Published in final edited form as:
Mol Cell Neurosci. 2008 April ; 37(4): 696–707.

Cre fate mapping reveals lineage specific defects in neuronal migration with loss of *Pitx2* function in the developing mouse hypothalamus and subthalamic nucleus

Jennifer M. Skidmore[1], **John D. Cramer**[1], **James F. Martin**[3], and **Donna M. Martin**[1,2,*]

1 *Department of Pediatrics, The University of Michigan Medical Center, Ann Arbor, Michigan, 48109*

2 *Department of Human Genetics, The University of Michigan Medical Center, Ann Arbor, Michigan, 48109*

3 *Institute of Biosciences and Technology, Texas A&M System Health Science Center, Houston, TX 77030*

Abstract

Establishment of neuronal diversity is a central topic in developmental neurobiology. Prior studies implicated *Pitx2*, a paired-like homeodomain transcription factor, in mouse subthalamic nucleus neuronal development, but precise stages of neuronal differentiation affected (migration, axon outgrowth, fate specification) and underlying mechanisms were unknown. Here we report lineage tracing experiments using *Pitx2*$^{cre/+}$, *Pitx2*$^{cre/null}$, and conditional nuclear *lacZ* reporter mice to track embryonic *Pitx2* expressing neurons. Migration of subthalamic nucleus and hypothalamic neurons was severely arrested in *Pitx2*$^{cre/null}$ embryos, and subclasses of subthalamic nucleus neurons identified by *Lmx1b*, *Foxp1*, and *Foxp2*-gene expression revealed differing sensitivities to *Pitx2* dosage. Interestingly, embryonic subthalamic nucleus development was unaffected in *Lmx1b* null mice, suggesting that *Pitx2* and *Lmx1b* act via independent genetic pathways. These data provide the first direct evidence for *Pitx2*-dependent neuronal migration in the developing hypothalamus, and demonstrate that complex transcriptional networks regulate regional specialization of distinct hypothalamic and subthalamic nucleus neurons.

Keywords

development; differentiation; mice; migration; mutant; transcription factor

Introduction

Generation of neuronal diversity in the mammalian brain requires coordinated expression of transcription factors and signaling molecules (Puelles and Rubenstein 2003; Sur and Rubenstein 2005; Lim and Golden 2006). Regional specialization of these complex neuronal populations also requires that differentiating neurons travel long, sometimes circuitous routes to their final destinations in the brain. Central questions in developmental neurobiology include how these distinct neuronal populations are formed and which molecular signals are used to guide their terminal differentiation. Recent advances in genetic fate mapping of cells with restricted gene expression have made it possible to explore cellular fates and phenotypes of

*Correspondence to: Donna M. Martin, 1150 W. Medical Center Dr. 3520A MSRB I, Ann Arbor, MI 48109-0652. Email donnamm@umich.edu, Phone (734) 647-4859. FAX (734) 763-9512.

specific neuronal lineages in the developing mouse brain (Branda and Dymecki 2004; Joyner and Zervas 2006).

Restricted expression of *Pitx2*, a paired-like homeodomain transcription factor, in the developing mouse hypothalamus occurs concomitant with or soon after terminal mitosis of neural progenitors around E9.5–E10.5 (Martin et al., 2002), and is necessary for normal development of neurons in the subthalamic nucleus (Martin et al., 2004). Due to complex central nervous system phenotypes and a lack of reporter alleles, earlier studies could not distinguish the effects of *Pitx2* deficiency on neural cell migration, axon outgrowth, or cell fate specification. Moreover, it was not possible to exclude altered *Pitx2* gene expression as an explanation for defects seen in *Pitx2* null embryos (Martin et al., 2004).

Pitx2 exhibits pleiotropic, tissue specific effects during development, with a short (30 minutes) mRNA half-life that is regulated by Wnt/Dvl/β-catenin signaling (Kioussi et al., 2002). *Pitx2* promotes migration of cultured HeLa cells (Wei and Adelstein 2002) and cells that give rise to craniofacial and cardiac structures (Liu et al., 2002; Liu et al., 2003), and *Pitx2* function is critical for survival of pituitary hormone-producing cells (Charles et al., 2005). Based on these observations, we hypothesized that *Pitx2* may be required for one or more aspects of neuronal differentiation including maintenance of gene expression, cell identity, neuronal migration, and axonal outgrowth. To explore the fates of *Pitx2* deficient neurons during mid-gestation, we designed a lineage tracing strategy using previously characterized $Pitx2^{\boldsymbol{null}}$ (Gage et al., 1999) and $Pitx2^{\boldsymbol{cre}}$ knock-in (Liu et al., 2003) alleles and transgenic mice containing a *Cre* conditional nuclear localized *lacZ* reporter under the control of the chicken β-actin promoter (*N-lacZ*) (Zinyk et al., 1998). Our analysis revealed that *Pitx2* is essential for lineage specific neuronal migration in the developing hypothalamus and subthalamic nucleus.

Results

Nuclear localized β-galactosidase labels Pitx2 mutant neurons

PITX2 protein and mRNA are expressed in differentiating neurons of the E9.5–E14.5 mouse brain around the time of terminal mitosis–(Martin et al., 2002) and unpublished data. Prior studies showed that homozygous *Pitx2* null embryos require *Pitx2* for normal gene expression and formation of neuronal projections in the developing subthalamic nucleus, but the lack of a permanent reporter made it impossible to track the fates of *Pitx2* mutant neurons (Martin et al., 2004). To evaluate for region specific defects in *Pitx2* gene expression or localized defects in cell migration or fate, we generated a system for indelibly marking *Pitx2* heterozygous mutant and compound heterozygous mutant cells with nuclear localized β-galactosidase. Matings between $Pitx2^{\boldsymbol{+/null}}$*; N-lacZ/N-lacZ* and $Pitx2^{\boldsymbol{cre/+}}$ mice were used to generate littermate embryos of genotype $Pitx2^{\boldsymbol{cre/+}}$*;N-lacZ* and $Pitx2^{\boldsymbol{cre/null}}$*;N-lacZ* (Fig. 1A). This approach allowed for direct comparison ofβ-galactosidase activity in embryos with one normal copy of *Pitx2* to those with complete (compound heterozygous) *Pitx2* deficiency.

Whole mount X-gal staining of E12.5 $Pitx2^{\boldsymbol{cre/+}}$*;N-lacZ* and $Pitx2^{\boldsymbol{cre/null}}$*;N-lacZ* embryos revealed β-galactosidase activity in known sites of *Pitx2* expression in the brain, eye, heart, proximal limb, and craniofacial regions (Fig. 1B), consistent with prior observations showing reporter expression in the developing brain, spinal cord, heart, and some skeletal muscles (Zinyk et al., 1998). Close analysis of sectioned E14.5 $Pitx2^{\boldsymbol{cre/+}}$*;N-lacZ+* embryos demonstrated β-galactosidase activity in brain regions known to express *Pitx2*, including the superior colliculus, subthalamic nucleus, mammillary region, posterior hypothalamus, zona limitans intrathalamica and first rhombomere. The pattern of β-galactosidase activity in the postnatal hypothalamus and subthalamic nucleus of $Pitx2^{\boldsymbol{cre/+}}$*;N-lacZ* embryos matched previously reported patterns of *Pitx2* mRNA and protein expression in wildtype and $Pitx2^{+/-}$ embryos, as illustrated with double immunofluorescence for β-gal and PITX2 (Suppl. Fig. 1)

(Martin et al., 2002). There were no ectopic regions of β-galactosidase activity in E12.5–E14.5 *Pitx2*$^{cre/+}$*;N-lacZ* brains (data not shown), indicating that *Pitx2* expression perdures in the mid-gestation developing mouse brain.

These observations confirm that labeling of *Pitx2*-expressing neurons with the βgal reporter in *Pitx2*$^{cre/+}$*;N-lacZ* mice exhibits high fidelity. Moreover, inherent delays in *Cre* expression, excision of the conditional reporter, and subsequent transcription and translation of β-galactosidase (as long as 24 hours) (Metzger et al., 1995; Zervas et al., 2004), do not appear to interfere with the ability ofβ-galactosidase to label *Pitx2* mutant neurons. Loss of only one copy of *Pitx2* does not disrupt *Pitx2* protein or mRNA expression in the brain relative to wildtype controls (Martin et al., 2004), and heterozygous *Pitx2*$^{cre/+}$*; N-lacZ* embryos exhibit no identifiable defects in neuronal location, thus validating the use of *Pitx2*$^{cre/+}$ mice as controls for comparisons with *Pitx2*$^{cre/null}$ neuronal phenotypes.

Pitx2$^{cre/null}$ embryonic neurons are mislocalized in the developing hypothalamus

Embryos with compound heterozygosity for *Pitx2* deficient alleles (*Pitx2*$^{cre/null}$*; N-lacZ*) did not survive beyond E14.5, with externalized heart and abdominal structures and distal turning defects similar to *Pitx2*$^{null/null}$ and *Pitx2*$^{cre/cre}$ embryos (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Liu et al., 2002; Liu et al., 2003). In order to determine the locations of *Pitx2* heterozygous and compound heterozygous *Pitx2* mutant neurons, we stained E12.5–E14.5 embryos for β-galactosidase activity and obtained vibratome sections throughout the developing hypothalamus (Fig. 2). In the E14.5 hypothalamus, *Pitx2*$^{cre/+}$*;N-lacZ* embryos expressed high levels of β-galactosidase (Fig. 2) in a pattern similar to *Pitx2* mRNA and protein expression (Martin et al., 2002; Martin et al., 2004). In contrast, β-galactosidase positive cells were absent from the *Pitx2*$^{cre/null}$*;N-lacZ* lateral hypothalamus, and the density of β-galactosidase label in the medial hypothalamus was increased (Fig. 2F–H). Similar observations were made in E13.5 *Pitx2*$^{cre/null}$*;N-lacZ* embryos, in which β-galactosidase positive cells were concentrated in medial regions of the hypothalamus, with no visible cells occupying more lateral positions (Fig. 2M, N). At E12.5, there were no visible differences between the locations of β-galactosidase positive cells in the *Pitx2*$^{cre/null}$*;N-lacZ* and *Pitx2*$^{cre/+}$*;N-lacZ* hypothalamus (Fig. 2K, L, O, P). Thus, hypothalamic *Pitx2* null neurons became mislocalized after E12.5, and are retained medially at E13.5–E14.5.

To test whether lateral hypothalamicβ-galactosidase label represents subthalamic nucleus neurons, we co-stained transverse vibratome E14.5 sections for β-galactosidase activity and Calretinin immunoreactivity (Fig. 3). These studies revealed co-expression of Calretinin and β-galactosidase in the lateral *Pitx2*$^{cre/+}$; *N-lacZ* hypothalamus, consistent with prior reports showing co-localization of PITX2 and Calretinin in subthalamic nucleus neurons (Martin et al., 2004). We observed absence of β-galactosidase and Calretinin in the lateral hypothalamus of *Pitx2*$^{cre/null}$; *N-lacZ* embryos, providing definitive evidence for loss of PITX2-lineage neurons from the subthalamic nucleus region.

In addition to the medial shift in β-galactosidase, we observed increased β-galactosidase expression at the ventral hypothalamic midline in E14.5 *Pitx2*$^{cre/null}$; *N-lacZ* mutants, suggesting aberrant midline positioning or crossing of these cells (brackets in Fig. 2, C–H). Wildtype E12.5–E14.5 mouse hypothalamic neurons migrate from medial to lateral, ventral to dorsal, and caudal to rostral as they progress toward their final destinations in the subthalamic nucleus (Suppl. Fig. 2) (Altman and Bayer 1986; Marchand 1987; Martin et al., 2004). This 3-dimensional route of subthalamic nucleus neuronal migration includes mixed radial and tangential components, and may also result from distinct waves of neurogenesis (Altman and Bayer 1986). The increased medial and aberrant midlineβ-gal label in *Pitx2*$^{cre/null}$; *N-lacZ* mutants suggests that neuronal migration might become arrested or disrupted shortly after E12.5.

Pitx2$^{cre/null}$ neurons exhibit defects in migration

To test for changes in hypothalamic cell number with loss of *Pitx2*, we counted β-galactosidase positive cells in the medial vs. lateral hypothalamus of transverse sections from *Pitx2*$^{cre/+}$*;N-lacZ* and *Pitx2*$^{cre/null}$*;N-lacZ* embryos (Fig. 4). Slightly increased numbers of β-galactosidase positive cells were located medially in the ventral hypothalamus of *Pitx2*$^{cre/null}$*;N-lacZ* embryos compared to *Pitx2*$^{cre/+}$*;N-lacZ* littermates, but this difference was not statistically significant (*Pitx2*$^{cre/+}$*;N-lacZ* = 590 vs. *Pitx2*$^{cre/null}$*;N-lacZ* = 746 in representative embryos from $N = 3$ pairs) (Fig. 4A, B). This trend toward increased *Pitx2*-expressing cells in the *Pitx2*$^{cre/null}$ hypothalamus might be due to extrinsic effects on neighboring cells. We also detected a shift in β-galactosidase positive cells toward locations farther away from the floor plate in *Pitx2*$^{cre/null}$*;N-lacZ* embryos (Fig. 4C, D).

To test for defects in neuronal migration, we performed BrdU immunohistochemistry on E14.5 embryos exposed to BrdU by maternal intraperitoneal BrdU injection at 1 hour, 1 day, or 2 days prior to embryo collection (Fig. 5). In embryos injected with BrdU 1 hour or 1 day prior to sacrifice, there were no differences in the locations of BrdU-positive cells in the developing hypothalamus of *Pitx2*$^{cre/null}$*;N-lacZ* mutants compared with *Pitx2*$^{cre/+}$*;N-lacZ* embryos (Fig. 5, A–B, E–F). However, in *Pitx2*$^{cre/+}$*;N-lacZ* embryos injected 2 days prior to sacrifice, BrdU-positive cells were distributed throughout the hypothalamus (including the laterally located subthalamic nucleus) (Fig. 5I), whereas in *Pitx2*$^{cre/null}$*;N-lacZ* mutants, BrdU-positive cells were restricted to more medial locations (Fig. 5J).

To identify *Pitx2*-expressing, BrdU-positive cells, we co-labeled transverse sections with anti-BrdU and anti-β-gal. These studies showed no cellular colocalization of β-gal and BrdU in embryos injected at E13.5 or E14.5 (Fig. 5C–D, G–H), consistent with prior evidence that *Pitx2* expression is restricted to postmitotic neurons (Martin et al., 2002). In contrast, there were numerous β-gal+/BrdU+ cells in *Pitx2*$^{cre/+}$*;N-lacZ* and *Pitx2*$^{cre/null}$*;N-lacZ* embryos injected at E12.5, indicating that some normally migrating and arrested cells are of the *Pitx2* lineage. Co-label of β-gal and BrdU in a subset of cells in *Pitx2*$^{cre/null}$*;N-lacZ* embryos injected at E12.5 is consistent with prior studies showing that subthalamic nucleus neurons are born over a five day period between E10.5 and E14.5 in the mouse (Altman and Bayer 1986; Marchand 1987; Martin et al., 2004). These data also provide evidence that *Pitx2*$^{cre/null}$ neurons undergo terminal mitosis normally but become arrested after E12.5 during their migration away from the third ventricle.

To test whether defects in *Pitx2*$^{cre/null}$ neuronal migration are associated with changes in cell survival, we performed TUNEL studies on E10.5 and E12.5 embryos. We found very few TUNEL-positive cells in the hypothalamus of both *Pitx2*$^{cre/+}$*;N-lacZ* and *Pitx2*$^{cre/null}$*;N-lacZ* embryos (Suppl. Fig. 3), suggesting normal cell survival. This observation also corroborates previous data showing normal cell survival in E14.5 *Pitx2* null mutants (Martin et al., 2004). Altogether, these data demonstrate that loss of *Pitx2* disrupts migration of hypothalamic neurons after E12.5, but does not impair neuronal survival or cell cycle exit.

As an initial step toward identifying a mechanism for arrested or delayed migration of *Pitx2* mutant neurons, we tested expression of candidate molecules implicated in neuronal migration based on their known expression in the embryonic mouse hypothalamus and midbrain. Immunohistochemistry for N-cadherin, neural cell adhesion molecule (NCAM), and doublecortin-like protein kinase DCAMKL1/DCLK (Lin et al., 2000) were unchanged in *Pitx2*$^{cre/null}$*; N-lacZ*+ mutant embryos compared to *Pitx2*$^{cre/+}$*; N-lacZ*+ littermates (data not shown), indicating that altered expression of these molecules does not likely explain the observed neuronal migration defects. Additional studies are necessary to determine the molecules and/or signaling factors that mediate PITX2-dependent hypothalamic neuronal migration.

Pitx2 mutant neurons retain some of their molecular fates

Pitx2 acts as a transcriptional regulator in other tissues including the heart, pituitary, craniofacial structures, skeletal muscle, and ocular tissues (Hjalt et al., 2001; Ganga et al., 2003; Charles et al., 2005; Berry et al., 2006; Diehl et al., 2006; Shih et al., 2007). We hypothesized that hypothalamic neuronal migration defects arise due to disrupted gene expression in hypothalamic neurons. To test this hypothesis, we analyzed expression of *Lmx1b*, a LIM homeodomain transcription factor expressed in the subthalamic nucleus and posterior hypothalamus which regulates development of serotonergic and dopaminergic neurons (Smidt et al., 2000; Asbreuk et al., 2002; Ding et al., 2003; Guo et al., 2007). We found, by double immunofluorescence, that PITX2 and LMX1B colocalize extensively in the E14.5 subthalamic nucleus and medial hypothalamus (data not shown). LMX1B expression in the hypothalamus is disrupted with loss of *Pitx2*, based on double immunofluorescence with antibodies againstβ-galactosidase and LMX1B (Fig. 6, A–H). Complete absence of LMX1B immunofluorescence was seen in the subthalamic nucleus region of E14.5 $Pitx2^{cre/null}$;*N-lacZ* mutants (Fig. 6F), whereas double-labeled (β-gal+/LMX1B+) and single-labeled LMX1B + neurons were preserved in the medial hypothalamus (Fig. 6F, G). Thus, LMX1B-expressing neurons destined to occupy the subthalamic nucleus require *Pitx2* for normal migration, whereas medial hypothalamic neurons express *Lmx1b* independent of *Pitx2* function.

We also tested expression of several members of the *forkhead* family of transcription factors, since they are highly expressed in the developing hypothalamus and subthalamic nucleus (Ferland et al., 2003; Teramitsu et al., 2004). Expression of *Foxb1* mRNA, which encodes a forkhead transcription factor required for formation of ventral diencephalon and mammillary bodies (Alvarez-Bolado et al., 2000), was expressed throughout the basal hypothalamus of E14.5 $Pitx2^{+/-}$ and $Pitx2^{-/-}$ embryos (Suppl. Fig. 3). In $Pitx2^{cre/+}$;*N-lacZ* embryos, some hypothalamic cells were double-labeled with anti-βgal and anti-FOXP1 (Fig. 6, K) or anti-FOXP2 (Fig. 6, S). In $Pitx2^{cre/null}$;*N-lacZ* mutants, FOXP1 and FOXP2 immunoreactivity were reduced in, but not absent from, the subthalamic nucleus region, suggesting that some FOXP1 +/β-gal-negative and FOXP2+/β-gal-negative subthalamic nucleus neurons escape the *Pitx2* mutant migration defect (Fig. 6, O, W). FOXP1+/β-gal+ and FOXP2+/β-gal+ cells were also present in the medial $Pitx2^{cre/null}$;*N-lacZ* hypothalamus, indicating that *Pitx2* is not required for FOXP1 or FOXP2 gene expression in these cells (Fig. 6, O, W).

Lmx1b is not required for subthalamic nucleus neuronal development

Certain *Lmx1b* neuronal lineages (subthalamic nucleus neurons) are sensitive to *Pitx2* deficiency, whereas others (dorsal midbrain and medial thalamic/hypothalamic neurons) are not. To test whether *Lmx1b* acts genetically upstream of *Pitx2* in the developing subthalamic nucleus, perhaps through coordinated activity with other transcription factors, we analyzed $Lmx1b^{n/n}$ embryos (Chen et al., 1998) at E14.5 for defects in *Pitx2* mRNA and protein and FOXP1/FOXP2 expression (Fig. 7) (Ferland et al., 2003; Martin et al., 2004; Tamura et al., 2004). Expression of each of these markers in the hypothalamus of E14.5 *Lmx1b* embryos remained intact, indicating that loss of *Lmx1b* does not recapitulate the *Pitx2* mutant phenotype in the subthalamic nucleus. Since *Lmx1b* expression is preserved in a subset of *Pitx2* mutant neurons of the developing hypothalamus, expression of *Pitx2* and *Lmx1b* are likely regulated by independent mechanisms in subthalamic nucleus neuronal development.

These observations suggested that specific populations of developing hypothalamic neurons (with unique PITX2/LMX1B/FOXP1/FOXP2 transcription factor expression profiles) exhibit variable sensitivities to *Pitx2* dosage. LMX1B-positive subthalamic nucleus neurons are highly disrupted by loss of *Pitx2*, whereas some FOXP1-positive and FOXP2-positive cells migrate normally. To test whether this reflects differences in *Pitx2*-lineage, we performed a *Pitx2*-lineage tracing experiment in postnatal day 1 $Pitx2^{cre/+}$;*N-lacZ* hypothalamus and subthalamic

nucleus (Fig. 8). Double label experiments revealed extensive but differential colocalization between β-gal and the following subthalamic nucleus markers: PITX2, Calretinin, LMX1B, FOXP1, and FOXP2. LMX1B+/β-gal-negative cells were present in the caudomedial subthalamic nucleus, whereas FOXP1+/β-gal-negative and FOXP2+/β-gal-negative cells were abundant in the rostral and caudomedial subthalamic nucleus (Fig. 8).

Discussion

We demonstrate, through the use of a *Cre* lineage tracing *Pitx2* deficiency allele, neuronal migration defects in the developing mouse hypothalamus with loss of *Pitx2* function. Our studies provide direct evidence of a genetic requirement for *Pitx2* in normal neuronal migration in the developing hypothalamus, which, unlike the cortex and cerebellum, has not been extensively explored (Ayala et al., 2007; Lim and Golden 2007).

A primary role for Pitx2 in region specific neuronal migration

Prior studies demonstrated a requirement for *Pitx2* in neuronal differentiation of the developing diencephalon, but the precise stages of differentiation (i.e. migration, axon outgrowth, cell specification) and causative mechanisms were not clear (Martin et al., 2004). A detailed understanding of the stages of neuronal differentiation that require *Pitx2* is critical, since these stages may involve separate molecular genetic mechanisms that are differentially susceptible to injury or developmental insult. *Pitx2* expression is high in postmitotic neurons of the hypothalamus (Martin et al., 2002), and data presented here demonstrate that defective neuronal migration is a primary phenotype in this brain region of *Pitx2* null mutants (Suppl. Fig. 2). Initial patterning of hypothalamic regions appears intact in embryos with complete loss of *Pitx2*, yet refined formation of subgroups of PITX2-positive neurons or neurons derived from PITX2-positive cells is impaired. Moreover, migration appears to be an early aspect of neuronal differentiation that is sensitive to *Pitx2* deficiency, but these defects in neuronal migration may influence later cell identity or axon outgrowth.

The primary neuronal migration defect in *Pitx2* deficient mice disrupts organization of the subthalamic nucleus. *Pitx2* mutant cells accumulate medially in the hypothalamus, and appear to be retained medially or aberrantly cross the ventral midline. These data are consistent with previous results showing midline crossing of cells in the chick diencephalon, where retroviral labeling produced bilateral clones on either side of the third ventricle (Golden and Cepko 1996).

Prior studies also raised the possibility that anatomical shifts in expression or stability of *Pitx2* mRNA might occur with *Pitx2* deficiency (Martin et al., 2004), but data presented here make that unlikely. In mouse pituitary cell lines, *Pitx2* mRNA is highly unstable, with an estimated half-life of 30 minutes (Briata et al., 2003). Wnt/β-catenin signaling in pituitary cells stabilizes *Pitx2* mRNA, via changes in interactions between 3′ UTR sequences in the *Pitx2* gene and other proteins (Briata et al., 2003). DNA sequences in the 3′ UTR of the *Pitx2* gene that regulate *Pitx2* mRNA stability are also present in the $Pitx2^{null}$ and $Pitx2^{cre}$ alleles; hence, any inherent difference in *Pitx2* mRNA stability between these alleles is not related to these 3′ mRNA sequences. Moreover, the $Pitx2^{cre}$ allele has the advantage of expressing *Cre* from IRES mediated bicistronic cassettes, allowing for distinction between indelibly marked *Pitx2* expressing mutant neurons and those *Pitx2* mutant neurons that are actively expressing *Pitx2* mRNA. Since our *Cre* lineage tracing approach uses a β-gal reporter that is expressed in *trans* (i.e. a different genomic locus) to *Pitx2* and permanently marks *Pitx2*-expressing neurons (Zinyk et al., 1998), the data provide definitive evidence for defects in migration of $Pitx2^{cre/null}$ neurons that cannot be explained by intrinsic differences in *Pitx2* mRNA expression or stability.

Pitx2 function in neurogenesis and cell fate

We found no evidence to support a major role for *Pitx2* in neural progenitor proliferation or cell cycle exit, indicating that neurogenesis likely proceeds independent of *Pitx2*. These results are similar to those obtained in earlier studies (Martin et al., 2004), and suggest that *Pitx2* may have tissue and cell type specific roles in progenitor proliferation. Canonical Wnt β-catenin signaling regulates *Pitx2* expression in cellular proliferation and survival of the developing pituitary gland and heart (Kioussi et al., 2002); however, our data show that mouse brain neurons are born and survive independent of *Pitx2* function. Developing mouse hypothalamic neurons seem to exhibit highly regionally specific requirements for *Pitx2* for normal migration. Knowledge about specific roles for *Pitx2* in neuronal cell fate are limited to a study in *C. elegans* showing that an orthologue of *Pitx2* (*unc-30*), is critical for formation of GABAergic neurons (Westmoreland et al., 2001). Identifying altered cell fates in the mouse brain with *Pitx2* loss of function remains a major challenge.

Our observations in neurons are also supported by results obtained in craniofacial tissues, where *Pitx2* mutant cells exhibit misdirected or arrested migration (Liu et al., 2003). In cultured cells, *Pitx2* may regulate one or more aspects of migration through changes in the cytoskeleton, as suggested by studies showing that *Pitx2* controls *Rho GTPase* activity of HeLa cells through regulation of *Trio*, a guanine nucleotide exchange factor (Wei and Adelstein 2002). Since *Rho GTPases* are implicated in non-canonical planar cell polarity (PCP) (Winter et al., 2001; Habas et al., 2003), these observations also support the possibility of a more generalized role for *Pitx2* in brain non-canonical *Wnt* signaling.

Genetic interactions in the developing hypothalamus

Expression of LMX1B and PITX2 in the developing subthalamic nucleus implied that these two proteins might actively cooperate or repress each other during neuronal development (Asbreuk et al., 2002). *Lmx1b* is required for proper expression of *Pitx3* in tyrosine hydroxylase positive neurons (Smidt et al., 2000) and for development of the tectum and cerebellum via regulation of *Wnt1* and *Fgf8* (Guo et al., 2007). Our studies identified colocalization of LMX1B and PITX2 in the subthalamic nucleus. We also found that *Lmx1b* expression is absent in the *Pitx2* mutant subthalamic nucleus, consistent with a failure in migration of subthalamic nucleus neurons (Martin et al., 2004). *Lmx1b* expression is retained in medially located hypothalamic *Pitx2* mutant neurons, suggesting that *Lmx1b* positive neurons require *Pitx2* for proper migration to the subthalamic nucleus but not for *Lmx1b* gene expression. Dorsal midline LMX1B-positive midbrain neurons are also unaffected by loss of *Pitx2*.

Lmx1b mutant embryos exhibited normal expression of multiple subthalamic nucleus markers in E14.5 embryos, ruling out an early developmental requirement for *Lmx1b* in *Pitx2* lineage neurons. Combinatorial actions between *Pitx* genes and *Lmx1b* may therefore be limited to *Pitx3* in mesencephalic dopaminergic neurons (Smidt et al., 2000). Our results are similar to data obtained in developing eye tissues, which demonstrate minimal or unchanged *Lmx1b* and *Pitx2* expression in developing mutant mice (Lu et al., 1999; Pressman et al., 2000). We also analyzed for differences in hypothalamic expression of *Ldb1*, a cofactor of *Lmx1b*, and found no changes between *Pitx2* mutant and wildtype sections (data not shown). These data indicate that subthalamic nucleus neurons are highly sensitive to *Pitx2* but not *Lmx1b* dosage for normal migration.

Expression of the transcription factors FOXP1 and FOXP2 in specific subthalamic nucleus neurons with unique sensitivities to PITX2 dosage indicates that complex transcriptional networks are required for subthalamic nucleus neuronal differentiation. Mutations in human *FOXP2* cause a severe language disorder (Balter 2001), and similar expression of FOXP1 and FOXP2 in humans and birds suggests these two proteins may cooperate to regulate

development of specific neuronal populations (Teramitsu et al., 2004). Expression of FOXP1/2 in the mouse cortex, basal ganglia, thalamus, and cerebellum is consistent with roles in postmigratory neurons (Ferland et al., 2003). Some FOXP1-positive and FOXP2-positive subthalamic nucleus neurons require *Pitx2* for proper migration, whereas others do not. These observations suggest that *Pitx2*-expressing subthalamic nucleus neurons exhibit cell intrinsic defects that do not influence the migratory capacities of neighboring neurons. As in other brain regions, functions of FOXP1/2 in the hypothalamus are not well understood, including whether FOXP1 and FOXP2 regulate transcription independently or in cooperation with PITX2 and LMX1B. The differential cellular overlap in FOXP1/2 expression with PITX2 and LMX1B suggests that complex and cell specific combinatorial codes between these four transcriptional regulators are critical for hypothalamic regional specificity and neuronal migration.

Our studies provide indirect evidence about the cell autonomous nature of *Pitx2* defects in the developing hypothalamus. Normal migration of some FOXP1-positive and FOXP2-positive subthalamic nucleus suggests cell autonomous *Pitx2* defects in migration, whereas the slight increase in hypothalamic *Pitx2*-expressing cells suggests cell extrinsic influences on gene expression. Further experiments using transplants, chimeras, or reporter tagged, tissue specific *Pitx2* knockouts will be helpful in addressing these questions.

Experimental Methods

Generation of mutant mice

We used a previously characterized *Pitx2* null allele, $Pitx2^{creneo}$, that exhibits *Cre* activity in areas of known *Pitx2* expression in the heart and craniofacial structures (Liu et al., 2002; Liu et al., 2003). $Pitx2^{creneo/+}$ mice are heterozygous for a *Pitx2* deficiency allele in which the homeodomain is replaced with an IRES-Cre-*neomycin* cassette (Liu et al., 2003). Since brain *Cre* activity patterns in these mice had not been explored in detail, we mated $Pitx2^{creneo/+}$ mice with FLPe recombinase expressing transgenic mice (Jackson Laboratory, Bar Harbor, Maine; stock #003946) to remove intervening neomycin sequences that might interfere with normal *Pitx2* gene expression. Offspring were PCR genotyped for $Pitx2^{cre}$ sequences using Chromo Taq (Denville, Metuchen, NJ) with primers GGTGGGGTGGGGGTGTCTGTAAAA, GCTAGGCGCGAAGGTTCTCCAGTG, and AGATATGGCCCGCGCTGGAGTTTC (Invitrogen, Carlsbad, CA). The resulting PCR product was verified by sequencing (University of Michigan DNA Sequencing Core) to confirm excision of the *neomycin* cassette. PCR reactions were performed with an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C × 30 sec, 59°C × 30 sec, 72°C × 2 min 30 sec, and 10 min incubation at 72°C. *In situ* hybridization with a *Cre* antisense RNA probe on $Pitx2^{cre/+}$ embryos (E10.5 and E12.5) showed *Cre* mRNA in sites of known *Pitx2* expression in the brain, including the developing hypothalamus, midbrain, and zona limitans (Mucchielli et al., 1996) (data not shown). Initial experiments for this study were performed by crossing $Pitx2^{cre/+}$ mice with Rosa26R mice that are homozygous for a *Cre* conditional *lacZ* reporter (Soriano 1999). This approach resulted in preferential β-galactosidase label in the cytoplasm, complicating anatomic definition within the histologically uniform neuroepithelium. To overcome this, we used a strain of mice (*N-lacZ*) that are transgenic for a *Cre* conditional, nuclear localized *lacZ* reporter (Zinyk et al., 1998). $Pitx2^{null/+}$ mice were as previously described (Gage et al., 1999), and were maintained to generation N11 on a C57BL/6J background. $Lmx1b^{+/n}$ mice were obtained from Iain McIntosh (Johns Hopkins University) and were genotyped as previously described (Chen et al., 1998).

N-lacZ doubly hemizygous mice (Jackson Laboratory, Bar Harbor, Maine; stock #002982) (Zinyk et al., 1998) were mated with $Pitx2^{+/null}$ mice (maintained at generation N9-11 on a C57BL/6J background). $Pitx2^{+/null}$; *N-lacZ*/+ mice were mated with *N-lacZ* doubly hemizygous mice, and $Pitx2^{+/null}$; *N-lacZ/N-lacZ* offspring identified by genotyping for

Pitx2 wildtype and *null* alleles as previously described (Gage et al., 1999), and for the *N-lacZ* transgene using quantitative PCR. In each Q-PCR reaction, doubly hemizygous and hemizygous genomic DNA samples were used for reference. DNA samples were assayed in triplicate at (100 ng/μl, 10 ng/μl, and 2 ng/μl). Each sample was assayed with primers 5′ GCCCATCTACACCAACGTAACC3′ and 5′AGTAACAACCCGTCGGATTCTC3′ and probe 6FAM-CGGTCAATCCGCCGTTTGTTCCTA-MRA (Applied Biosystems, Foster City, CA) for detecting *LacZ* and Assay ID: Mm99999915-g1 (Applied Biosystems, Foster City, CA) for detecting *GAPDH* with Platinum qPCR SuperMix-UDG with Rox (Invitrogen, Carlsbad, CA). Samples were amplified and detected using an ABI Prism 7000.

Embryo preparation

Timed pregnancies were established using $Pitx2^{cre/+}$, $Pitx2^{+/null}$;*N-lacZ/N-lacZ*, $Pitx2^{+/null}$, or $Lmx1b^{+/neo}$ mice. The morning of plug identification was designated as E0.5. Litters of embryos were dissected into PBS from pregnant females following cervical dislocation and hysterectomy. From each embryo, an amniotic sac, tail, or limb was retained for genotyping. Embryos were fixed in 4.0% formaldehyde (Fisher, Waltham, MA) for 30 min to 4 hours depending on age, embedded in paraffin and sectioned at 7 μm thickness.

β-galactosidase assay

Litters of E10.5–E14.5 embryos were dissected and fixed in 4% formaldehyde for 20–30 minutes. Embryos were then washed in PBS and X-gal Wash Buffer, as described (Sclafani et al., 2006). Whole embryos were cleared in a glycerol gradient and photographed on a Leica MZ10F dissection microscope. For preparation of vibratome sections, embryos were then post-fixed in 4% formaldehyde overnight at 4° C, craniofacial tissues removed, and brains embedded in 4% low melt Gene Pure agarose (BioExpress, Kaysville, UT) and sectioned at 100–150 μm. Some sections were then double labeled with rabbit anti-Calretinin (Chemicon) at 1:2000 and detected with diaminobenzidine (DAB), as previously described (Martin et al., 2004), then cleared in glycerol and photographed.

Immunohistochemistry, in situ hybridization, and TUNEL staining

Cell proliferation studies with 5-bromo-2′-deoxyuridine (BrdU, Sigma, St. Louis, MO) were done as previously described (Martin et al., 2002; Martin et al., 2004), with intraperitoneal injections of BrdU (300 μl of 1 mg/ml in PBS) at 1 hour, 1 day or 2 days prior to embryo collection. Immunohistochemistry or immunofluorescence on paraffin embedded tissues was also done as described (Martin et al., 2002; Martin et al., 2004), with rat anti-β-galactosidase at 1:1000 (from Tom Glaser), guinea pig anti-LMX1B at 1:10,000 (from Tom Jessell), rabbit anti-FOXP1 at 1:250 (Ferland et al., 2003), rabbit anti-FOXP2 at 1:2000 (Abcam, Cambridge, MA), rabbit anti-DCAMKL1(DCLK) at 1:50 (Lin et al., 2000), rabbit anti-NCAM at 1:500 (Millipore, Billerica, MA), rabbit anti-N-cadherin (Abcam, Cambridge, MA) at 1:100, rabbit anti-PITX2 at 1:400 (Hjalt et al., 2000), rat anti-BrdU at 1:200 (GeneTex, San Antonio, TX), and rabbit anti-Calretinin (Chemicon) at 1:2000. Secondary antibodies were from Vector Laboratories (Burlingame, CA) or Invitrogen (Carlsbad, CA). *In situ* hybridization was performed as previously described (Martin et al., 2002; Martin et al., 2004), using cRNA probes for *Pitx2*, *Cre*, *Ldb1*, and *Foxb1*. Cell death assays were done by TUNEL labeling (Oncogene Research Products, Boston, MA) with methyl green counterstain.

Cell counts

For quantification of hypothalamic neurons, we performed β-galactosidase immunofluorescence on at least three transverse sections from $Pitx2^{cre/+}$; *N-lacZ* and $Pitx2^{cre/null}$; *N-lacZ* E14.5 embryos. Sections were photographed on a Leica DMRB microscope and with Photoshop vCS2 software. β-galactosidase positive cells were counted

by superimposing an orthogonal grid with lines spaced 100 μm apart, scoring cells for nuclear β-galactosidase fluorescence, and tallying the cumulative total number of cells in each column or row over three sections spaced 300 μm apart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Ben Novitch and Yasushi Nakagawa for critical comments on the manuscript. The following people provided reagents: Iain McIntosh (*Lmx1b* mice), Tord Hjalt (PITX2 antibody), Tom Glaser (β-galactosidase antibody), Tom Jessell (LMX1B antibody), Chris Walsh (CAMKL1 antibody), and Edward Morrissey (FOXP1 antibody). Margaret Lomax and Bob Lyons assisted with Q-PCR for genotyping *N-lacZ* mice. These data were presented in part at the 2006 annual meetings of the Society for Developmental Biology and the Society for Neuroscience. This work was supported by funds to DMM (NIH KO8 HD40288, R01 NS054784, a Children's Health Research Center Award, and an award from Janette Ferrantino) and NIH RO1 DE16329 to JFM.

References

Altman J, Bayer SA. The development of the rat hypothalamus. Adv Anat Embryol Cell Biol 1986;100:1–178. [PubMed: 3788679]

Alvarez-Bolado G, Zhou X, Voss AK, Thomas T, Gruss P. Winged helix transcription factor Foxb1 is essential for access of mammillothalamic axons to the thalamus. Development 2000;127(5):1029–38. [PubMed: 10662642]

Asbreuk CH, Vogelaar CF, Hellemons A, Smidt MP, Burbach JP. CNS expression pattern of lmx1b and coexpression with ptx genes suggest functional cooperativity in the development of forebrain motor control systems. Mol Cell Neurosci 2002;21(3):410–20. [PubMed: 12498783]

Ayala R, Shu T, Tsai LH. Trekking across the brain: the journey of neuronal migration. Cell 2007;128 (1):29–43. [PubMed: 17218253]

Balter M. Genetics. First gene linked to speech identified. Science 2001;294(5540):32. [PubMed: 11588230]

Berry FB, Lines MA, Oas JM, Footz T, Underhill DA, Gage PJ, Walter MA. Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. Hum Mol Genet 2006;15(6):905–19. [PubMed: 16449236]

Branda CS, Dymecki SM. Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev Cell 2004;6(1):7–28. [PubMed: 14723844]

Briata P, Ilengo C, Corte G, Moroni C, Rosenfeld MG, Chen CY, Gherzi R. The Wnt/beta-catenin-->Pitx2 pathway controls the turnover of Pitx2 and other unstable mRNAs. Mol Cell 2003;12(5):1201–11. [PubMed: 14636578]

Charles MA, Suh H, Hjalt TA, Drouin J, Camper SA, Gage PJ. PITX genes are required for cell survival and Lhx3 activation. Mol Endocrinol. 2005

Charles MA, Suh H, Hjalt TA, Drouin J, Camper SA, Gage PJ. PITX genes are required for cell survival and Lhx3 activation. Mol Endocrinol 2005;19(7):1893–903. [PubMed: 15761027]

Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet 1998;19(1):51–5. [PubMed: 9590288]

Diehl AG, Zareparsi S, Qian M, Khanna R, Angeles R, Gage PJ. Extraocular muscle morphogenesis and gene expression are regulated by Pitx2 gene dose. Invest Ophthalmol Vis Sci 2006;47(5):1785–93. [PubMed: 16638982]

Ding YQ, Marklund U, Yuan W, Yin J, Wegman L, Ericson J, Deneris E, Johnson RL, Chen ZF. Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci 2003;6(9):933–8. [PubMed: 12897786]

Ferland RJ, Cherry TJ, Preware PO, Morrisey EE, Walsh CA. Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. J Comp Neurol 2003;460(2):266–79. [PubMed: 12687690]

Gage PJ, Suh H, Camper SA. Dosage requirement of Pitx2 for development of multiple organs. Development 1999;126(20):4643–51. [PubMed: 10498698]

Ganga M, Espinoza HM, Cox CJ, Morton L, Hjalt TA, Lee Y, Amendt BA. PITX2 Isoform-specific Regulation of Atrial Natriuretic Factor Expression: SYNERGISM AND REPRESSION WITH Nkx2.5. J Biol Chem 2003;278(25):22437–45. [PubMed: 12692125]

Golden JA, Cepko CL. Clones in the chick diencephalon contain multiple cell types and siblings are widely dispersed. Development 1996;122(1):65–78. [PubMed: 8565854]

Guo C, Qiu HY, Huang Y, Chen H, Yang RQ, Chen SD, Johnson RL, Chen ZF, Ding YQ. Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice. Development 2007;134(2):317–25. [PubMed: 17166916]

Habas R, Dawid IB, He X. Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. Genes Dev 2003;17(2):295–309. [PubMed: 12533515]

Hjalt TA, Amendt BA, Murray JC. PITX2 regulates procollagen lysyl hydroxylase (PLOD) gene expression: implications for the pathology of Rieger syndrome. J Cell Biol 2001;152(3):545–52. [PubMed: 11157981]

Hjalt TA, Semina EV, Amendt BA, Murray JC. The Pitx2 protein in mouse development. Dev Dyn 2000;218(1):195–200. [PubMed: 10822271]

Joyner AL, Zervas M. Genetic inducible fate mapping in mouse: establishing genetic lineages and defining genetic neuroanatomy in the nervous system. Dev Dyn 2006;235(9):2376–85. [PubMed: 16871622]

Kioussi C, Briata P, Baek SH, Rose DW, Hamblet NS, Herman T, Ohgi KA, Lin C, Gleiberman A, Wang J, Brault V, Ruiz-Lozano P, Nguyen HD, Kemler R, Glass CK, Wynshaw-Boris A, Rosenfeld MG. Identification of a Wnt/Dvl/beta-Catenin --> Pitx2 Pathway Mediating Cell-Type-Specific Proliferation during Development. Cell 2002;111(5):673–85. [PubMed: 12464179]

Kitamura K, Miura H, Miyagawa-Tomita S, Yanazawa M, Katoh-Fukui Y, Suzuki R, Ohuchi H, Suehiro A, Motegi Y, Nakahara Y, Kondo S, Yokoyama M. Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development 1999;126(24):5749–58. [PubMed: 10572050]

Lim Y, Golden JA. Patterning the developing diencephalon. Brain Res Brain Res Rev. 2006

Lim Y, Golden JA. Patterning the developing diencephalon. Brain Res Rev 2007;53(1):17–26. [PubMed: 16876871]

Lin CR, Kioussi C, O'Connell S, Briata P, Szeto D, Liu F, Izpisua-Belmonte JC, Rosenfeld MG. Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. Nature 1999;401(6750):279–82. [PubMed: 10499586]

Lin PT, Gleeson JG, Corbo JC, Flanagan L, Walsh CA. DCAMKL1 encodes a protein kinase with homology to doublecortin that regulates microtubule polymerization. J Neurosci 2000;20(24):9152–61. [PubMed: 11124993]

Liu C, Liu W, Palie J, Lu MF, Brown NA, Martin JF. Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions. Development 2002;129(21):5081–91. [PubMed: 12397115]

Liu W, Selever J, Lu MF, Martin JF. Genetic dissection of Pitx2 in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. Development 2003;130(25):6375–85. [PubMed: 14623826]

Lu MF, Pressman C, Dyer R, Johnson RL, Martin JF. Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. Nature 1999;401(6750):276–8. [PubMed: 10499585]

Marchand R. Histogenesis of the subthalamic nucleus. Neuroscience 1987;21(1):183–95. [PubMed: 3601074]

Martin DM, Skidmore JM, Fox SE, Gage PJ, Camper SA. Pitx2 distinguishes subtypes of terminally differentiated neurons in the developing mouse neuroepithelium. Dev Biol 2002;252(1):84–99. [PubMed: 12453462]

Martin DM, Skidmore JM, Philips ST, Vieria C, Gage PJ, Condie BG, Raphael Y, Martinez S, Camper SA. PITX2 is required for normal development of neurons in the mouse subthalamic nucleus and midbrain. Dev Biol 2004;267(1):93–108. [PubMed: 14975719]

Metzger D, Clifford J, Chiba H, Chambon P. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci U S A 1995;92(15):6991–5. [PubMed: 7624356]

Mucchielli ML, Martinez S, Pattyn A, Goridis C, Brunet JF. Otlx2, an Otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. Mol Cell Neurosci 1996;8 (4):258–71. [PubMed: 9026314]

Pressman CL, Chen H, Johnson RL. LMX1B, a LIM homeodomain class transcription factor, is necessary for normal development of multiple tissues in the anterior segment of the murine eye. Genesis 2000;26 (1):15–25. [PubMed: 10660670]

Puelles L, Rubenstein JL. Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci 2003;26(9):469–76. [PubMed: 12948657]

Sclafani A, Skidmore J, Ramaprakash H, Trumpp A, Gage P, Martin D. Nestin-Cre mediated deletion of *Pitx2* in the mouse. Genesis 2006;44:336–344. [PubMed: 16823861]

Shih HP, Gross MK, Kioussi C. Cranial muscle defects of Pitx2 mutants result from specification defects in the first branchial arch. Proc Natl Acad Sci U S A 2007;104(14):5907–12. [PubMed: 17384148]

Smidt MP, Asbreuk CH, Cox JJ, Chen H, Johnson RL, Burbach JP. A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 2000;3(4):337–41. [PubMed: 10725922]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain [letter]. Nature Genetics 1999;21(1):70–1. [PubMed: 9916792]

Sur M, Rubenstein JL. Patterning and plasticity of the cerebral cortex. Science 2005;310(5749):805–10. [PubMed: 16272112]

Tamura S, Morikawa Y, Iwanishi H, Hisaoka T, Senba E. Foxp1 gene expression in projection neurons of the mouse striatum. Neuroscience 2004;124(2):261–7. [PubMed: 14980377]

Teramitsu I, Kudo LC, London SE, Geschwind DH, White SA. Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. J Neurosci 2004;24(13):3152–63. [PubMed: 15056695]

Wei Q, Adelstein RS. Pitx2a expression alters actin-myosin cytoskeleton and migration of HeLa cells through Rho GTPase signaling. Mol Biol Cell 2002;13(2):683–97. [PubMed: 11854422]

Westmoreland JJ, McEwen J, Moore BA, Jin Y, Condie BG. Conserved function of Caenorhabditis elegans UNC-30 and mouse Pitx2 in controlling GABAergic neuron differentiation. J Neurosci 2001;21(17):6810–9. [PubMed: 11517269]

Winter CG, Wang B, Ballew A, Royou A, Karess R, Axelrod JD, Luo L. Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 2001;105(1):81–91. [PubMed: 11301004]

Zervas M, Millet S, Ahn S, Joyner AL. Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. Neuron 2004;43(3):345–57. [PubMed: 15294143]

Zinyk DL, Mercer EH, Harris E, Anderson DJ, Joyner AL. Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Curr Biol 1998;8(11):665–8. [PubMed: 9635195]

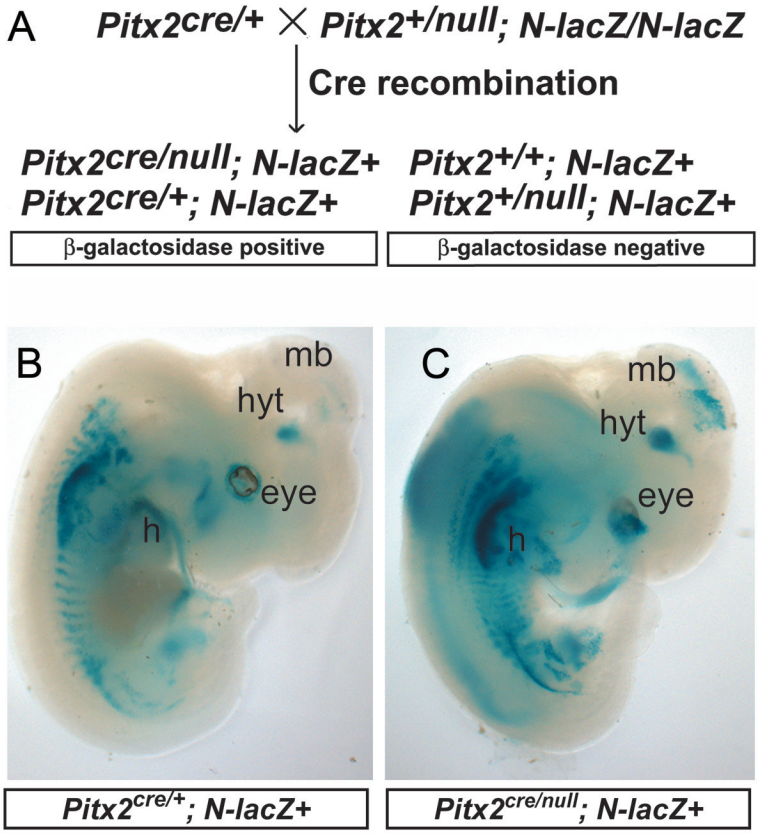

Figure 1.

Pitx2^{cre}; *N-lacZ* lineage tracing approach labels *Pitx2* mutant neurons. (A) *Pitx2*$^{cre/+}$ embryos were mated with *Pitx2*$^{+/null}$; *N-lacZ/N-lacZ* mice to yield embryos of four possible genotypes. *Pitx2*$^{cre/+}$;*N-lacZ* (B) and *Pitx2*$^{cre/null}$:*N-lacZ* (C) embryos express β-galactosidase in *Pitx2*-expressing tissues at E12.5, including the eye, hypothalamus (hyt), midbrain (mb), heart (h), craniofacial structures, and somites. *Pitx2*$^{cre/null}$;*N-lacZ* embryos exhibit lethality after E14.5, likely due to cardiac and abdominal defects that are not well visualized in this sagittally oriented embryo (C).

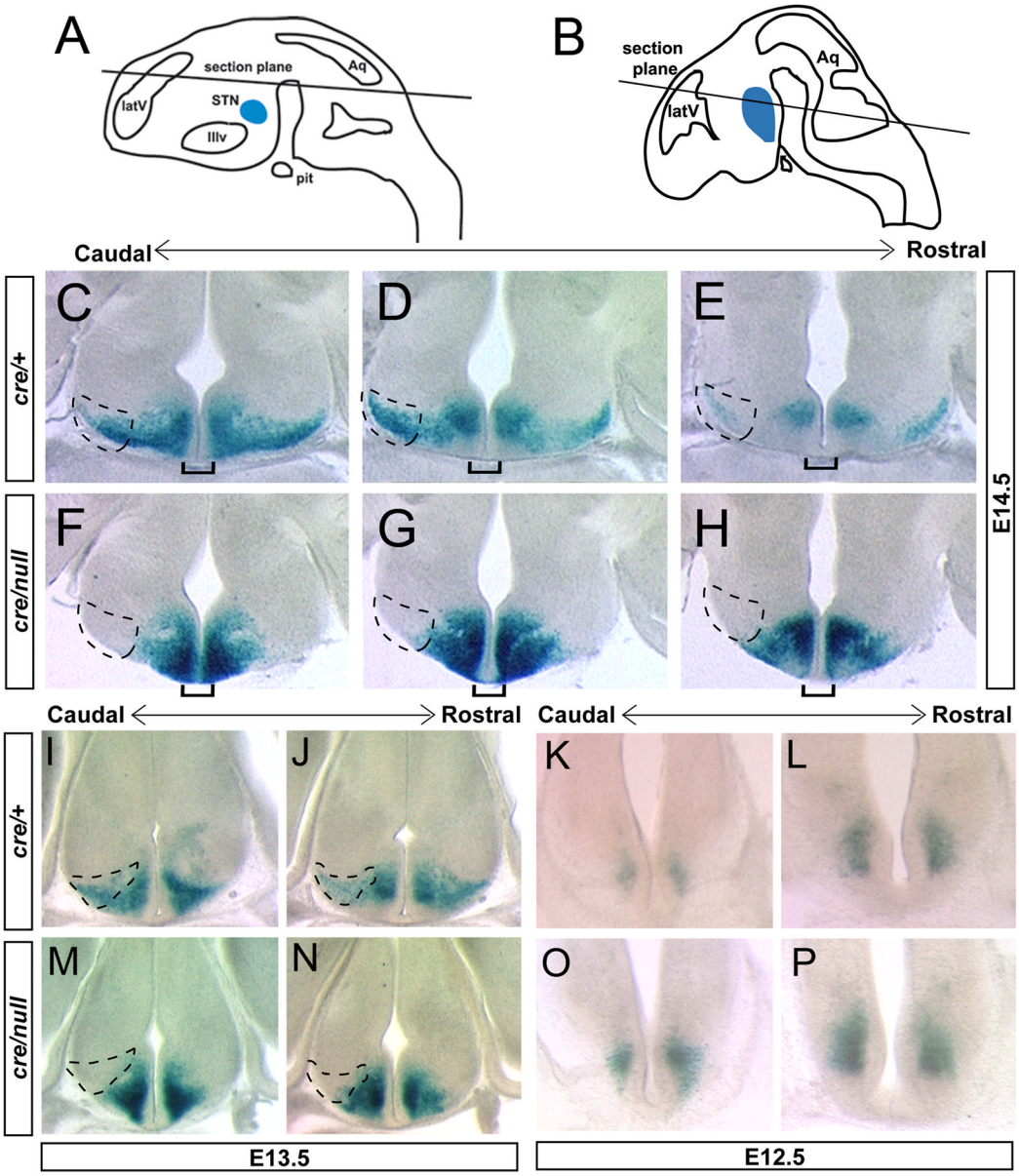

Figure 2.

Pitx2$^{cre/null}$; *N-lacZ* mutants exhibit defects in hypothalamic neuronal location. Littermate embryos of genotype *Pitx2*$^{cre/+}$; *N-lacZ* or *Pitx2*$^{cre/null}$; *N-lacZ* were fixed, stained for β-galactosidase activity, and vibratome sectioned at 100 μm in the plane of section indicated in A (for C–H) and 150 μm in the plane of section shown in B (for I–P). Anterior is up for each section. Hatched areas depict the lateral hypothalamus where subthalamic nucleus neurons are located. Serial adjacent sections of caudal to rostral hypothalamus from E14.5 embryos (C–H) reveal absence of β-galactosidase positive cells in the lateral hypothalamus of *Pitx2*$^{cre/null}$; *N-lacZ* mutants and a medial abundance of β-galactosidase positive cells. *Pitx2*$^{cre/null}$ mutants also exhibit increased β-galactosidase expression at the ventral midline (brackets), suggesting a failure of these cells to respect the midline boundary. Serial sections from E13.5 embryos (I, J, M, N) also exhibit medially shifted β-galactosidase positive cells in *Pitx2*$^{cre/null}$; *N-lacZ* embryos, whereas locations of β-galactosidase expressing cells are similar in E12.5

Pitx2$^{cre/+}$; *N-lacZ* (K, L) and *Pitx2*$^{cre/null}$; *N-lacZ* (O, P) mutants. Abbreviations: latV, lateral ventricle; STN, subthalamic nucleus; IIIv, third ventricle; Aq, cerebral aqueduct; pit, pituitary.

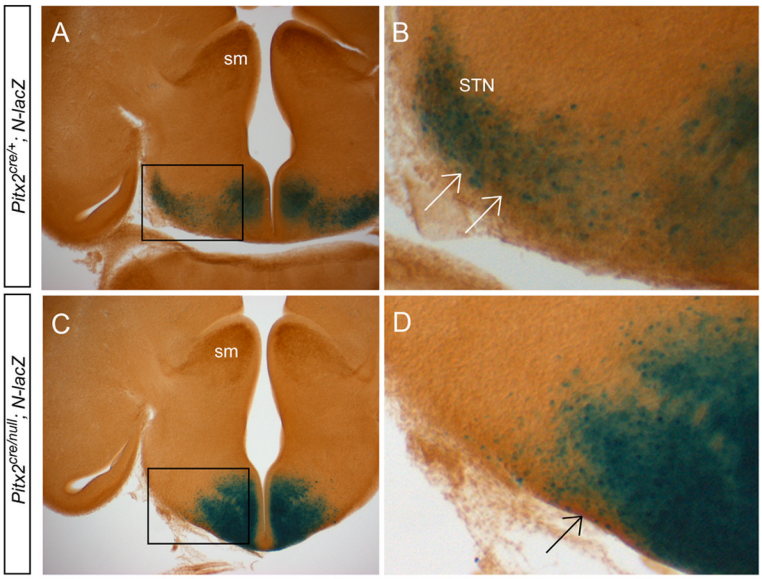

Figure 3.

Pitx2$^{cre/+}$; *N-lacZ* embryos lack subthalamic nucleus neurons. Vibratome sections of E14.5 *Pitx2*$^{cre/+}$; *N-lacZ* (A, B) and *Pitx2*$^{cre/null}$; *N-lacZ* (C, D) littermate embryos were processed for X-gal immunohistochemistry and Calretinin immunoreactivity using DAB. Single X-gal stained images are shown in figure 3 (D, G). Images in B and D are enlarged from boxes shown in A and C. Calretinin immunolabel is abundant in the stria medullaris (sm), subthalamic nucleus (STN), and hypothalamus of *Pitx2*$^{cre/+}$; *N-lacZ* embryos, but is missing from the *Pitx2*$^{cre/null}$; *N-lacZ* subthalamic nucleus. These data show that PITX2-lineage cells (labeled by Calretinin; arrows in B, D) are absent from the *Pitx2*$^{cre/null}$; *N-lacZ* mutant subthalamic nucleus.

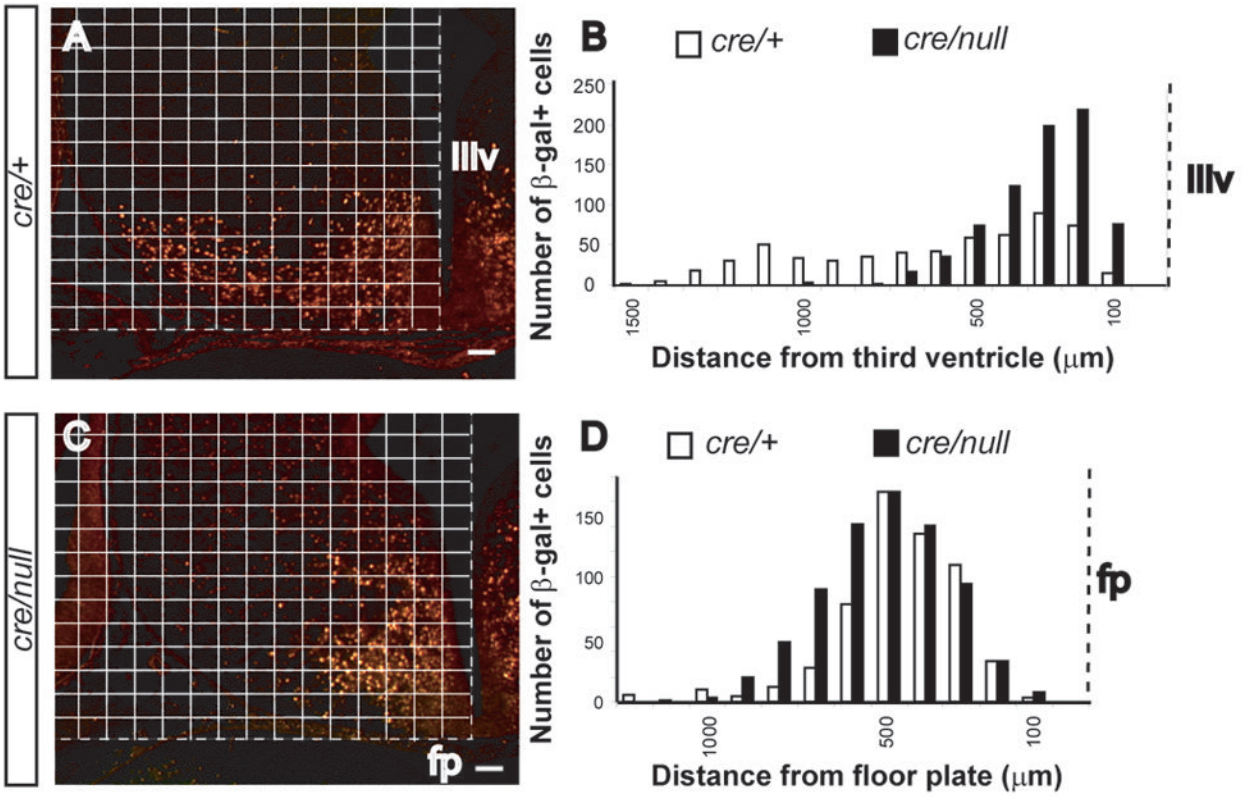

Figure 4.

Hypothalamic *Pitx2*$^{cre/null}$ neurons are shifted medially and away from the floor plate. β-galactosidase positive cells were counted in three different transverse sections from *Pitx2*$^{cre/+}$; *N-lacZ* (A) and *Pitx2*$^{cre/null}$; *N-lacZ* (C) littermate embryos at the level of the hypothalamus (same sections as in figure 7 I, M). β-galactosidase positive cells were more abundant in medial regions of the hypothalamus toward the third ventricle (IIIv) of *Pitx2*$^{cre/null}$; *N-lacZ* embryos compared to *Pitx2*$^{cre/null}$; *N-lacZ* embryos. The overall number of β-galactosidase positive cells was slightly increased in the mutant (*N* = 590 cells for *Pitx2*$^{cre/+}$; *N* = 746 cells for *Pitx2*$^{cre/null}$). There was also a minimal shift of βgal+ cells away from the floor plate (fp) in *Pitx2*$^{cre/null}$; *N-lacZ* cells compared to *Pitx2*$^{cre/+}$; *N-lacZ* embryos (C, D). Scale bar = 100 μm.

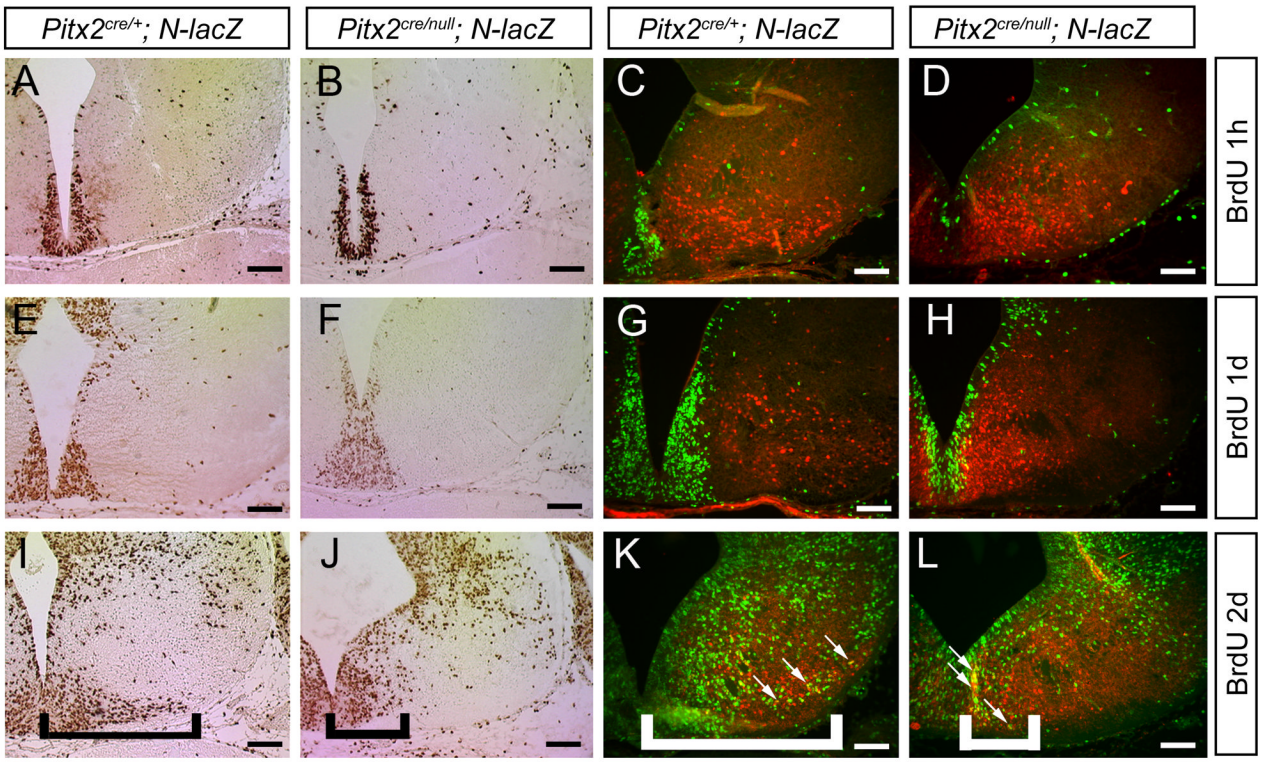

Figure 5.

Hypothalamic *Pitx2*$^{cre/null}$; *N-lacZ* neurons fail to migrate. E14.5 *Pitx2*$^{cre/+}$; *N-lacZ* (A, C, E, G, I, K) and *Pitx2*$^{cre/null}$; *N-lacZ* (B, D, F, H, J, L) embryos were exposed to BrdU by intraperitoneal injection 1 hour (A–D), 1 day (E–H), or 2 days (I–L) prior to collection, and transverse sections processed for anti-BrdU immunohistochemistry (A, B, E, F, I, J) or double-label immunofluorescence with anti-BrdU and anti-β-galactosidase (C, D, G, H, K, L). There were no differences in BrdU labeling between *Pitx2*$^{cre/+}$ and *Pitx2*$^{cre/null}$ mutants injected with BrdU 1 hour (A–D) or 1 day (E–H) prior to analysis. *Pitx2*$^{cre/null}$ mutants injected with BrdU 2 days prior to analysis (I–L) exhibited fewer BrdU positive cells in the lateral hypothalamus (subthalamic nucleus region) compared with *Pitx2*$^{cre/+}$ embryos (bracketed areas). There was no cellular colocalization between BrdU and β-gal in embryos injected at E14.5 (C, D) or E13.5 (G, H), whereas double-labeled cells were present in embryos injected at E12.5 (white arrows in K and L). Scale bars = 100 μm.

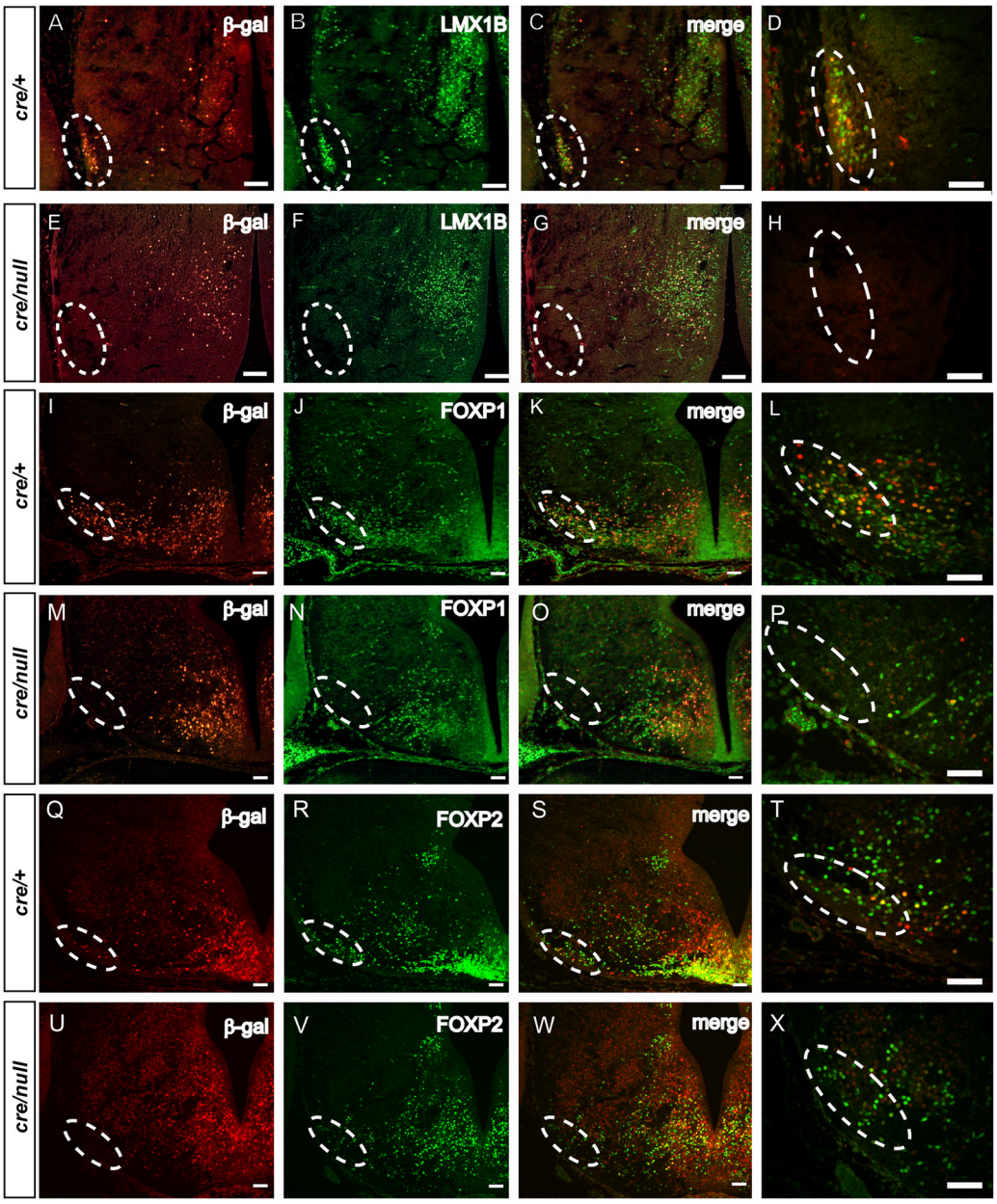

Figure 6.

Subclasses of subthalamic nucleus neurons require *Pitx2* for normal migration. Sections of E14.5 *Pitx2*$^{cre/+}$; *N-lacZ* (A–D, I–L, Q–T) and *Pitx2*$^{cre/null}$; *N-lacZ* (E–H, M–P, U–X) embryos in the coronal (A–H) or transverse plane (I–X) at the level of the subthalamic nucleus were double labeled with anti-β-galactosidase and anti-LMX1B, anti-FOXP1, or anti-FOXP2. Merged images are in C, G, K, O, S, and W, with enlarged subthalamic nucleus regions shown in D, H, L, P, T, and X. Hatched ovals show the lateral hypothalamic region where the subthalamic nucleus normally resides. Loss of β-galactosidase (E, M, and U), and LMX1B (F) in the lateral hypothalamus of *Pitx2*$^{cre/null}$; *N-lacZ* mutants is consistent with absence of these subthalamic nucleus neurons. In contrast, some FOXP1+/β-gal-negative and FOXP2+/β-gal-negative cells remain in the lateral *Pitx2*$^{cre/null}$; *N-lacZ* hypothalamus (P and X), suggesting that these cells escape the migration defect. Some medially located neurons co-expressβ-

galactosidase and LMX1B, FOXP1, or FOXP2, indicating that *Pitx2* is not globally required for expression of these genes. Scale bars = 100μm (A–C, E–G, I–K, M–O, Q–R, and U–W) or 50 μm (D, H, L, P, T, and X).

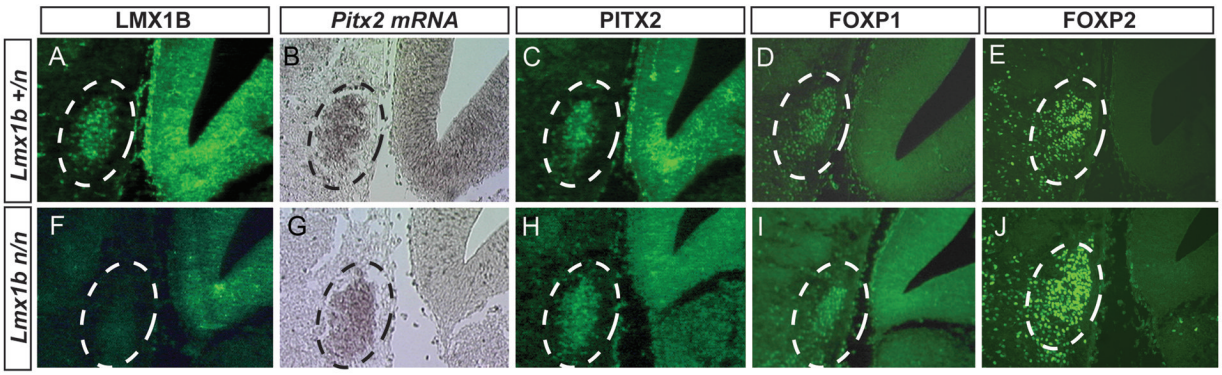

Figure 7.

Lmx1b is dispensable for subthalamic nucleus development. Coronal sections of E14.5 $Lmx1b^{+/n}$ (A–E) and $Lmx1b^{n/n}$ (F–J) paraffin embedded embryos were labeled for anti-LMX1B (A, F), *Pitx2* mRNA (B, G), anti-PITX2 (C, H), anti-FOXP1 (D, I), or anti-FOXP2 (E, J). Absence of LMX1B confirms the null status of $Lmx1b^{n/n}$ embryos. *Pitx2* mRNA and immunofluorescence and FOXP1/FOXP2 immunofluorescence are unchanged in $Lmx1b^{n/n}$ embryos, indicating *Lmx1b* is not required for subthalamic nucleus development.

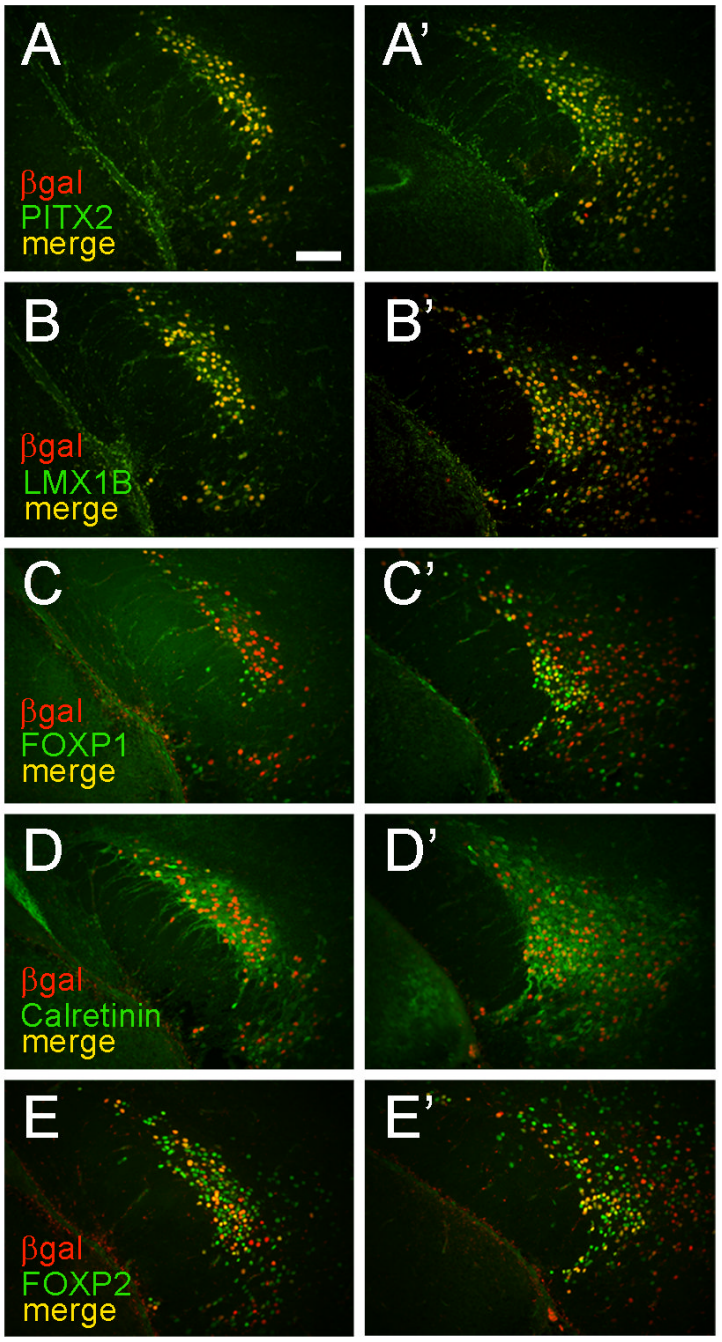

Figure 8.

Neurons in the postnatal subthalamic nucleus exhibit heterogenous lineages. Adjacent coronal sections from rostral (A–E) or caudal (A″–E″) hypothalamus of postnatal day 1 *Pitx2*$^{cre/+}$; *N-lacZ* embryos were co-labeled with anti-β-gal and anti-PITX2 (A, A″), anti-LMX1B (B, B″), anti-FOXP1 (C, C″), anti-Calretinin (D, D″), or anti-FOXP2 (E, E″). Medial is to the right for each section. Extensive colocalization of β-gal is seen with all five markers; however, anti-FOXP1 and anti-FOXP2 both label numerousβ-gal negative subthalamic nucleus cells, suggesting they derive from non *Pitx2*-expressing lineages. Scale bar in A (50 μm) applies to all panels.