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RESEARCH ARTICLE

GABAergic and Glutamatergic Identities of Developing Midbrain *Pitx2* Neurons

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***Pitx2*, a paired-like homeodomain transcription factor, is expressed in post-mitotic neurons within highly restricted domains of the embryonic mouse brain. Previous reports identified critical roles for PITX2 in histogenesis of the hypothalamus and midbrain, but the cellular identities of PITX2-positive neurons in these regions were not fully explored. This study characterizes *Pitx2* expression with respect to midbrain transcription factor and neurotransmitter phenotypes in mid-to-late mouse gestation. In the dorsal midbrain, we identified *Pitx2*-positive neurons in the stratum griseum intermedium (SGI) as GABAergic and observed a requirement for PITX2 in GABAergic differentiation. We also identified two *Pitx2*-positive neuronal populations in the ventral midbrain, the red nucleus, and a ventromedial population, both of which contain glutamatergic precursors. Our data suggest that PITX2 is present in regionally restricted subpopulations of midbrain neurons and may have unique functions that promote GABAergic and glutamatergic differentiation. *Developmental Dynamics 240:333–346,* 2011.** © **2011 Wiley-Liss, Inc.**

Key words: differentiation; transcription factor; nucleogenesis; superior colliculus; red nucleus

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INTRODUCTION

The midbrain is an important relay center that receives and processes sensory inputs and transmits signals to motor outputs in the hindbrain and spinal cord (Wickelgren, 1971; Meredith and Stein, 1986). The dorsal and ventral midbrain are divided by anatomic location and have distinct functional roles and developmental programs. The developing midbrain can be subdivided into three medio-lateral zones: a deeply localized ventricular zone, an intermediate zone, and a superficial mantle zone. Along the dorso-ventral axis, the midbrain is comprised of seven domains (m1–m7), each characterized by unique combinations of transcription factors, signaling molecules, and neurotransmitter expression (Nakatani et al., 2007; Kala et al., 2009). The dorsal domains (m1–m3) make up the superior colliculus and are organized into layers, whereas the ventral domains (m4–m7) are organized into distinct nuclei.

The superior colliculus receives multisensory inputs from the retina, cortex, and spinothalamic pathway (Mehler et al., 1960; Garey et al., 1968; Valverde, 1973). These inputs are important for movement of the head and limbs in response to stimuli, attention, and mediating saccades (Sparks and Mays, 1990; Kustov and Robinson, 1996; Lunenburger et al., 2001). Dorsal midbrain layers develop in an inside-out manner, whereby early born neurons migrate radially to reach a predestined layer, then migrate tangentially to their final rostro-caudal destinations (Edwards et al., 1986; Tan et al., 2002). In this fashion, older neurons occupy deeper layers and younger neurons are located more superficially (Altman and Bayer, 1981). Neurogenesis in the dorsal mouse midbrain occurs between E11.5–E14.5 (Edwards et al., 1986). Early born neurons migrate and differentiate such that by E18.5, all seven layers of the superior colliculus (stratum zonale [SZ], stratum griseum superficiale [SGS], stratum

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opticum [SO], stratum griseum intermedium [SGI], stratum album intermedium [SAI], stratum griseum profundum [SGP], and stratum album profundum [SAP]) are established (Altman and Bayer, 1981; Edwards et al., 1986). Between E18.5 and P6, collicular layers expand radially, become better defined, and undergo refinement of fiber bundles (Edwards et al., 1986).

The ventral midbrain is important for control of limb movement and locomotor coordination, and for mediating reward and stress responses (Le Moal and Simon, 1991; Feenstra et al., 1992; Sinkjaer et al., 1995). The ventral midbrain consists of domains m4–m7 and, unlike the layered dorsal midbrain, is comprised of distinct nuclei (red nucleus, oculomotor nucleus, Edinger-Westphal nucleus, reticular formation, ventral tegmental area, and substantia nigra) that are organized in a stereotypic pattern (Hasan et al., 2010). During development, the ventral midbrain is divided into five morphogenetic arcs,

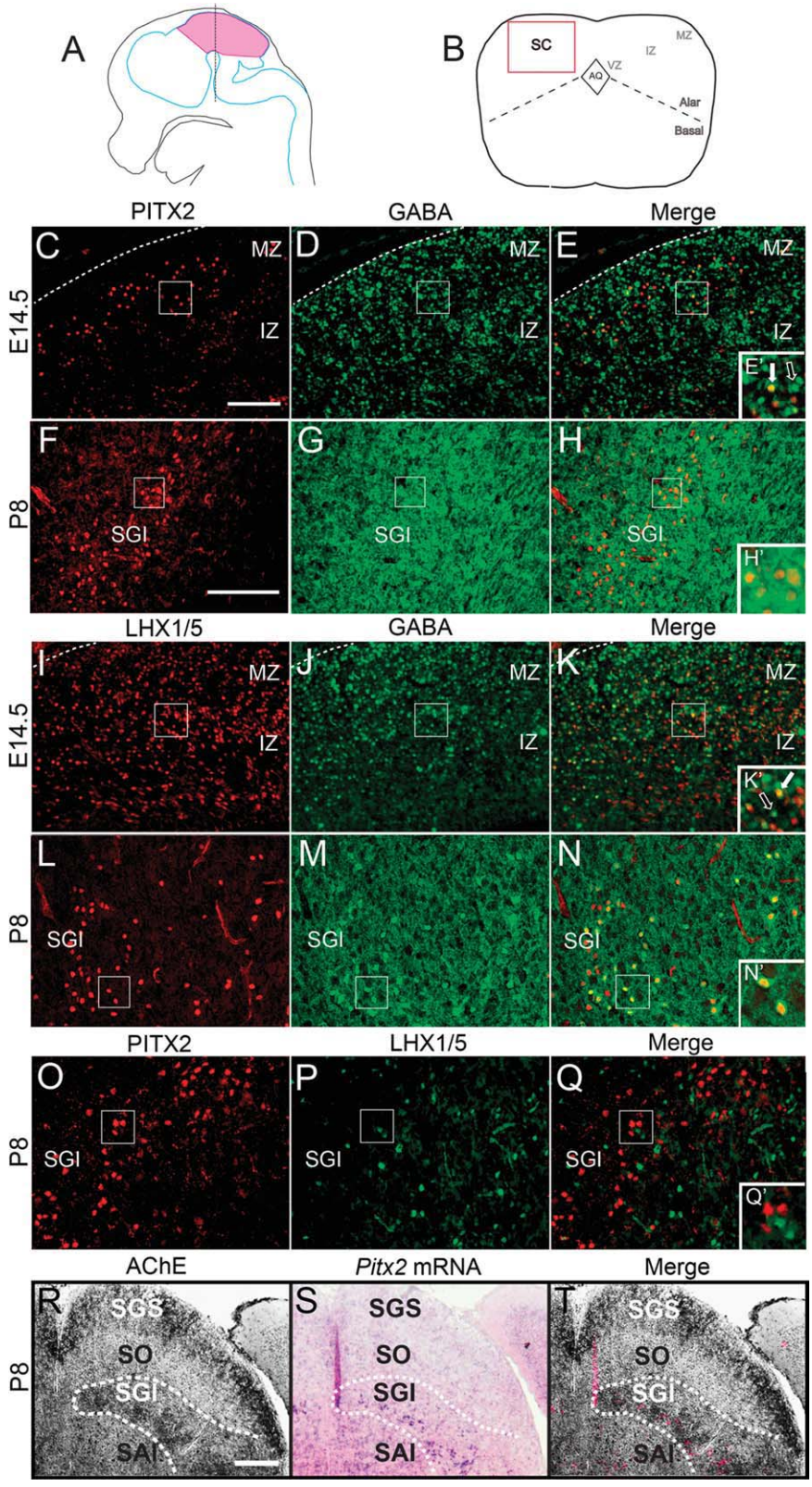

Fig. 1.

Fig. 1. PITX2 identifies GABAergic interneurons in an intermediate layer of the dorsal midbrain. **A:** Cartoon showing sagittal view of an embryonic mouse brain with midbrain highlighted in pink and a dotted line indicating the location of coronal sections shown in C–T. **B:** Cartoon of a coronal midbrain section highlighting the superior colliculus (SC), aqueduct (AQ), alar-basal boundary, and the ventricular (VZ), intermediate (IZ), and mantle zones (MZ). **C–H:** E14.5 and P8 midbrains processed for immunofluorescence for PITX2 (red) and GABA (green). **E′, H′:** Enlarged boxes in E and H. At E14.5, PITX2-positive and GABA-positive cells are located in the intermediate and mantle regions of the superior colliculus, where most PITX2-positive cells are also GABA-positive (E′). White arrow in E′ indicates co-localization of PITX2 and GABA. Open arrow indicates a GABA-positive, PITX2-negative neuron. F–H′: At P8, PITX2-positive cells occupy an intermediate GABAergic layer of cells where GABA-positive cytoplasm surrounds PITX2-positive nuclei. **I–N:** E14.5 and P8 midbrains processed for immunofluorescence for LHX1/5 and GABA. **K′, N′:** Enlarged boxes in K and N. At E14.5, LHX1/5-positive cells are distributed throughout the superior colliculus, whereas GABA-positive cells reside in the intermediate and mantle zones. At P8, some LHX1/5-positive cells are GABA-positive (white arrow in K′). The open arrow in K′ indicates a GABA-positive, LHX1/5-negative neuron. **O–Q′:** At P8, PITX2-positive neurons are localized superficial to the LHX1/5-positive population. **R–T:** Adjacent sections from P8 brains processed for acetylcholinesterase (AChE) staining and *Pitx2* in situ hybridization shows *Pitx2* mRNA and AChE strongly expressed in an intermediate superior colliculus layer, the stratum griseum intermedium (SGI). Dotted lines indicate the outline of the AChE-positive layer. Scale bar in C = 100 μm and applies to C–E and I–K; in F = 100 μm and applies to F–H and L–Q; in R = 250 μm and applies to R–T. L–N′ were imaged using confocal microscopy. MZ, mantle zone; IZ, intermediate zone; SGS, stratum griseum superficiale; SO, stratum opticum; SGI, stratum griseum intermedium; SAI, stratum album intermedium.

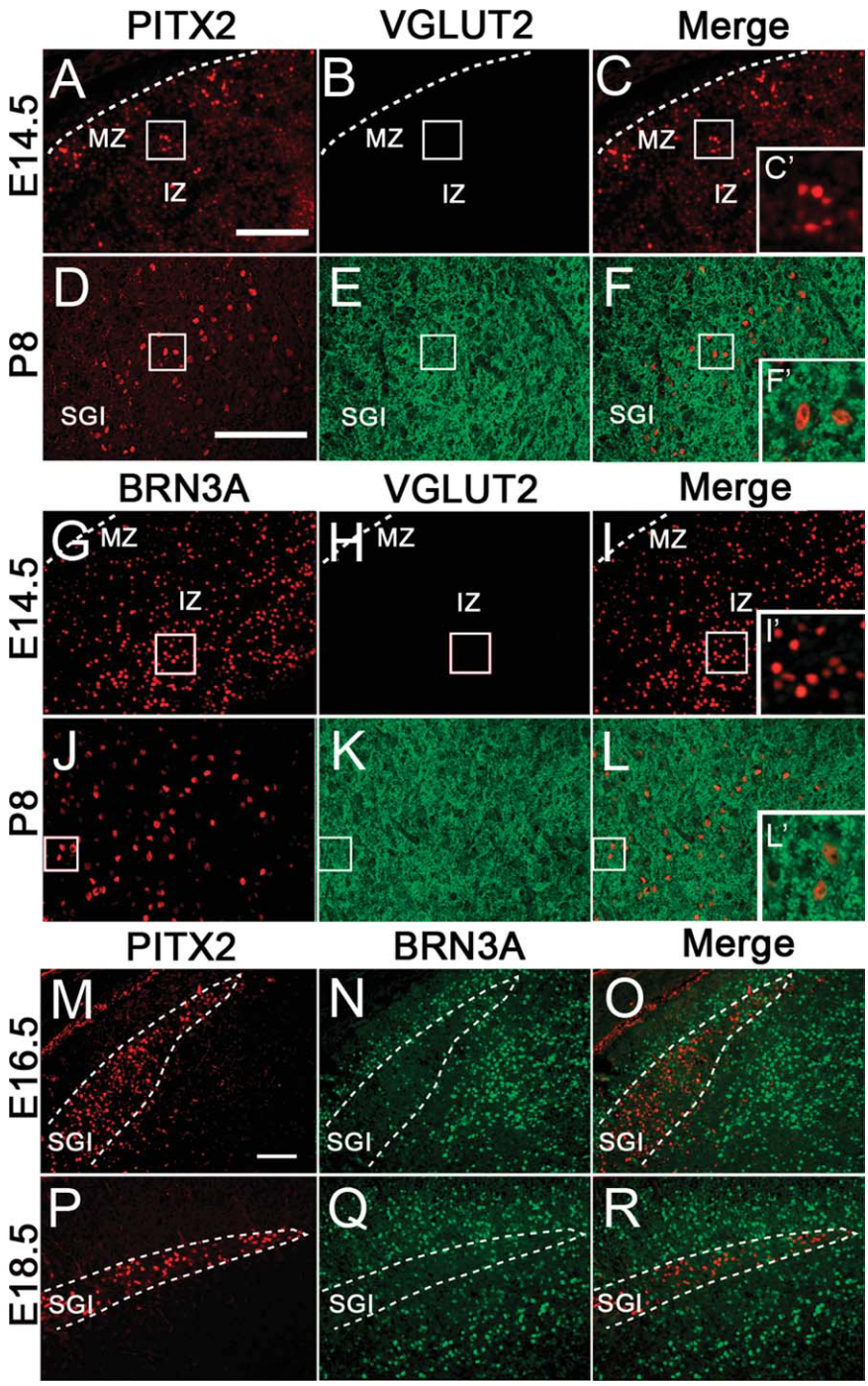

Fig. 2. Collicular glutamatergic neurons are BRN3A-positive and PITX2-negative. Coronal sections of E14.5 and P8 midbrains were processed for immunofluorescence for PITX2 and VGLUT2 (A–F). Boxes in C and F are enlarged in C′ and F′. Dotted lines indicate the midbrain pial surface. **A–C′:** At E14.5, VGLUT2 is absent from the dorsal midbrain. **D–F′:** At P8, PITX2-positive cells are located in the SGI and VGLUT2-positive neurons occupy the intermediate and deep layers of the superior colliculus; however, VGLUT2-positive staining does not circumscribe the PITX2-positive nuclei. **G–L′:** Coronal sections of E14.5 and P8 midbrains processed for immunofluorescence for BRN3A and VGLUT2. Boxes in I and L are enlarged in I′ and L′. At E14.5, BRN3A-positive cells are located in the deep and intermediate superior colliculus, and VGLUT2 is absent. J–L′: At P8, VGLUT2 circumscribes collicular BRN3A-positive nuclei. **M–R:** Coronal sections from E16.5 (M–O) and E18.5 (P–R) embryos labeled with antibodies against PITX2 and BRN3A show that PITX2 is present in an intermediate layer positioned between two BRN3A-positive layers. P–R: At E18.5, PITX2 and BRN3A continue to be localized in separate tectal layers and the PITX2-positive layer appears more compact. Dotted lines indicate the outline of the PITX2-positive collicular layer. Scale bar in A = 100 μm and applies to A–C and G-1; in D = 100 μm and applies to D–F and J–L; in M = 100 μm and applies to M–R. D–F′ and J–L′ were imaged using confocal microscopy. MZ, mantle zone; IZ, intermediate zone; SGI, stratum griseum intermedium.

each distinguishable by a unique pattern of transcription factor expression (Agarwala and Ragsdale, 2002; Sanders et al., 2002). Cells in these arcs are postulated to undergo nucleogenesis, during which cells receive specific signals to differentiate and migrate to form distinct anatomic nuclei based on their location within each arc (Agarwala and Ragsdale, 2002). Nucleogenesis in the ventral midbrain requires precise temporal and spatial control of transcription factor expression (Bayly et al., 2007; Andersson et al., 2008), although the unique contributions of these transcription factors have not been fully characterized.

Previous studies showed that the transcription factor PITX2 is required for proper midbrain development (Martin et al., 2004). *Pitx2* is expressed in both dorsal and ventral midbrain subpopulations and is required for proper migration of collicular neurons into the intermediate zone and mantle zone (Martin et al., 2004). In the superior colliculus, a subpopulation of post-mitotic PITX2-positive neurons was identified as GABAergic (Martin et al., 2002). Ventral PITX2-positive populations have not been characterized. Other researchers have begun to map midbrain transcription factors by domain and factor co-expression (Nakatani et al., 2007; Kala et al., 2009), but PITX2 has not been incorporated into these maps. Here, we characterized dorsal and ventral midbrain *Pitx2*-positive cells for their neurotransmitter identities, localization within the neuroepithelium, and early co-expression with other transcription factors and signaling molecules. We also placed PITX2 within the emerging paradigm of m1–m7 dorso-ventral midbrain domains. Our results suggest that PITX2 may have unique roles in the development of midbrain GABAergic versus glutamatergic neurons.

RESULTS

PITX2 Is Expressed in GABAergic Neurons of the Intermediate Superior Colliculus

To determine the identity of PITX2-positive neurons in the dorsal midbrain, we used double immunofluorescence with antibodies against PITX2

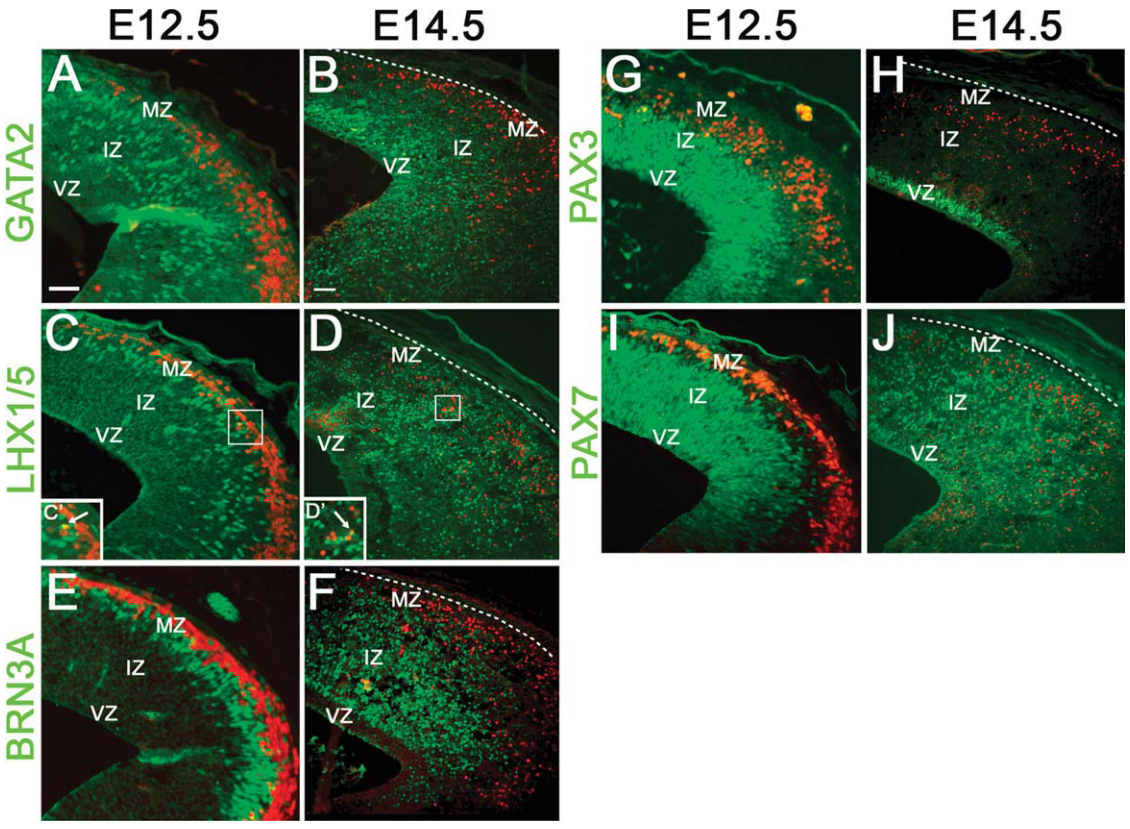
E12.5
E14.5
E12.5
E14.5
GATA2
PAX3
LHX1/5
PAX7
BRN3A
A
B
C
D
E
F
G
H
I
J
MZ
IZ
VZ
C'
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Fig. 3.

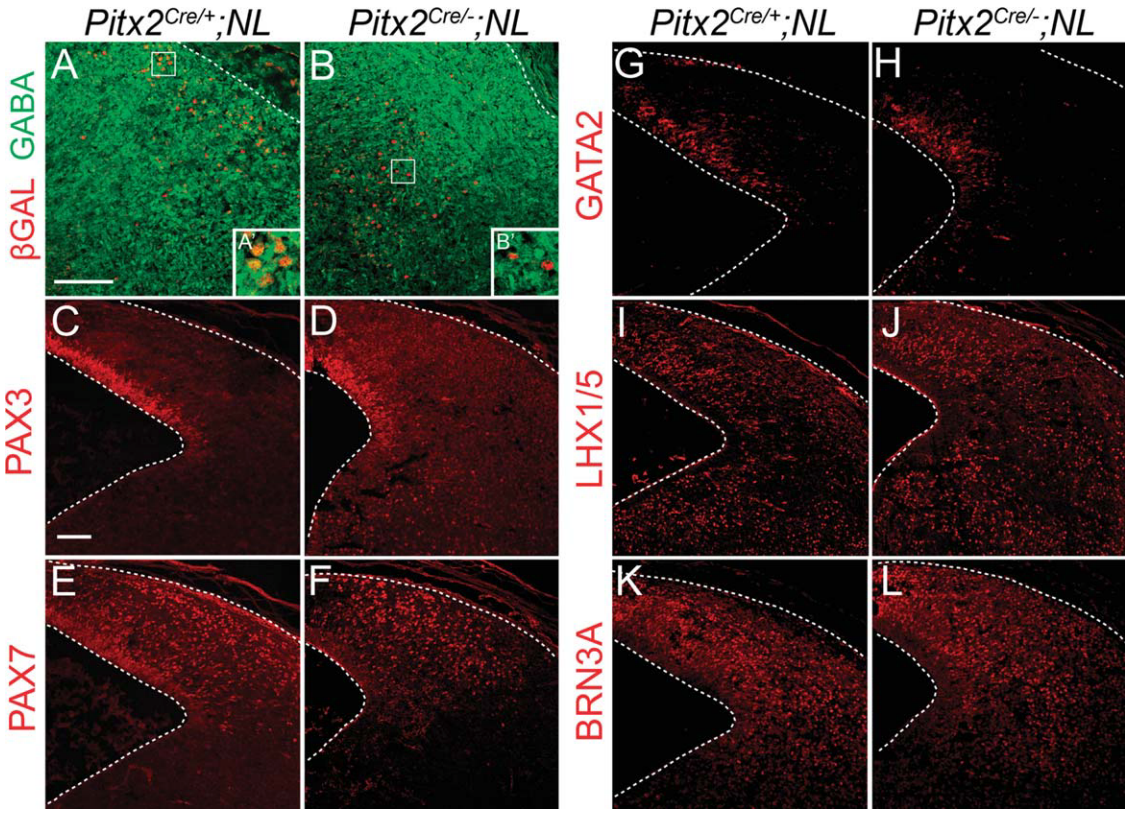
Pitx2Cre/+;NL
Pitx2Cre/-;NL
Pitx2Cre/+;NL
Pitx2Cre/-;NL
βGAL GABA
PAX3
PAX7
GATA2
LHX1/5
BRN3A
A
B
C
D
E
F
G
H
I
J
K
L
A'
B'

Fig. 4.

and markers of specific neurotransmitters. In the E14.5 superior colliculus, most PITX2-positive cells were positive for GABA (Fig. 1C–E′). At postnatal day 8 (P8), all PITX2-positive neurons in the superior colliculus had undergone GABAergic differentiation and were surrounded by GABAergic cytoplasm (Fig. 1F–H′). In order to determine whether GABAergic neurons could be identified by LHX1, a transcription factor expressed during collicular GABAergic differentiation (Kala et al., 2009), we analyzed collicular neurons for LHX1/5 and GABA co-localization. At E14.5, many intermediate collicular neurons were positive for both LHX1/5 and GABA (Fig. 1I–K′). Near the pial surface, the majority of GABA-positive neurons were LHX1/5-negative. At P8, densely labeled GABA-positive neurons continued to express LHX1/5 (Fig. 1L–N′). PITX2-positive cells were located superficial to LHX1/5-positive cells (Fig. 1O–Q′), indicating that PITX2 and LHX1/5 mark different GABAergic subpopulations of the superior colliculus.

GABAergic neurons are abundant in the midbrain and are especially prevalent in the superficial layers and the intermediate layer (SGI) of the colliculus (Lee et al., 2007). The SGI receives numerous cholinergic inputs and can be identified by staining for the cholinergic enzyme acetylcholinesterase (AChE) (McHaffie et al., 1991). To determine whether collicular PITX2-positive cells are located in the SGI, we analyzed dorsal midbrains for *Pitx2* and AChE expression. At P8, the SGS and SGI were easily identified by strong AChE staining (Fig. 1R) and *Pitx2*-expressing cells were identified within the intermediate AChE positive layer (Fig. 1S,T), indicating that collicular *Pitx2*-positive neurons are located in the SGI.

Because the SGI layer is rich in glutamatergic afferents, we analyzed the expression of PITX2 and vesicular glutamate transporter 2 (VGLUT2), a membrane transport protein responsible for glutamate uptake into vesicles. At E14.5, VGLUT2 was absent in the dorsal midbrain (Fig. 2A–C′). At P8, VGLUT2 was present throughout the intermediate and deep layers of the superior colliculus and was not co-localized at the cellular level with PITX2 (Fig. 2D–F′). This is consistent with known glutamatergic afferents projecting to the SGI including cortico-collicular, retino-collicular, and colliculo-collicular pathways (Woo et al., 1985; Mize and Butler, 1996; Olivier et al., 2000). To further characterize glutamatergic neurons in the colliculus, we analyzed expression of the transcription factor BRN3A, which marks the nuclei of glutamatergic precursor neurons (Fedtsova and Turner, 1995; Nakatani et al., 2007). BRN3A-positive cells were distributed throughout the E14.5 intermediate and medial superior colliculus (Fig. 2G–I′). At P8, BRN3A-positive nuclei were tightly associated with VGLUT2-positive label throughout the colliculus (Fig. 2J–L′), suggesting these BRN3A-positive cells are glutamatergic. We also examined PITX2 and BRN3A expression at E16.5 and E18.5. At E16.5, the PITX2-positive cell layer was tightly situated between two BRN3A layers with minimal intermingling among the cells (Fig. 2M–O), suggesting that BRN3A-positive cells are situated in the SAI and a sublayer within the SGI or SO. At E18.5, the BRN3A and PITX2-positive layers were more defined and no intermingling among cells in these layers occurred (Fig. 2P–R). Thus, collicular layers can be identified by unique transcription factor patterns.

Fig. 3. PITX2-positive cells represent a unique population of GABAergic dorsal midbrain precursors. Coronal sections of E12.5 and E14.5 midbrains were processed for double immunofluorescence with antibodies against markers of neuronal precursors. At E12.5 and E14.5, PITX2-positive cells (red) reside in the mantle layer of the superior colliculus. **A,B:** GATA2-positive cells are located intermedially at E12.5 and extend throughout the superior colliculus at E14.5, but do not overlap with PITX2-positive cells. **C,D:** LHX1/5 is located deep to PITX2 at E12.5 and by E14.5 LHX1/5-positive cells are found throughout the superior colliculus. At both timepoints, only a few neurons show co-localization of both markers (insets in **C′,D′**). White arrows indicate cells with co-localization. **E,F:** BRN3A and PITX2 are present in distinct cells at E12.5 and E14.5. **G,H:** PAX3-positive cells are found throughout dorsal ventricular and intermediate zones and are PITX2-negative at E12.5. H: At E14.5, PAX3-positive cells are PITX2-negative and ventricularly restricted. **I,J:** PAX7-positive cells are broadly distributed throughout the superior colliculus and expanded superficially at E14.5 but do not co-localize with PITX2. Dotted lines indicate the pial surface. Scale bars in A and B = 100 μm and apply to A, C, E, G, I and B, D, F, H, J, respectively. MZ, mantle zone; IZ, intermediate zone; VZ, ventricular zone.

Fig. 4. PITX2 is required for GABAergic differentiation. Coronal sections of E14.5 *Pitx2*$^{Cre/+}$;NL and *Pitx2*$^{Cre/-}$;NL midbrains were processed for double immunofluorescence with antibodies against βGAL and GABA (A,B). Boxes in A and B are enlarged in A′ and B′. **A,A′:** PITX2-lineage cells in the *Pitx2*$^{Cre/+}$;NL embryo are located near the pial surface in a strongly GABAergic layer and are GABA-positive. **B,B′:** PITX2-lineage cells in the *Pitx2*$^{Cre/-}$;NL embryo are medially mislocalized and are GABA-negative. C–L: *Pitx2*$^{Cre/+}$;NL and *Pitx2*$^{Cre/-}$;NL coronal E14.5 midbrain sections were processed for immunofluorescence with antibodies against PAX3, PAX7, GATA2, LHX1/5, or BRN3A. **C,D:** PAX3 is restricted to the ventricular zone in both *Pitx2*$^{Cre/+}$;NL and *Pitx2*$^{Cre/-}$;NL embryos. **E,F:** PAX7-positive cells are distributed throughout the colliculus of both *Pitx2*$^{Cre/+}$;NL and *Pitx2*$^{Cre/-}$;NL midbrains. **G,H:** GATA2 is restricted to deep collicular cells and loss of PITX2 does not affect GATA2 patterning. **I–L:** In both *Pitx2*$^{Cre/+}$;NL and *Pitx2*$^{Cre/-}$;NL midbrains, LHX1/5- and BRN3A-positive cells are spread throughout the superior colliculus. A–L were imaged using confocal microscopy. Scale bars in A and C = 100 μm and apply to A,B and C–L, respectively.

Collicular PITX2-Positive GABAergic Neurons Have Unique Molecular Signatures

Since *Pitx2* is expressed in GABAergic neurons in the dorsal midbrain during early collicular differentiation, we reasoned it might be co-expressed with dorsal GABAergic precursor markers such as LHX1/5 and GATA2. GATA2 is a transcription factor expressed in post-mitotic neurons in the early stages of GABAergic differentiation and LHX1 is expressed downstream of GATA2 (Kala et al., 2009). At E12.5, GATA2-positive cells occupied the intermediate superior colliculus, whereas PITX2-positive neurons were found in the mantle zone (Fig. 3A). At E14.5, GATA2 was restricted to cells located superficially to the ventricular zone and did not co-localize with PITX2 (Fig. 3B). At E12.5, LHX1/5-positive cells were localized to the mantle zone, more superficially than GATA2, and found

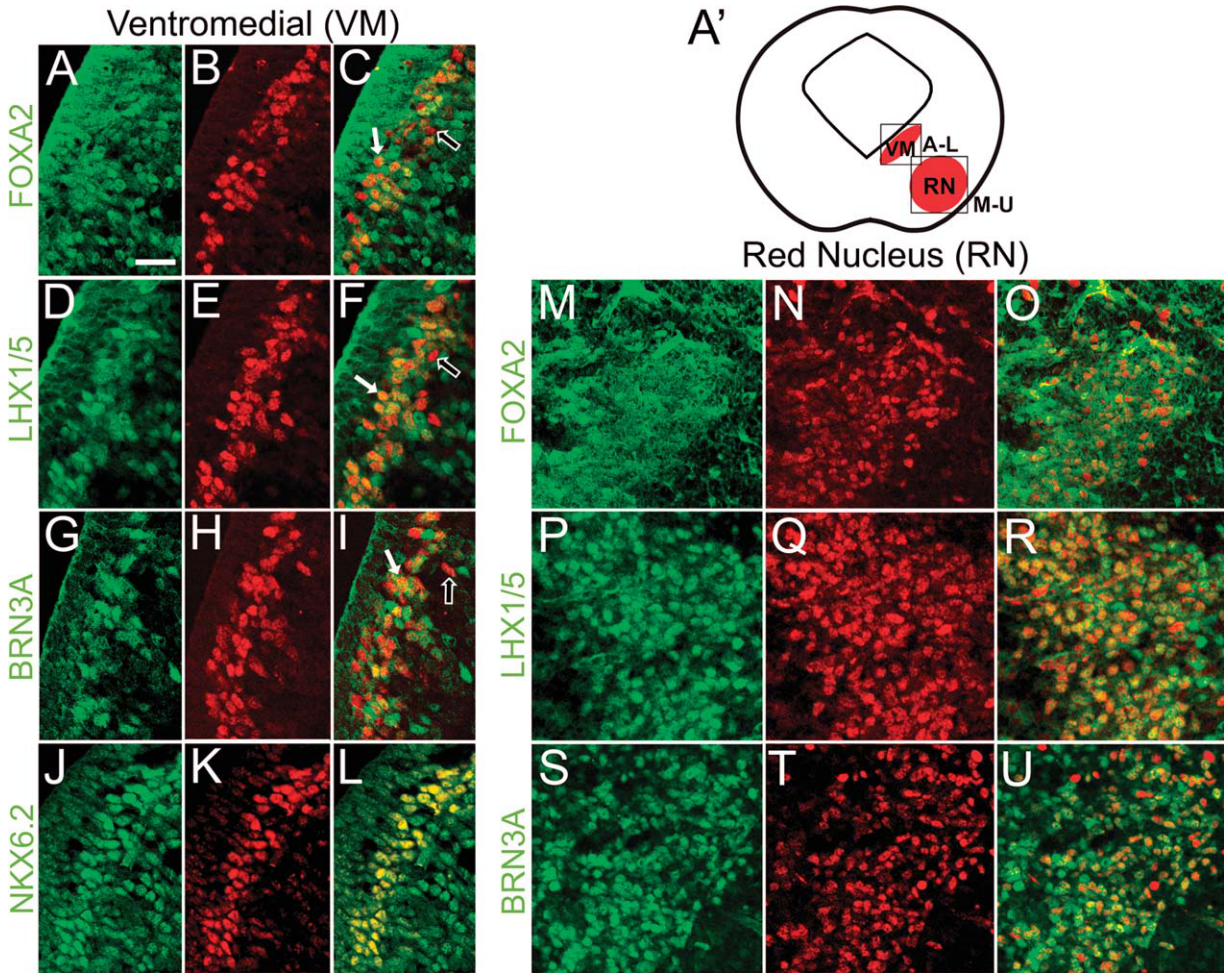

Fig. 5.

deeper than PITX2-positive neurons (Fig. 3C). At E14.5, LHX1/5-positive cells extended from the ventricular zone to the sub-pial surface (Fig. 3D). At both E12.5 and E14.5, only a few cells were positive for both PITX2 and LHX1/5 (Fig. 3C′,D′). Thus, PITX2, GATA2, and LHX1/5 appear to mark distinct subpopulations of GABAergic collicular neurons. At E12.5, BRN3A-positive cells were located adjacent and deeper than PITX2-positive cells, whereas at E14.5 BRN3A was expressed throughout the ventral and intermediate colliculus (Fig. 3E,F). Collicular PITX2-positive cells are thus negative for BRN3A during early development, providing further evidence against glutamatergic identity of PITX2-positive cells.

Unlike GATA2, LHX1/5, and BRN3A, which are required for cell-type differentiation, PAX3 and PAX7 transcription factors are necessary for early superior colliculus establishment

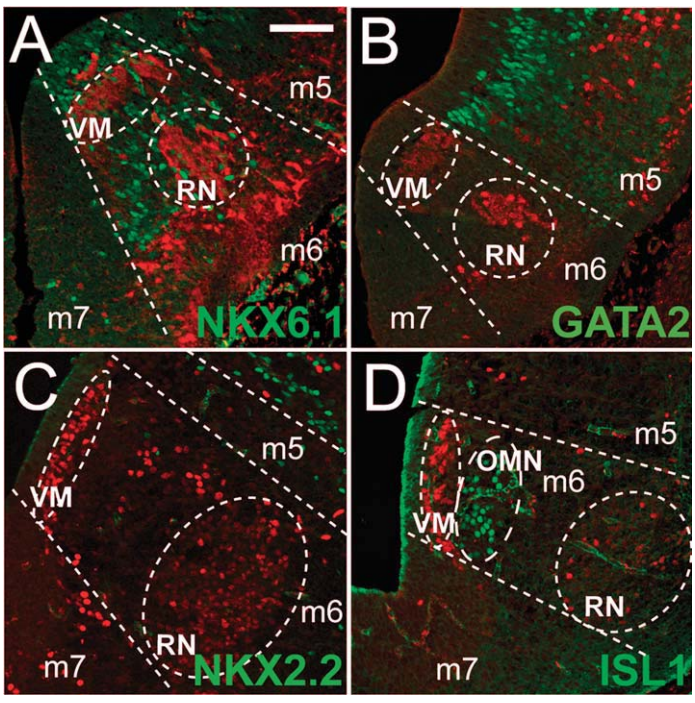

Fig. 6.

(Thompson et al., 2004, 2008). *Pax3* is expressed transiently in all early collicular cells and expression disappears by birth (Thompson et al., 2008). *Pax7* is also expressed in early collicular cells, but continues to be expressed in the mature colliculus where it regulates maintenance of superficial collicular layers (Thompson et al., 2008). We found no overlap between PITX2 and PAX3 or PAX7 at E12.5–E14.5 in the dorsal midbrain (Fig. 3G–J). Together, these data indicate that PITX2-positive neurons represent a subpopulation of superior colliculus cells with unique molecular signatures.

PITX2 Is Required for GABAergic Differentiation, But Not Early Collicular Patterning

Previous studies showed that PITX2 is downstream of the transcription factor GATA2, which is necessary for collicular GABAergic differentiation (Kala et al., 2009). Additionally, in vitro studies suggested that mouse PITX2 activates the promoter of *Gad1*, which encodes the enzyme for GABA synthesis (Westmoreland et al., 2001). To determine whether PITX2 is required for collicular GABAergic differentiation, we crossed $Pitx2^{Cre/+}$ mice to a nuclear *LacZ* (*NL*) reporter strain (Skidmore et al., 2008). $Pitx2^{Cre/+}$;*NL* mice permanently express β-galactosidase (βGAL) in the nuclei of PITX2-lineage neurons (Skidmore et al., 2008). We compared E14.5 $Pitx2^{Cre/+}$;*NL* and $Pitx2^{Cre/-}$;*NL* littermate midbrains for GABAergic differentiation of *Pitx2*-lineage cells. In $Pitx2^{Cre/+}$;*NL* midbrains, βGAL-positive cells were positive for GABA and localized in the mantle zone in a highly GABAergic layer (Fig. 4A,A′). In $Pitx2^{Cre/-}$;*NL* midbrains, βGAL-positive cells were medially mislocalized in a GABA-poor layer and were GABA-negative (Fig. 4B,B′). Interestingly, the $Pitx2^{Cre/-}$;*NL* colliculus also appeared to have fewer βGAL-positive cells compared to $Pitx2^{Cre/+}$;*NL*, suggesting there may be reduced neurogenesis or increased cell death of this population. These data suggest that PITX2 is required for both cellular migration and GABAergic differentiation in the superior colliculus.

To determine whether PITX2 is also necessary for early collicular patterning, we analyzed the expression patterns of early midbrain transcription factors in PITX2 mutant embryos. Loss of PITX2 did not disrupt the pattern of the general collicular precursor markers PAX3 and PAX7 (Fig. 4C–F; see Supp. Fig. S1, which is available online). Additionally, the GABAergic precursor markers GATA2 and LHX1/5 and the glutamatergic precursor marker BRN3A were correctly localized in $Pitx2^{Cre/-}$;*NL* midbrains (Fig. 4G–L). This indicates that although PITX2 is required for the GABAergic differentiation of a subpopulation of collicular neurons, it is not necessary for general early patterning of the superior colliculus.

Ventral Midbrain m6 Domain PITX2-Positive Precursors Have Distinct Transcriptional Profiles

To characterize the molecular profiles of m6 ventromedial and red nucleus PITX2-positive neurons, we analyzed early transcription factor co-localization with PITX2. At E12.5, many ventromedial PITX2-positive cells were also positive for FOXA2, LHX1/5, and BRN3A (Fig. 5A–I). In the m1–m5 domains, LHX1/5-positive neurons become GABAergic, whereas in the m6 domain, LHX1/5-positive cells become glutamatergic (Nakatani et al., 2007). Thus, PITX2 co-localization with LHX1/5 and BRN3A suggests a glutamatergic fate for many ventromedial m6 PITX2-positive cells. FOXA2 is present in the m6 and m7 domains, where it inhibits GABAergic differentiation via regulation of Nkx family transcription factors and repression of early factors necessary for GABAergic fates such as *Helt* (Ferri et al., 2007; Lin et al., 2009). Many ventromedial PITX2-positive cells were also positive for NKX6.2 (Fig. 5J–L), a transcription factor necessary for m6 identity (Prakash et al., 2009). Because all ventromedial PITX2-positive cells were also NKX6.2 positive, it is possible that ventromedial PITX2 marks a previously uncharacterized NKX6.2 subpopulation (Prakash et al., 2009). At E12.5, the majority of PITX2-positive red nucleus cells were also positive for FOXA2, LHX1/5, and BRN3A (Fig. 5M–U). These results suggest that PITX2 marks two heterogeneous cell populations in the ventral midbrain, a ventromedial one and a more lateral red nucleus population, both of which contain glutamatergic precursors that have distinct molecular signatures.

To further characterize *Pitx2* expression in the ventral midbrain, we co-labeled PITX2-positive cells with additional ventral midbrain markers. In the ventral midbrain, *Nkx2.2* is expressed in m5 progenitors

Fig. 5. PITX2 identifies restricted populations of ventromedial midbrain precursors. Immunofluorescence of E12.5 midbrain coronal sections processed with antibodies against PITX2 (red) and other ventral midbrain markers (green) and imaged with confocal microscopy. **A′:** Cartoon showing coronal view of an embryonic mouse midbrain identifying two ventral PITX2-positive populations. Boxes indicate the location of the ventromedial (VM) and red nucleus PITX2-positive populations magnified in A–L and M–U, respectively. **A–C:** FOXA2 marks precursors in the m6 domain and is co-localized with PITX2 in deep m6. **D–I:** Most ventromedial PITX2-positive cells are positive for LHX1/5 and BRN3A, which mark glutamatergic precursors in the m6 domain. **J–L:** NKX6.2-positive cells in deep m6 are also PITX2-positive. White arrows indicate transcription factor co-localization with PITX2, whereas open arrows indicate PITX2-positive, FOXA2, LHX1/5, or BRN3A-negative cells. M–U focus on PITX2-positive cells in the red nucleus. **M–R:** PITX2-positive cells in the red nucleus are FOXA2 and LHX1/5-positive. **S–U:** Most but not all BRN3A-positive red nucleus cells are PITX2-positive. Scale bar in A applies to A–U and = 25 μm.

Fig. 6. Ventral midbrain domains are delineated by transcription factor patterning. E12.5 coronal sections were processed for immunofluorescence with antibodies against PITX2 (red) and other ventral midbrain markers (green) and imaged with confocal microscopy. **A:** NKX6.1 is restricted to m6 progenitors and a PITX2-negative region between deep m6 and the PITX2-positive red nucleus. **B:** Ventromedial PITX2-positive cells in m6 reside near GATA2-positive cells in the m5 domain. **C:** NKX2.2-positive cells occupy m4 and m5, but not the m6 domain. **D:** ISL1-positive oculomotor neurons (OMN) are located superficially to the deep PITX2 population in m6. Scale bar in A = 50 μm and applies to A–D. VM, ventromedial population; RN, red nucleus; OMN, oculomotor nucleus.

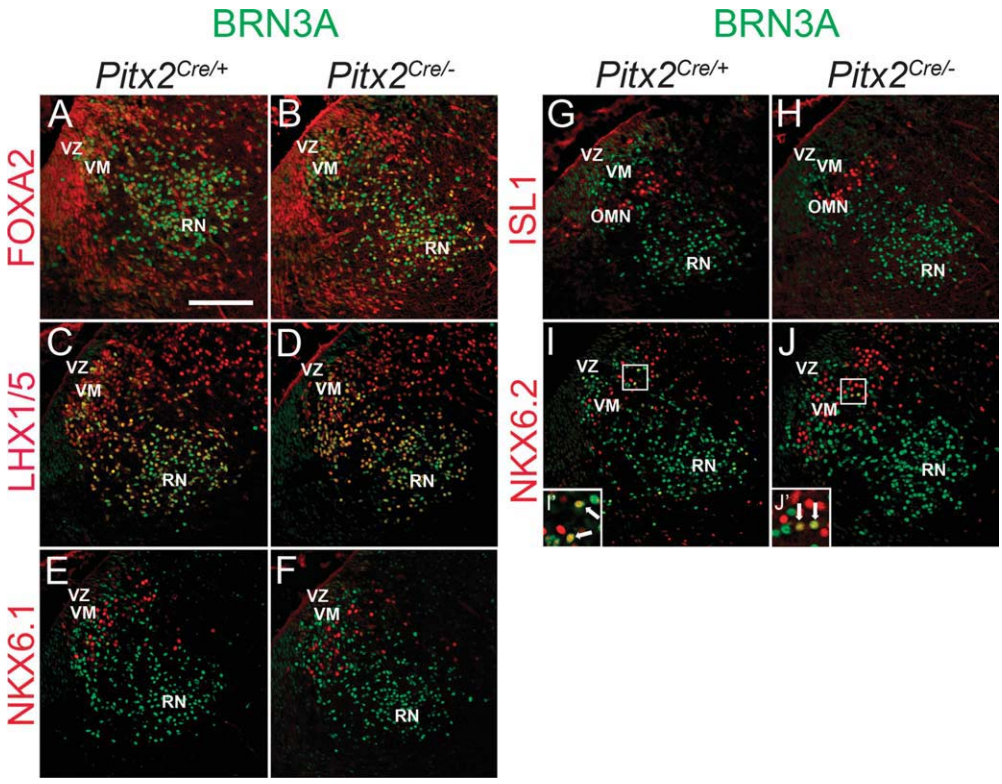

Fig. 7.

and m4 post-mitotic cells (Nakatani et al., 2007), whereas NKX6.1 and NKX6.2 are expressed in m6. *Nkx6.1* is expressed in m6 progenitors and in post-mitotic oculomotor neurons and is required for proper fate of cells in the red nucleus and oculomotor nucleus (Prakash et al., 2009). We found that NKX6.1-positive cells were located in the m6 ventricular zone and positioned between the two PITX2-positive populations, although no co-localization was observed between PITX2 and NKX6.1 (Fig. 6A). *Nkx2.2* and *Gata2* are expressed in m5 GABAergic progenitors and precursors, respectively (Nakatani et al., 2007; Joksimovic et al., 2009; Kala et al., 2009). Neither NKX2.2 nor GATA2 co-localized with PITX2 (Fig. 6B,C), further suggesting that ventral midbrain PITX2-positive neurons do not undergo GABAergic differentiation. ISLET1 (ISL1) marks the oculomotor nucleus in m6 and did not co-localize with PITX2, indicating that PITX2-positive cells do not contribute to the oculomotor nucleus (Fig. 6D).

To determine whether PITX2 is necessary for early transcription factor

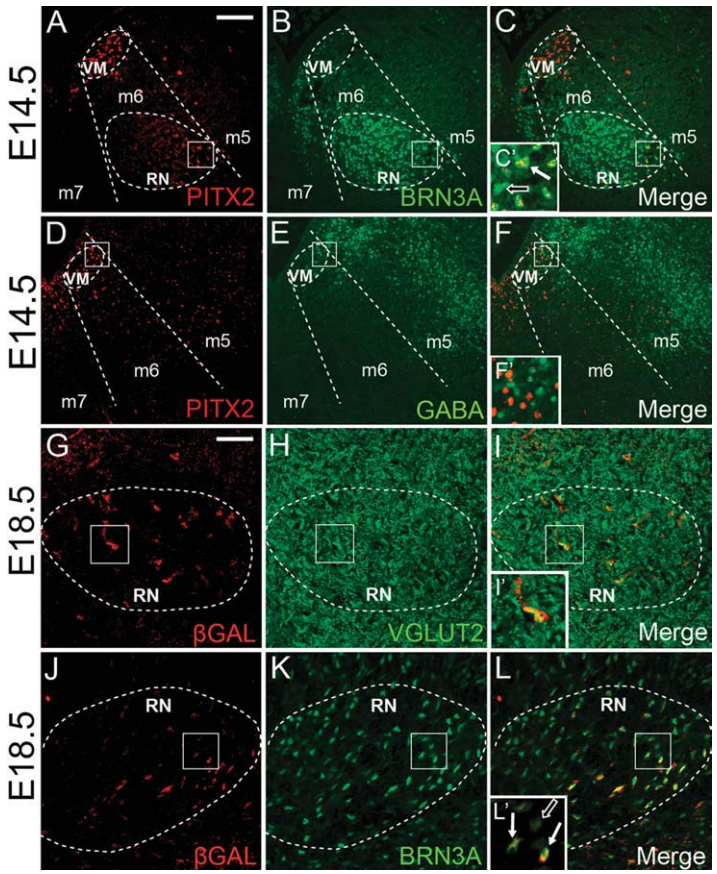

Fig. 8.

patterning of the ventral midbrain, we compared gene expression in E12.5 $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ littermate midbrains (Fig. 7). Transcription factor patterning in the midbrain was similar in $Pitx2^{Cre/+}$ and wildtype embryos (Fig. 5). BRN3A (green) was dispersed throughout the ventromedial zone and red nucleus in both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ midbrains (Fig. 7A–J). FOXA2 was present in the ventricular zone, ventromedial population, and the red nucleus and was unchanged in $Pitx2^{Cre/-}$ embryos (Fig. 7A,B). LHX1/5-positive cells were distributed throughout the ventromedial population and the red nucleus in both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ midbrains (Fig. 7C,D). Loss of PITX2 did not affect the localization of NKX6.1 cells, which are BRN3A-negative (Fig. 7E,F). The oculomotor nucleus marker, ISL1, also appeared normal in $Pitx2^{Cre/-}$ midbrains (Fig. 7G,H). The majority of NKX6.2-positive cells were negative for BRN3A, with only a few double-labeled cells in the intermediate zone of the ventral midbrain (Fig. 7I,I′); this pattern of expression was unchanged in $Pitx2^{Cre/-}$ embryos (Fig. 7J,J′). Transcription factor patterns also appeared unchanged in $Pitx2^{Cre/-}$ mutants in the rostral-caudal plane (Supp. Fig. S1). Together, these data indicate that PITX2 is not necessary for early ventral midbrain patterning.

PITX2 Is Transient in Glutamatergic Red Nucleus Neurons

In order to establish whether ventral PITX2-positive neurons are GABAergic or glutamatergic, we analyzed E14.5 ventral midbrains for PITX2 and BRN3A, VGLUT2, or GABA. We found that only a few red nucleus cells were PITX2-positive at E14.5 (Fig. 8A), in contrast with the E12.5 PITX2-positive red nucleus (Fig. 5). At E14.5, the few ventral PITX2-positive cells were also BRN3A-positive, suggesting that these neurons become glutamatergic (Fig. 8B–C′). In contrast, most GABA immunofluorescence was localized to the m5 domain and was not present in PITX2-positive cells (Fig. 8D–F′). Although previous studies suggested the presence of GABAergic neurons in the red nucleus (Katsumaru et al., 1984; Vuillon-Cacciuttolo et al., 1984), we did not observe GABA in midbrain red nucleus neurons during development (Fig. 8F).

We next asked whether PITX2-lineage red nucleus neurons are glutamatergic by analyzing $Pitx2^{Cre/+}$;*NL* embryos for βGAL and VGLUT2 immunofluorescence. At E18.5, βGAL-positive red nucleus neurons were VGLUT2-positive, suggesting that red nucleus PITX2-lineage cells are glutamatergic (Fig. 8G–I′). We also identified co-localization between βGAL and BRN3A in the red nucleus of $Pitx2^{Cre/+}$;*NL* embryos (Fig. 8J–L). This co-localization with BRN3A further suggests that red nucleus PITX2-lineage neurons adopt a glutamatergic fate.

In the ventral midbrain, precise spatial and temporal transcription factor expression is critical for proper development (Sanders et al., 2002; Kele et al., 2006; Prakash and Wurst, 2006). Our studies on E12.5 and E14.5 ventral midbrains suggested that PITX2 may be downregulated in the red nucleus during development (Figs. 5M–U, 8A–C). To test this, we characterized midbrain *Pitx2* expression at both the protein and mRNA levels using *Cre* lineage tracing, in situ hybridization, and immunofluorescence in $Pitx2^{Cre/+}$;*NL* midbrains. From E14.5 through E18.5, PITX2-lineage βGAL-positive neurons were abundant in the superior colliculus and sparse in the red nucleus (Fig. 9A,E,I). *Pitx2* mRNA expression was maintained in the superior colliculus, red nucleus, and ventromedial populations through E18.5 (Fig. 9C,G,K and data not shown). However, very few red nucleus neurons were labeled with PITX2 antibody at E14.5 and by E16.5 the red nucleus was devoid of PITX2-positive neurons (Fig. 9D,H,L).

Previous studies showed that *Pitx2* mRNA is auto-regulated (Briata et al., 2003), which may partially explain the discrepancy in red nucleus *Pitx2* mRNA and protein. It is also possible that translation or splicing of *Pitx2* in this region is uniquely regulated in red nucleus neurons. Consistent with these data, some pituitary cell lines appear to regulate *Pitx2* at the translational level (Tremblay et al., 1998). In order to determine whether the low number of βGAL-positive PITX2-lineage neurons in the red nucleus was due to low *LacZ* reporter expression, we also crossed $Pitx2^{Cre/+}$ mice with *ZsGreen* mice, which express the green fluorescent molecule ZsGreen (ZsGrn) upon *Cre* recombination (Madisen et al., 2009). Midbrains of E14.5 $Pitx2^{Cre/+}$;*ZsGrn* embryos displayed few ZsGrn-positive

Fig. 7. Early ventral midbrain patterning is PITX2-independent. E12.5 coronal sections were processed for immunofluorescence with antibodies against BRN3A (green) and FOXA2, LHX1/5, NKX6.1, ISL1, or NKX6.2 (red) and imaged with confocal microscopy. **A,B:** FOXA2-positive cells in m6 are localized in the ventricular zone and the BRN3A-positive red nucleus in both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ midbrains. **C,D:** LHX1/5-positive cells are distributed from deep m6 to the red nucleus, where all LHX1/5-positive cells are also BRN3A-positive. LHX1/5 patterning appears unchanged in $Pitx2^{Cre/-}$ midbrains. **E,F:** Cells in the ventricular zone are weakly NKX6.1-positive and a second, more lateral population is strongly NKX6.1-positive. Neither NKX6.1-positive population displays co-localization with BRN3A and both are unchanged in the $Pitx2^{Cre/-}$ midbrain. **G,H:** In both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ tissues, ISL1 marks cells in the oculomotor nucleus, which is surrounded by BRN3A-positive cells. **I–J′:** NKX6.2-positive cells intermingle with BRN3A-positive cells and a few cells are also BRN3A-positive (white arrows) in both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ embryos. Scale bar in A = 100 μm and applies to A–J. VZ, ventricular zone; VM, ventromedial population; RN, red nucleus.

Fig. 8. PITX2-lineage neurons are glutamatergic and sparse in the red nucleus. A–F: Coronal sections of E14.5 midbrains processed for PITX2 and BRN3A (A–C) or GABA (D–F) immunofluorescence. Dotted areas demarcate cells in the PITX2-positive deep ventromedial population and the red nucleus. Boxes in C, F, I, and L are enlarged in C′, F′, I′, and L′. **A–C′:** At E14.5, the red nucleus is composed of BRN3A-positive neurons, a few of which are PITX2-positive. White arrows indicate co-localization of BRN3A and PITX2, whereas open arrows indicate BRN3A-positive, PITX2-negative neurons. **D–F′:** Ventral GABAergic neurons are generally restricted to the m5 domain, and are PITX2-negative. G–L′: E18.5 $Pitx2^{Cre/+}$;*NL* coronal sections were processed for immunofluorescence for β-galactosidase (βGAL) and VGLUT2 or BRN3A and visualized with confocal microscopy. **G–I′:** At E18.5, PITX2-lineage neurons are present in the red nucleus and are VGLUT2-positive. **J–L′:** E18.5 red nucleus PITX2-lineage neurons are also BRN3A-positive. White arrows indicate co-localization of βGAL and BRN3A, whereas the open arrow indicates BRN3A-positive, βGAL-negative neurons. Dotted areas delimit the boundary of the red nucleus. Scale bars in A and G = 100 and 50 μm and apply to A–F and G–L, respectively. VM, ventromedial population; RN, red nucleus.

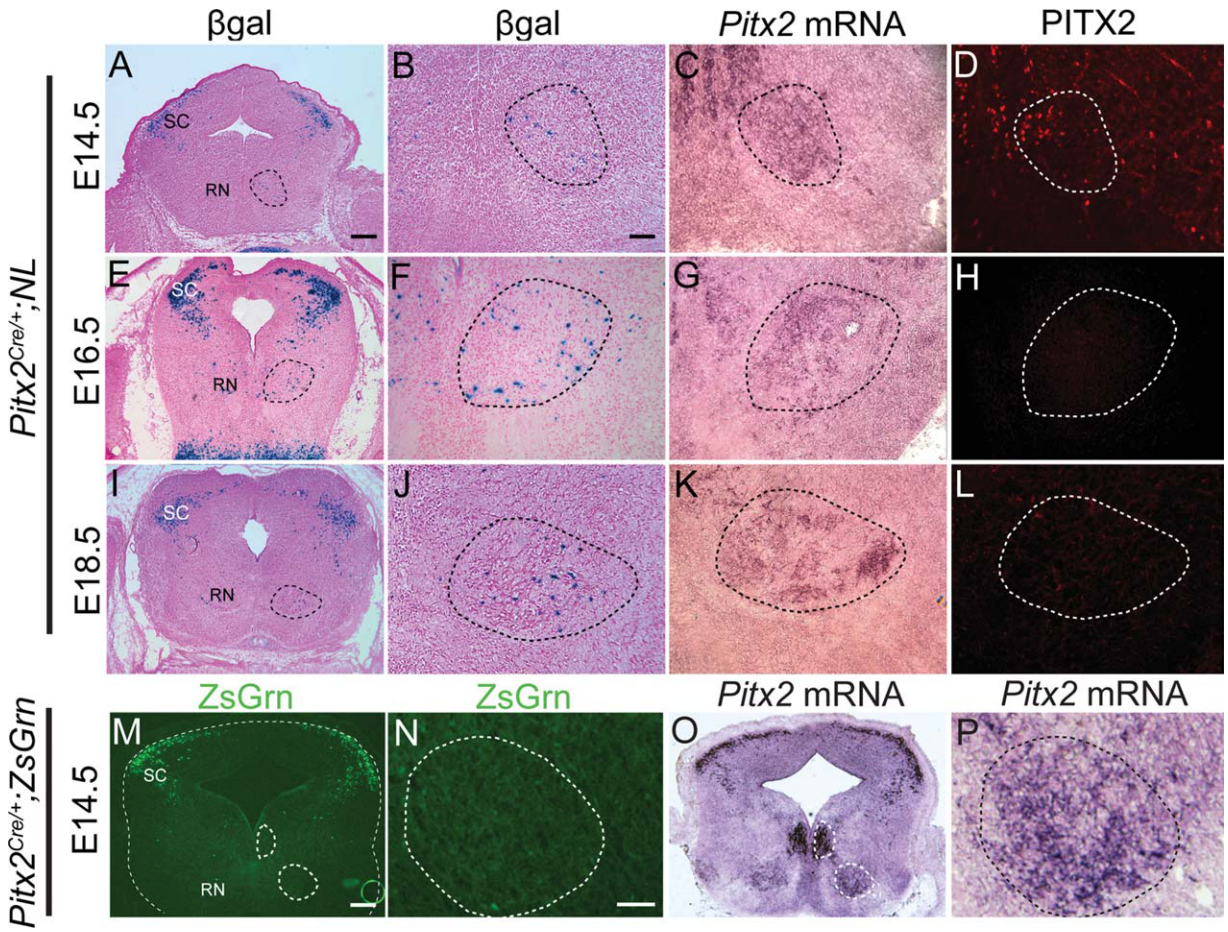

Fig. 9.

cells in the ventromedial population and red nucleus (Fig. 9M,N), even though neighboring sections showed significant *Pitx2* mRNA expression in both populations (Fig. 9O,P). At E12.5, many PITX2-positive cells in the red nucleus can be identified by immunofluorescence (Fig. 5), whereas very few red nucleus neurons are βGAL-positive by lineage tracing in $Pitx2^{Cre/+}$;*NL* embryos (data not shown). Since different *Cre* reporter systems (*NL* and *ZsGrn*) showed low $Pitx2^{Cre}$ activity in the E14.5 ventral midbrain, we speculate that *Cre* expression is regulated between E12.5 and E14.5 in the red nucleus at the transcriptional or translational level.

DISCUSSION

Through use of co-expression studies and *Cre* lineage tracing, we have identified GABAergic PITX2-positive cells in the SGI, an intermediate layer of the superior colliculus, and in

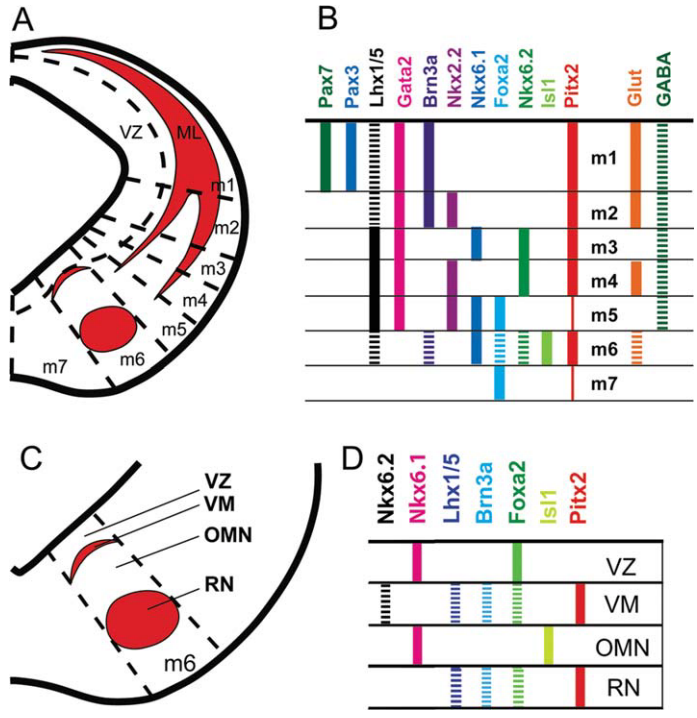

Fig. 10.

glutamatergic PITX2-positive cells in the ventral midbrain. Additionally, we characterized the expression of dorsal and ventral midbrain transcription factors in reference to temporal and spatial *Pitx2* expression and determined a role for PITX2 in dorsal midbrain GABAergic differentiation. We also discovered that PITX2 protein is transient during development of the red nucleus.

Studies on the developing midbrain have begun to map transcription factor expression by dorso-ventral domain (Nakatani et al., 2007; Kala et al., 2009). Our data on *Pitx2* expression in relation to other transcription factors can now be incorporated into these maps to produce a detailed summary of transcription factors during midbrain development (Fig. 10). We identified PITX2-positive cells in all seven midbrain domains, with strongest expression in the m1–m4 and m6 domains (Fig. 10A,B). In the m1–m2 domains, *Pitx2* is co-expressed with *Lhx1/5* and GABA (Fig. 10B) and marks unique subpopulations of GABAergic neurons. This is consistent with previous studies showing distinct transcription factor requirements for *Ascl1* and *Gata2* in midbrain GABAergic differentiation (Peltopuro et al., 2010). The m6 midbrain PITX2-positive neurons can be divided into distinct regions: a ventromedial population and the red nucleus (Fig. 10C,D). In the m6 domain, *Pitx2* is co-expressed with *Lhx1/5, Brn3a, Foxa2, Nkx6.2*, and *Vglut2* (Fig. 10B,D). Interestingly, ventromedial and red nucleus PITX2-positive populations both express *Foxa2, Lhx1/5*, and *Brn3a*, whereas ventromedial PITX2-positive neurons also express *Nkx6.2*. This map suggests that *Pitx2* marks distinctive midbrain subpopulations that have unique transcription factor expression patterns.

Midbrain Development Requires Distinct Expression Patterns of Transcription Factors and Signaling Molecules

Our studies suggest that superior colliculus layers in the mouse can be identified based on transcription factor expression. We also demonstrated that PITX2 marks the intermediate layer (SGI) of the superior colliculus and previous studies showed that superficial and intermediate layers can be identified by expression of *Pax7* and *Brn3a* (Fedtsova et al., 2008). Our studies suggest BRN3A marks the SO/SGI and SAI, consistent with previous studies on collicular glutamatergic localization (Mooney et al., 1990). We showed that PITX2 and BRN3A-positive populations occupy separate layers. Additionally, previous studies showed that both the SGI and the SGP can be identified by expression of the *Forkhead-5* (*Foxb1*) transcription factor (Alvarez-Bolado et al., 1999). Thus, developing superior colliculus layers can be identified by unique combinations of transcription factor expression.

We have also shown that *Pitx2* is expressed upstream of and is necessary for GABAergic differentiation in PITX2-positive superior colliculus cells. Our results are consistent with previous studies showing PITX2 is downstream of the GABAergic differentiation factor GATA2 (Kala et al., 2009). This positions PITX2 late in a cascade of transcription factors necessary for GABAergic differentiation. The earliest fate-choice factors, *Ascl1* and *Helt*, promote GABAergic differentiation (Miyoshi et al., 2004; Kala et al., 2009). In turn, *Helt* is necessary for the expression of *Gata2*, which is required for both *Lhx1/5* and *Pitx2* expression.

In the ventral midbrain, we identified *Pitx2* expression in a ventromedial population and in the red nucleus. Ventral midbrain populations form arcs, each of which expresses a specific combination of transcription factors necessary for nucleogenesis. Arc formation requires *Sonic Hedgehog* (*Shh*) signaling from the notochord and loss of *Shh* signaling results in disruption of arc structure and patterning (Bayly et al., 2007). *Shh* signaling contributes to the entire ventral midbrain and is required for repression of the dorsalization factors *Pax7, En2*, and *Fgf8* (Nomura and Fujisawa, 2000; Watanabe and Nakamura, 2000). SHH is also responsible for inducing *Foxa2* expression, which regulates Nkx family members and ventral midbrain specification (Perez-Balaguer et al., 2009). In addition to general ventral midbrain determination, studies in other tissues have shown that SHH indirectly regulates *Pitx2* expression (Logan et al., 1998; Ryan et al., 1998), further suggesting a requirement for

Fig. 9. *Pitx2* expression is transient in the ventral midbrain. A–L: Adjacent coronal sections of *Pitx2*$^{Cre/+}$*;NL* midbrains processed for βGAL histochemistry (A, B, E, F, I, J), *Pitx2* mRNA (C, G, K), or PITX2 immunofluorescence (D, H, L). The dotted line demarcates the red nucleus. **A:** At E14.5, *Pitx2*-lineage neurons are located in the superior colliculus (SC) and red nucleus (RN). **B:** High magnification of A shows only a few *Pitx2*-lineage cells in the red nucleus, despite high red nucleus *Pitx2* mRNA (**C**). **D:** Immunofluorescence indicates only a few PITX2-positive red nucleus cells. **E, F, I, J:** At E16.5–E18.5, β-galactosidase activity shows *Pitx2*-lineage neurons in the superior colliculus and red nucleus. **G, H, K, L:** The E16.5–E18.5 red nucleus continues to express *Pitx2* mRNA, although PITX2 protein is absent. **M:** Coronal section of an E14.5 *Pitx2*$^{Cre/+}$*;ZsGrn* midbrain showing ZsGrn fluorescence in the superior colliculus. **N:** Few cells are fluorescent in the ventral midbrain as seen in a high-magnification image of the red nucleus. **O:** A neighboring section to M processed for *Pitx2* in situ hybridization shows *Pitx2* mRNA in the superior colliculus, ventromedial population, and red nucleus. **P:** Higher magnification of the red nucleus in O. Dotted areas denote the ventromedial population and red nucleus. Scale bar in A = 125 μm and applies to A, E, and I; in B = 32 μm and applies to B–D, F–H, and J–L; in M and N = 100 and 50 μm and applies to M,O and N,P, respectively.

Fig. 10. Summary of *Pitx2* expression in the developing dorsal and ventral midbrain. Schematic is based on previously published models (Nakatani et al., 2007; Kala et al., 2009), wherein early developmental transcription factors were mapped by domain. **A,C:** Cartoons of typical E12.5 coronal sections showing PITX2-positive cells mapped onto the domain-delineated midbrain. PITX2-positive cells (red) are abundant in m1–m4 domains in the superior colliculus (SC) and in the m6 domain (C) containing a ventromedial (VM) population and the red nucleus (RN). PITX2-positive cells are sparse in m5 and m7. **B,D:** Solid bars show areas with no overlap in marker expression with PITX2; hatched bars show areas of co-localization with PITX2. In m1–m4, PITX2-positive neurons express some markers of GABAergic precursors and neurons (LHX1/5 and GABA, respectively). In m6, PITX2 co-localizes with glutamatergic markers (D). PITX2 is co-localized with the m6 precursor markers FOXA2, LHX1/5, BRN3A, and NKX6.2 in the ventromedial population. PITX2 neurons in the early red nucleus are positive for FOXA2, LHX1/5, and BRN3A, and red nucleus PITX2-lineage neurons are glutamatergic. DA, dopaminergic neurons; GLUT, glutamatergic neurons; GABA, GABAergic neurons; VZ, ventricular zone; VM, ventromedial population; OMN, oculomotor nucleus; RN, red nucleus; ML, mantle layer.

SHH signaling in PITX2-neuronal development.

Neurotransmitter Identity Is Heterogeneous in PITX2-Positive CNS Neurons

We characterized dorsal PITX2-positive neurons as GABAergic, consistent with previous studies (Martin et al., 2004), and ventral PITX2-positive neurons as glutamatergic. Studies in the spinal cord have identified PITX2-positive neurons as cholinergic and glutamatergic interneurons that are responsible for modulating the frequency of motor neuron firing (Zagoraiou et al., 2009). Together, these observations suggest that PITX2 may regulate neurotransmitter choice based on rostral-caudal positioning. This reliance on regional or axial-level factors for midbrain development is consistent with earlier studies showing that dorsal and ventral midbrain neurons are derived from different progenitor pools (Tan et al., 2002) and respond to distinct developmental signals.

In conclusion, we report that *Pitx2* is expressed in GABAergic neurons occupying the SGI and that PITX2 is necessary for their GABAergic differentiation and migration, but not early patterning. In contrast, most ventral *Pitx2*-lineage neurons are glutamatergic and are located in ventromedial and red nucleus populations. Additionally, each PITX2-positive population appears to be characterized by a unique combination of transcription factors, suggesting locally regulated mechanisms are important for glutamatergic and GABAergic differentiation. Further research into the developmental requirements of these neuronal subpopulations may facilitate diagnosis and insights into mechanisms of diseases/disorders and therapies for midbrain-related neurological conditions.

EXPERIMENTAL PROCEDURES

Mice

Wild type mice were on a C57BL/6J background (JAX 000664). $Pitx2^{Cre/+}$ mice (Liu et al., 2002) were crossed with FlpeR mice (JAX 003946) to excise the neomycin cassette. $Pitx2^{Cre/+}$;*NL* and $Pitx2^{Cre/-}$;*NL* embryos were generated as previously described (Skidmore et al., 2008). *ZsGrn* reporter mice were obtained from Jackson Laboratories (Bar Harbor, ME) and are on a C57BL/6J background (JAX 007906) (Madisen et al., 2009).

Embryo Tissue Preparation for Cryosectioning or Paraffin Embedding

Timed pregnancies were established with the morning of plug identification designated as E0.5. Pregnant dams were euthanized using cervical dislocation. Embryos were fixed in 4% paraformaldehyde for 30 min to 2 hr depending on age and genotype. Embryos for cryosectioning were cryoprotected in 30% sucrose-PBS overnight and frozen in O.C.T. embedding medium (Tissue Tek, Torrance, CA), and sectioned at 12 μm. Paraffin-embedded embryos were sectioned at a thickness of 7 μm. From each embryo and pup, an amniotic sac or tail was retained for genotyping. All procedures were approved by the University Committee on Use and Care for Animals at the University of Michigan.

β-Galactosidase Staining of Frozen Sections

To collect embryonic tissue for X-Gal staining, $Pitx2^{Cre/+}$ females were crossed with $Pitx2^{+/+}$;*NL* males. Pregnant dams were anesthetized with 250 mg/kg body weight tribromoethanol and perfusion fixed with 4% paraformaldehyde (Fisher, Waltham, MA). E14.5 whole embryos and E16.5–E18.5 brains were isolated and further fixed in 4% paraformaldehyde at 4°C for 20 min to 3 hr. P8 pups were anesthetized as described above and perfusion fixed. Brains were removed and fixed at 4°C for 3 hr in 4% paraformaldehyde. Samples were washed with PBS, cryoprotected in 30% sucrose-PBS overnight, and frozen in O.C.T. embedding medium (Tissue Tek, Torrance, CA) for cryosectioning. Frozen sections all were postfixed with 0.5% glutaraldehyde fixative, washed in X-Gal Wash Buffer, and stained with X-Gal Staining Solution overnight at 37°C as previously described (Sclafani et al., 2006). Slides were washed in PBS and X-Gal Wash Buffer, eosin counterstained, and mounted with Permount (Fisher).

Acetylcholinesterase (AChE) Staining of Frozen Sections

To collect postnatal tissue for AChE staining, wild type P8 tissue was prepared and frozen as described above. Sections were post-fixed in 4% paraformaldehyde and incubated in 0.1% H_2O_2. AChE staining was performed as previously described (Tago et al., 1986).

Immunofluorescence and In Situ Hybridization

Immunofluorescence on frozen and paraffin-embedded tissue was performed as described (Martin et al., 2002, 2004) with rabbit anti-PITX2 at 1:8,000 (Zagoraiou et al., 2009), rabbit anti-PITX2 at 1:4,000 (Capra Science, Ångelholm, Sweden), guinea pig anti-BRN3A at 1:400 (Fedtsova and Turner, 1995), rabbit-anti VGLUT2 at 1:1000 (Millipore, Billerica, MA), rabbit anti-GABA at 1:1,000 (Sigma, St. Louis, MO), guinea pig anti-GATA2 at 1:500 (Peng et al., 2007), guinea pig anti-NKX6.2 at 1:8000 (Vallstedt et al., 2001), chicken anti-β-galactosidase at 1:200 (Abcam, Cambridge, MA) and the following mouse antibodies from the Developmental Studies Hybridoma Bank: anti-LHX1/5 (4F2) at 1:100, anti-FOXA2 (4C7) at 1:100, anti-ISLET1 (40.2D6) at 1:500, anti-NKX2.2 (74.5A5) at 1:500, anti-PAX3 at 1:100, anti-PAX7 at 1:100, or anti-NKX6.1 (F64A6B4) at 1:250. In situ hybridization was performed as previously described (Martin et al., 2002, 2004) using a cRNA probe for *Pitx2*.

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