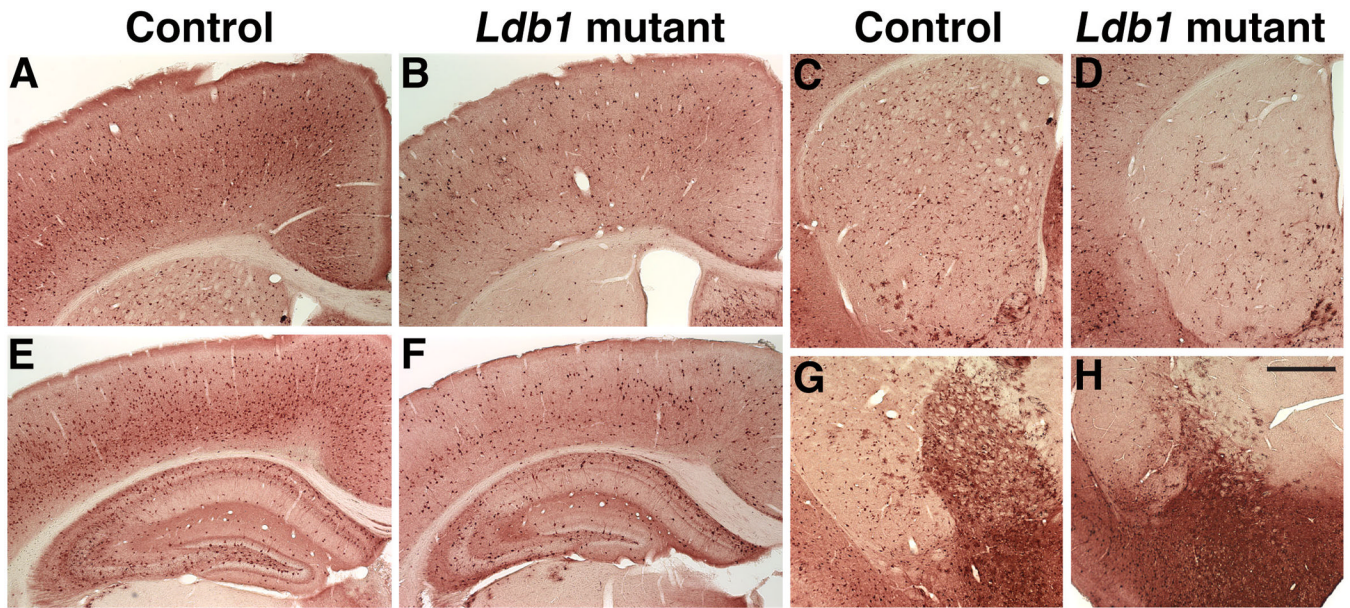
neocortex; *Dlx1* (J-L, J'-L'), black arrowhead and white arrowhead show reduced interneurons in the neocortex and paleocortex, respectively; *ErbB4* (M-O, M'-O'), black arrowhead and arrow show reduced interneurons in the neocortex and striatum, respectively; *Mafb* (P-R, P'-R'), black arrowhead, white arrowhead and black arrow show reduced interneurons in the neocortex, paleocortex and striatum, respectively; *Npy* (S-U, S'-U'), white arrowhead and black arrow show reduced interneurons in the paleocortex and MGE, respectively; *Som* (V-X, V'-X'), black arrowhead and white arrowhead show reduced interneurons in the neocortex and paleocortex, respectively. Abbreviations: CGE: caudal ganglionic eminence, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence; PCx: paleocortex; Str: striatum.



**Fig. 8.** Anti-YFP staining shows abnormal distribution of Nkx2.1-lineage (YFP<sup>+</sup>) cells in the telencephalon of *Ldb1* mutant (*Ldb1*<sup>f/f-/-</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+/flox-stop-flox</sup>YFP) compared to the control (*Ldb1*<sup>f/f+</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+/flox-stop-flox</sup>YFP). At E14.5 (A–D), less YFP<sup>+</sup> cells are present in the dorsal cortex (arrowheads) of the mutant (B) as compared to the control (A). The YFP<sup>+</sup> cells in the globus pallidus (arrows) are less densely packed in the mutant (D) compared to the control (C). By E18.5 (E–N), in both the lateral (E, F) and the medial (E', F') cortex (Ctx), there are less YFP<sup>+</sup> cells (indicated by arrowheads) in the superficial layers of the mutant (F, F') as compared to the control (E, E'). In contrast, more YFP<sup>+</sup> cells

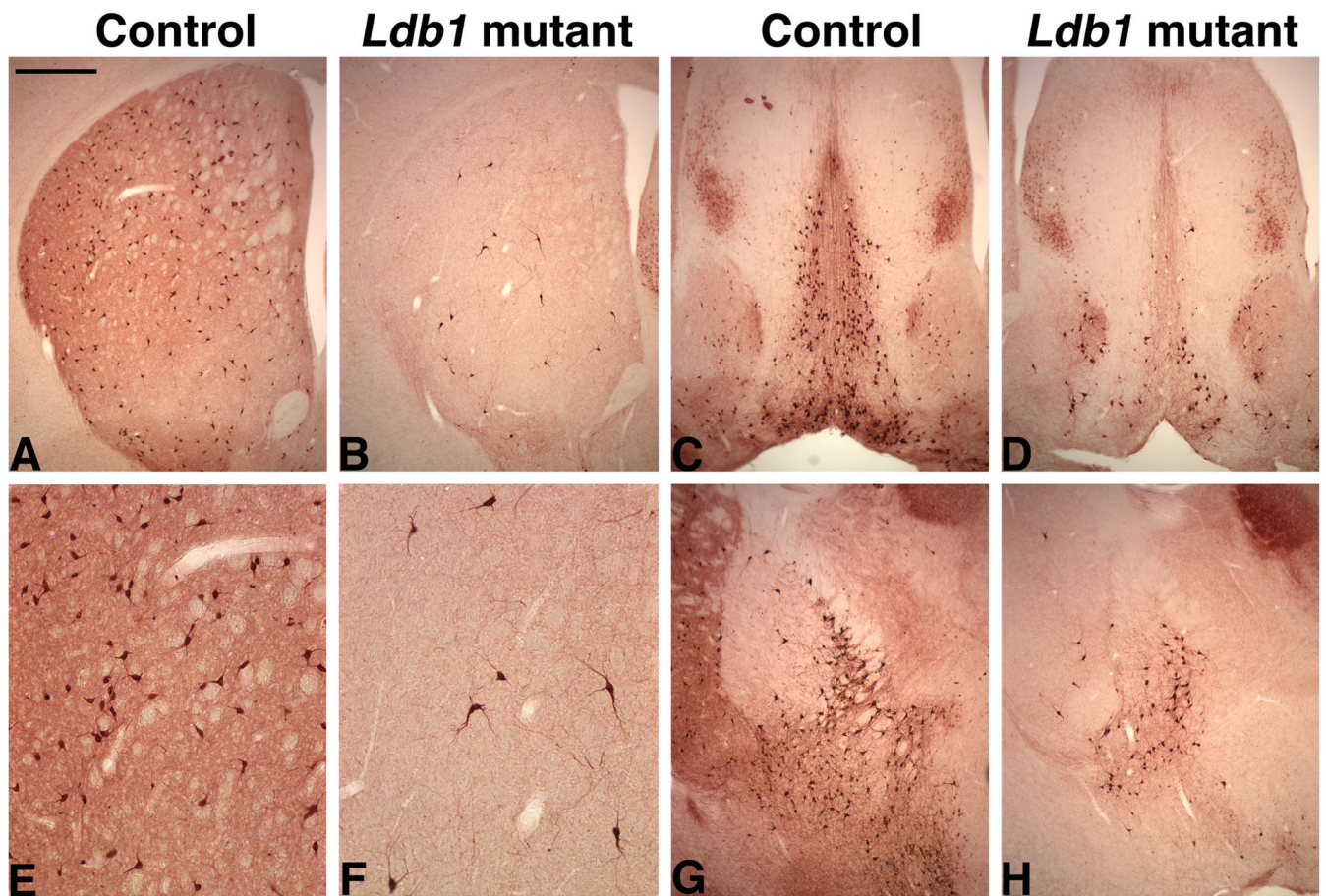
(indicated by arrows) are present close to the ventricular zone of the cortex in the mutant (F) compared to the control (E). G–H, YFP<sup>+</sup> cells are present in the developing hippocampus (Hip) of the *Ldb1* mutant, but the cells appear more scattered in the stratum oriens (indicated by arrows) in the mutant (H) as compared to the control (G), in which the cells form a more compact layer adjacent to the pyramidal cell layer (arrowheads). I–J, ectopic (Ectp) YFP<sup>+</sup> cells accumulate (indicated by arrows) in the dorsal region of the MGE of the *Ldb1* mutant (J) as compared to the control (I). K–L, reduction of YFP<sup>+</sup> cells in the striatum (St) of the *Ldb1* mutant (L) compared to the control (K). M–N, Disorganization of YFP<sup>+</sup> cells in the globus pallidus (GP) of the *Ldb1* mutant (N) compared to the control (M). Bar in N represents 114  $\mu\text{m}$  for A–D and 100  $\mu\text{m}$  for E–N.



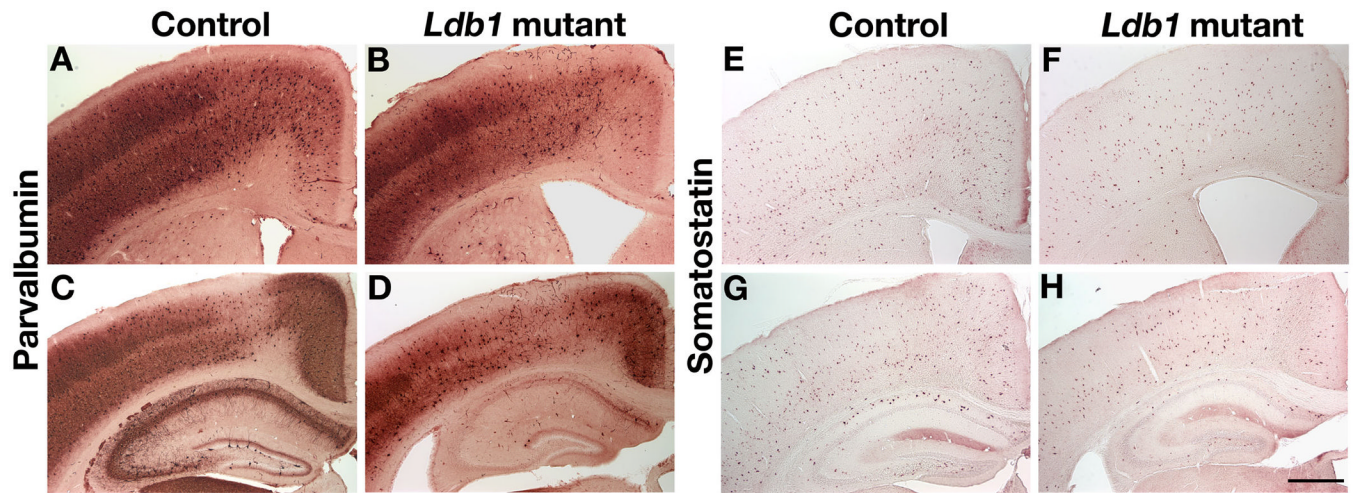


**Fig. 9.**

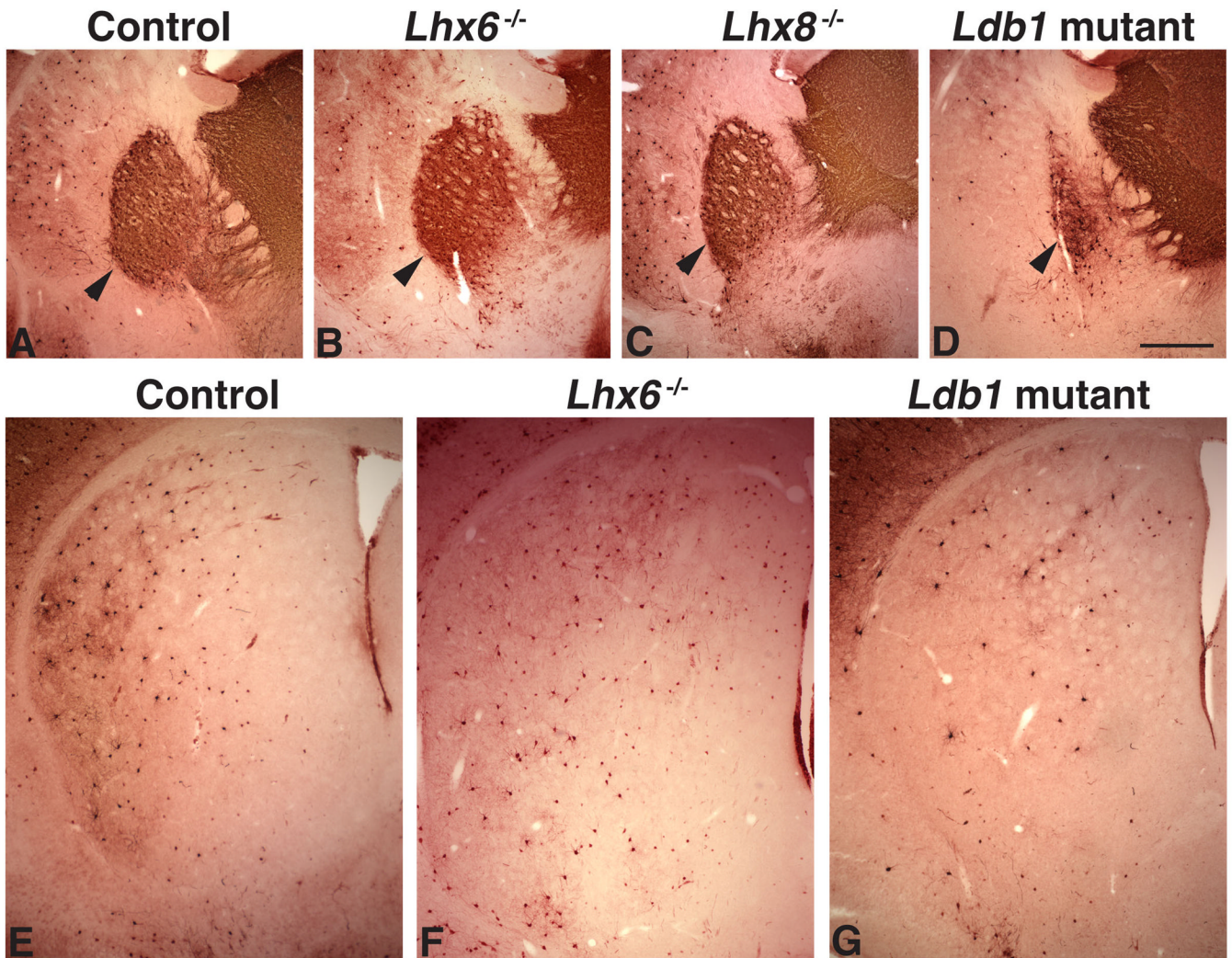
Anti-YFP staining shows a reduction in number of Nkx2.1-lineage (YFP<sup>+</sup>) cells in the telencephalon of the postnatal (P16) *Ldb1* mutant (*Ldb1*<sup>f/f-/-</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+/flox-stop-floxYFP</sup>, B, D, F, H) compared to the control (*Ldb1*<sup>f/f+/+</sup>; *Nkx2.1-Cre*<sup>+</sup>; *R26*<sup>+/flox-stop-floxYFP</sup>, A, C, E, G). A–B, cortex at a rostral level. C–D, striatum. E–F, cortex at a caudal level and the hippocampus. G–H, the globus pallidus. Bar in H represents 500  $\mu$ m for all panels.



**Fig. 10.** Reduction in number of cholinergic neurons in the telencephalon of the *Ldb1* conditional mutant. Anti-ChAT staining shows a reduction in the number of cholinergic neurons in the striatum (A–B, E–F), the diagonal bands of the septum (C–D), and the nucleus Basalis (G–H) in a P18 *Ldb1<sup>f/f</sup>; Nkx2.1-Cre<sup>+</sup>* mutant (B, D, F, H) as compared to the control (A, C, E, G). E and F show enlargement of a part of A and B, respectively. Bar in A represents 500  $\mu\text{m}$  for A–D, G–H, and 200  $\mu\text{m}$  for E–F.



**Fig. 11.** Reduction in number of GABAergic interneurons in the cortex and hippocampus of the *Ldb1* conditional mutant. Immuno-staining of parvalbumin (A–D) and somatostatin (E–H) shows a reduction in number of parvalbumin<sup>+</sup> and somatostatin<sup>+</sup> interneurons in the cortex and hippocampus of a P18 *Ldb1*<sup>f/f</sup>; *Nkx2.1-Cre*<sup>+</sup> mutant (B, D, F, H) as compared to the control (A, C, E, G). A, B, E, F show sections of the cortex from a rostral level. C, D, G, H show sections of the cortex and hippocampus from a caudal level. Bar in H represents 500  $\mu$ m for all panels.



**Fig. 12.**

Defect of the globus pallidus and the striatal parvalbumin interneurons in the *Ldb1* conditional mutant. A–D, anti-parvalbumin staining shows that the globus pallidus (pointed by arrowheads) is severely reduced in size in a postnatal (P18) *Ldb1*<sup>f/f</sup>; *Nkx2.1-Cre*<sup>+</sup> mutant (D) as compared to the control (A), while it appears normal in either *Lhx6*<sup>-/-</sup> (B) or *Lhx8*<sup>-/-</sup> (C) single mutant. E–G, reduction in number of parvalbumin<sup>+</sup> interneurons in the striatum of the *Ldb1* mutant (G) as compared to the control (E) and a *Lhx6*<sup>-/-</sup> mutant. Bar in D represents 500  $\mu$ m for A–D and 370  $\mu$ m for E–G.