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Distinct populations of GABAergic neurons in mouse rhombomere 1 express but do not require the homeodomain transcription factor PITX2

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Abstract

Hindbrain rhombomere 1 (r1) is located caudal to the isthmus, a critical organizer region, and rostral to rhombomere 2 in the developing mouse brain. Dorsal r1 gives rise to the cerebellum, locus coeruleus, and several brainstem nuclei, whereas cells from ventral r1 contribute to the trochlear and trigeminal nuclei as well as serotonergic and GABAergic neurons of the dorsal raphe. Recent studies have identified several molecular events controlling dorsal r1 development. In contrast, very little is known about ventral r1 gene expression and the genetic mechanisms regulating its formation. Neurons with distinct neurotransmitter phenotypes have been identified in ventral r1 including GABAergic, serotonergic, and cholinergic neurons. Here we show that PITX2 marks a distinct population of GABAergic neurons in mouse embryonic ventral r1. This population appears to retain its GABAergic identity even in the absence of PITX2. We provide a comprehensive map of markers that places these PITX2-positive GABAergic neurons in a region of r1 that intersects and is potentially in communication with the dorsal raphe.

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1. Introduction

The hindbrain region of the central nervous system is responsible for coordination of motor control and regulation of autonomic processes such as respiration, heart rate, blood pressure, and arousal (Saper, 2000). The developing hindbrain is subdivided along the rostral-caudal axis into eight rhombomeres (r1–r8) which are distinguished by boundaries of gene expression, patterns of cell differentiation, and morphology (Lumsden and Keynes, 1989). Rhombomere 1 (r1), located caudal to the midbrain and isthmus and rostral to r2, is easily distinguishable morphologically in the mouse brain by E10.5. Dorsal r1 gives rise to the cerebellum and neurons of the locus coeruleus whereas ventral r1 contributes to the sensory vestibular nuclei, trigeminal and trochlear nuclei, pedunculopontine tegmental nucleus, parabrachial nucleus, Kölliker-Fuse nucleus, dorsal nucleus of the lateral lemniscus and neurons of dorsal raphe (Alder et al., 1996; Aroca et al., 2006; Chatonnet et al., 2007; Ding et al., 2003; Eddison et al., 2004; Jensen et al., 2008; Lin et al., 2001; Machold and Fishell, 2005; Marin and Puelles, 1995; Wingate and Hatten, 1999).

Much attention has been directed toward understanding the molecular markers and mechanisms of development of the cerebellum, the most prominent r1 derivative (Herrup and Kuemerle, 1997; Wang and Zoghbi, 2001). The cerebellum is one of the first brain structures to differentiate and one of the last to mature; it is estimated to contain as many as 80–85% of all human neurons and is an important center for many processes critical for life (Wang and Zoghbi, 2001). In contrast, the definition and origins of the neuronal populations in ventral r1 have received relatively little attention. This lack of molecular information on developing r1 has impeded progress in the characterization of mouse mutants and therapies for individuals with hindbrain defects.

Here, we test the hypothesis that *Pitx2*, a paired-like homeodomain transcription factor, specifies distinct GABAergic neurons that derive from ventral r1, using loss-of-function, conditional, and *Cre* knock-in alleles. We also present data from analyses of gene expression patterns in embryonic mouse ventral r1. Our results suggest that PITX2-positive neurons in r1 are GABAergic and can be divided into two unique subpopulations based on expression of unique combinations of transcription factors, including LHX1/5, NKX6.1/6.2, PAX2, and SOX2. Both PITX2-positive GABAergic populations occupy specific bilaterally symmetric regions of ventro-medial r1. The GABAergic identity of these cells is not disrupted by loss of PITX2. These observations provide a framework for ongoing studies aimed at exploring the functional molecular genetic pathways that regulate r1 neuronal development.

2. Materials and Methods

2.1 Mice

C57BL/6J mice were obtained from the Jackson Laboratory (JAX 000664). GAD67-GFP embryos were generated by crossing GAD67-GFP males with C57BL/6J females (Tamamaki et al., 2003). $Pitx2^{Cre/+}$ mice (Liu et al., 2002) were crossed with *FlpeR* mice (JAX 003946) to excise the neomycin cassette. $Dbx1^{Cre};R26^{YFP}$ tissues were obtained by Frédéric Causeret by crossing $Dbx1^{Cre/+}$ mice (Bielle et al., 2005) with a $ROSA26^{loxP\text{-}stop\text{-}loxP\text{-}YFP}$ strain (Srinivas et al., 2001). $Pitx2^{Cre/+};ZsGrn$ embryos were generated by crossing $Pitx2^{Cre/+}$ mice to *ZsGrn* reporter mice obtained from Jackson Laboratories (JAX 007006) (Madisen et al., 2010). $Pitx2^{+/-}$ mice were as previously

described (Gage et al., 1999). *Nestin-Cre* transgenic (Tronche et al., 1999) mice were bred to *Pitx2*$^{tlz/+}$ mice which are heterozygous for a null allele that expresses β-*galactosidase* under the control of *Tau* (manuscript in preparation). *Nestin-Cre;Pitx2*$^{tlz/+}$ mice were then bred to *Pitx2*$^{flox/flox}$ (Gage et al., 1999) mice to generate embryos for analysis.

2.2 Tissue Preparation

Timed pregnancies were established with the morning of plug identification designated as E0.5. Embryos were dissected into PBS from pregnant females following cervical dislocation and hysterectomy. Embryos were fixed and processed for antibody staining or paraffin *in situ* hybridization histochemistry as previously described (Novitch et al., 2001; Skidmore et al., 2008). For frozen *in situ* hybridization, sections were fixed in 4% PFA for 40 minutes, washed in PBS, then incubated in TEA/acetic anhydride for 10 minutes (50 ml DPC-H_20, 580 µl TEA 0.1M pH 8, 150 µl acetic anhydride). The remaining steps were performed as previously described (Martin et al., 2002).

Embryos were fixed in 4% paraformaldehyde for 1–2 hours depending on age and genotype. For frozen sections, embryos were cryoprotected overnight in 30% sucrose, flash frozen in O.C.T. embedding compound (Tissue Tek, Torrance, CA), and stored at −80°C until sectioning at 12–30 µm. For paraffin sections, tissues were embedded in paraffin and sectioned at 7 µm thickness. 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.

2.3 Immunofluorescence and in situ hybridization

Immunofluorescence on paraffin embedded tissues was done as previously described (Martin et al., 2002; Martin et al., 2004). Immunofluorescence on frozen sections was done as previously described (Novitch et al., 2001). Antibodies used were guinea pig anti-phosphohistone H3 at 1:200 (Upstate Biotechnology, Inc., Lake Placid, NY), rabbit anti-PITX2 at 1:8000 (provided by Dr. Thomas Jessell, Columbia University), rabbit anti-PITX2 at 1:4000 (Capra Science, Ängelholm, Sweden), rabbit anti-VGLUT2 at 1:1000 (Millipore), rabbit anti-GABA at 1:1000 (Sigma), rabbit anti-5-hydroxytryptamine (5-HT) at 1:5000 (Sigma), goat anti-ChAT at 1:100 (Millipore), rabbit anti-LBX1 at 1:10000 (provided by Thomas Müller, Max-Delbrück Center of Molecular Medicine, Berlin), guinea pig anti-LMX1B at 1:5000 (provided by Dr. Thomas Müller), rabbit anti-SOX2 (Millipore) at 1:250, guinea pig anti-NKX6.2 at 1:8000 (provided by Dr. Thomas Jessell), guinea pig anti-BHLHB5 at 1:32,000 (provided by Dr. Ben Novitch), and the following mouse antibodies from Developmental Studies Hybridoma Bank at 1:100–1:500: anti-PAX7, anti-LHX1/5 (4F2), anti EVX1 (3A2), anti-EN1 (4G11), anti-NKX6.1 (F64A6B4), and anti-ISL1 (39.4D5). *In situ* hybridization on frozen and paraffin sections was done as previously described (Martin et al., 2002; Martin et al., 2004) using cRNA probes for *Pitx2, Gbx2, Hoxa2, Otx2, Fgf8, Phox2a, Phox2b,* and *Lmx1a*.

2.4 Microscopy and cell counts

Confocal fluorescent images were taken using a Leica TCS SP5 X Supercontinuum Confocal System with Upright Fluorescent Microscope. For single *in situ* and X-gal-stained slides, sections were photographed in brightfield. For pseudocolored neighboring merged images, sections were photographed in brightfield and converted into pseudo-fluorescent color, then overlaid in Photoshop. Digital images were processed with Adobe Photoshop CS2 v9.0 software. For quantification of double labeled cells, cells in r1 were counted in a minimum of 3 sections from E12.5 (NKX6.1, PAX2, SOX2, LHX1/5) or E14.5 (GABA) embryos.

3. Results

Pitx2-positive neuronal populations are localized in the forebrain, midbrain, hindbrain, and spinal cord (Mucchielli et al., 1996; Zagoraiou et al., 2009). At E11.5, *Pitx2*-positive cells were located in ventral r1 which was bordered rostrally by the *Otx2*-positive midbrain (Fig. 1C, D) and *Fgf8*-positive isthmic organizer (Fig. 1E, F) and caudally by *Hoxa2*-expressing r2 (Fig. 1G–H). Interestingly, at E11.5 most *Pitx2*-expressing cells were located in r1, however a few *Pitx2*-positive cells appeared to localize at the r1/r2 boundary (Fig. 1A). By E12.5, all *Pitx2*-positive cells were in a single population in mid-r1 (Fig. 1K). At this timepoint, r1 continued to be bordered rostrally by the *Otx2*-positive midbrain and *Fgf8*-positive isthmic organizer (Fig. 1L, M) and caudally by the *Hoxa2/Gbx2*-positive r2 (Fig. 1N, O).

Adult ventral r1 contains several different cell populations, including trochlear motor neurons, branchiomotor neurons of the trigeminal nucleus, locus coeruleus neurons derived from the alar plate, GABAergic neurons of the pedunculopontine nucleus and laterodorsal tegmental nucleus, and the GABAergic, serotonergic, and glutamatergic neurons of the dorsal raphe (Aroca et al., 2006; Fu et al., 2010; Jensen et al., 2008; Marin and Puelles, 1995; Martin et al., 2002; Wang and Morales, 2009). As an early step toward characterizing the transcriptional profiles of ventral r1 neurons, we focused on cells expressing the paired-like transcription factor PITX2, which prior studies suggested were GABAergic interneurons (Martin et al., 2002). Here, we asked whether some PITX2-positive cells in r1 adopt neurotransmitter fates other than GABAergic, and whether their neurotransmitter identity requires functional PITX2.

Through double immunofluorescence, we found that 100% (+/−0%) of E14.5 PITX2-positive cells were positive for GABA and GAD67-GFP (Fig. 2B–C’). Importantly, most GAD67-GFP-positive neurons in r1 were also positive for GABA immunoreactivity (Fig. 2D, D’), confirming that GABA is an accurate marker of GABAergic neurons in this tissue. PITX2-positive cells were negative for VGLUT2 immunofluorescence (Fig. 2E, E’), which likely marks glutamatergic tracts passing through the hindbrain, such as ponto-tegmental afferents (Geisler et al., 2007). There was no co-localization between PITX2 and 5-HT (5-hydroxytryptamine) (Fig. 2F), suggesting that r1 PITX2-positive neurons do not contribute to serotonergic cells of the raphe nuclei or locus coeruleus. Interestingly, some serotonin- and PITX2-positive cells intermingle at the ventromedial limits of *Pitx2* expression, and these may represent GABAergic neurons that contribute to the raphe nuclei, pedunculopontine nucleus, or laterodorsal tegmental nucleus (Fig. 2F) (Torterolo et al., 2000; Wang and Morales, 2009). The close proximity of PITX2-positive cells to the 5-HT-positive population also raises the possibility that these neurons may communicate with each other (Jensen et al., 2008).

To better understand the distribution of developing neurons in the ventral hindbrain, we performed an extensive analysis of gene expression in the E12.5 mouse brain, a period of active neurogenesis (Figs. 3 and 4). The Engrailed homeobox genes *En1/2* are expressed in a broad region encompassing the midbrain and anterior hindbrain and are critical for midbrain and dorsal r1 development (Sgaier et al., 2007; Zervas et al., 2005). We found that, similar to PITX2-positive cells, many EN1-positive cells in E12.5 ventral r1 were also GABAergic (Fig. 3A–C’). At E12.5 there was no significant co-expression of PITX2 and EN1 in ventral r1 cells (Fig. 3D–F’). These data suggest a mixed population of PITX2-positive and EN1-positive GABAergic neurons.

The LIM-homeodomain transcription factors are expressed by many neuronal subtypes throughout the developing central nervous system (Hunter and Rhodes, 2005). *Lhx1* and

Lhx5 are widely expressed in GABAergic cells throughout the midbrain, r1, and the developing cerebellum, and are required for normal cerebellar development (Morales and Hatten, 2006; Zhao et al., 2007). We sought to determine whether PITX2-positive cells in ventral r1 also express *Lhx1/5* using an antibody that marks both LHX1 and LHX5, but does not distinguish between the two. We found that at E12.5, 100% (+/−0%) of the PITX2-positive cells were also LHX1/5-positive (Fig. 3G–I' and Fig. 4Q), whereas LHX1/5-positive cells comprised a much larger population of cells, most of which were negative for PITX2. In addition, all EN1-positive cells were also positive for LHX1/5 (Fig. 3J–L') and some were also positive for GABA (Fig. 3A–C'), providing evidence that r1 GABAergic neurons express unique and specific combinations of *Pitx2*, *En1*, and *Lhx1/5*. Additionally, many LHX1/5-positive cells were negative for GABA (Fig. 3M–O'). Thus, the LHX1/5-positive population of cells in ventral r1 appears to represent a heterogeneous group of neurons, and combinations of GABA, *Pitx2* or *En1* expression may be used to define distinct subpopulations within this group.

PITX2-positive cells in r1 at E12.5 occupy a region of the neural tube that contains non-mitotic cells that were negative for phospho-histone H3 (Fig. 4B). This is consistent with expression of PITX2 in post-mitotic developing neurons of the hypothalamus and midbrain (Martin et al., 2002). Early developing neurons in r1 can be identified by expression of several different transcription factors. LMX1B is a LIM homeodomain transcription factor involved in initiation and maintenance of the isthmic organizer and is important for midbrain and hindbrain patterning (Jacob et al., 2009; Matsunaga et al., 2002; Mishima et al., 2009). *Lmx1b* is expressed in the principal sensory nucleus of the trigeminal nerve, the Kölliker-Fuse nucleus, the dorsal raphe, and the parabrachial nuclei (Dai et al., 2008; Jacob et al., 2009; Matsunaga et al., 2002; Prakash et al., 2009; Zervas et al., 2005). LMX1B is also required for the differentiation of midbrain dopaminergic neurons and hindbrain serotonergic neurons (Ding et al., 2003; Jacob et al., 2009). We observed two groups of LMX1B-positive cells in E12.5 r1, one medial and one more laterally positioned (Fig. 4C). The PITX2-positive neurons were located between these two groups of LMX1B-positive cells (Fig. 4C).

Islet1 (*Isl1*) is expressed by motor neurons, including the oculomotor and trochlear nuclei that contain cell bodies of the third and fourth cranial nerves, respectively (Agarwala and Ragsdale, 2002; Prakash et al., 2009). In our study, ISL1-positive cells marking the trochlear nucleus were located at the dorsal-ventral axis border of r1 and lateral to PITX2-positive neurons (Fig. 4D). The lack of ISL1 co-localization with PITX2 suggests that PITX2-positive cells do not contribute to the r1-derived trochlear nucleus (see Fig. 9).

GATA2, a transcription factor implicated in GABAergic development in both dorsal and ventral midbrain and required for serotonergic neuron development in the hindbrain, is highly expressed in a superficial ventral domain of r1 (Craven et al., 2004). In our studies, this GATA2-positive population was distinct from the PITX2-positive cell population (Fig. 4E). PAX7, a paired-like transcription factor, is widely expressed in ventral r1, but did not mark PITX2-positive cells (Fig. 4F). EVX1, a transcription factor that is highly expressed in spinal cord interneurons, was also expressed in ventral r1 cells in a region adjacent to but distinct from PITX2-positive cells (Fig. 4G). LBX1 marked a distinct population of neurons dorsal to the PITX2-expressing population (Fig. 4H). Thus, PITX2-positive neurons are negative for the transcription factors GATA2, PAX7, EVX1, and LBX1.

In addition to *Isl1*, we tested whether *Pitx2* neurons express other markers of trochlear and parabrachial nuclei. Trochlear nucleus motor neurons in rostral r1 can also be identified by expression of *Phox2a* and *Phox2b* (Pattyn et al., 1997). We determined the expression patterns of *Phox2a* and *Phox2b* in E12.5 ventral r1 in relation to *Pitx2*-expressing cells (Fig.

4I–L). Analysis of neighboring sections showed no overlap between *Phox2a* or *Phox2b* with *Pitx2* mRNA, providing further evidence that PITX2-positive cells do not contribute to the trochlear nucleus. *Lmx1a* encodes a LIM homeodomain transcription factor that is expressed in the parabrachial nucleus and rhombic lip and is necessary for proper cerebellar development (Mishima et al., 2009; Zou et al., 2009); however, its role in ventral r1 has not been explored. *Lmx1a* was weakly expressed lateral and dorsal to the LMX1B population of cells in the LHX1/5-positive region (Fig. 4I–J and Fig. 3H, K). Together, these data suggest that PITX2-positive r1 neurons do not express *Phox2a*, *Phox2b*, or *Lmx1a* and thus do not contribute to trochlear or parabrachial nuclei.

In the caudal hindbrain and spinal cord, NKX6.1 and NKX6.2 are expressed in visceral motor neurons and interneurons where they are required for neural identity (Briscoe et al., 2000; Pattyn et al., 2003; Sander et al., 2000). NKX6.1 is also implicated in serotonergic neuronal specification in r1 (Craven et al., 2004). NKX6.1 and NKX6.2 were both broadly expressed in ventral r1 and co-localized with some PITX2-positive cells (Fig. 4M, N). Quantitative analysis showed that 27.0% (+/−3.3%) of PITX2-positive cells were NKX6.1-positive (Fig. 4Q). PAX2, a paired-like homeodomain transcription factor and marker of early midbrain/hindbrain (Rowitch and McMahon, 1995), was expressed in ventral r1 in a domain that partially overlapped with PITX2 positive cells wherein 43.7% (+/−7.8%) of PITX2-positive cells were PAX2-positive (Fig. 4O). We also observed 61.6% (+/−6.8%) overlap between PITX2-positive cells and SOX2 (Fig. 4P). Co-expression of some PITX2-positive cells with NKX6.1, NKX6.2, and PAX2 is consistent with the idea that PITX2-positive cells may adopt interneuron fates. In the developing spinal cord, PAX2 and NKX6.1 mark separate post-mitotic populations with distinct progenitor populations (Lebel et al., 2001). Similarly, PAX2 and NKX6.1 in ventral r1 mark separate populations (Fig. 4R, S), suggesting the presence of at least two subpopulations of PITX2-positive cells that arise from separate progenitor populations.

In order to identify whether *Pitx2* is expressed in progenitor populations, we looked for early *Pitx2* expression in r1 at E10.5. Although *Pitx2* is expressed in the E10.5 midbrain, there was no detectable *Pitx2* mRNA in r1 at E10.5 (data not shown). At E11.5, PITX2-positive cells were located lateral to the progenitor zone and negative for the transcription factors BHLHB5, which marks V1 and V2 populations in the spinal cord (Liu et al., 2007), and SOX2 (Fig. 5A, B). Like the E12.5 PITX2-positive population, PITX2- positive E11.5 cells were also positive for LHX1/5, PAX2, and NKX6.1 (Fig. 5C–E). Again, PAX2 and NKX6.1 marked separate r1 populations with PAX2-positive cells medially bordering the NKX6.1-positive population (Fig. 5F–F“), indicating PITX2-positive cells form at least two subpopulations. To determine whether PITX2-positive cells derive from a single progenitor population, we analyzed E11.5 $Dbx1^{Cre};R26^{YFP}$ embryos for PITX2 patterning. In the spinal cord, the *Dbx1*-lineage marks V0 populations and was chosen for analysis because *Pax2* positive, *En1*-negative cells in the spinal cord are known to derive from V0 *Dbx1*-positive progenitors (Lanuza et al., 2004), whereas *Nkx6.*1-expressing cells are more ventral and constitute V2–V3 populations (Sander et al., 2000). Interestingly, most ventral r1 PITX2-positive cells were also positive for YFP, although there were several cells which were YFP-negative (Fig. 5G–G”), which likely indicates separate progenitor populations, but may also be due to inefficient *Cre* recombination (Teissier et al., 2010). Thus, r1 PITX2-positive cells appear to derive from at least two different ventral r1 progenitor populations that can be divided into subpopulations based on transcription factor expression patterns.

Next we asked whether loss of PITX2 affects neuronal distribution or early patterning by analyzing transcription factor expression in E12.5 $Pitx2^{+/-}$ and $Pitx2^{-/-}$ littermate embryos. Loss of *Pitx2* did not affect overall distribution of *Pitx2*-expressing cells (Fig. 6A, B) as determined by *in situ* hybridization using a cRNA probe that is expressed from both the wild

type and null alleles (Martin et al., 2004). The increased density of *Pitx2* mRNA in null embryos compared to controls could signify increased transcription or mRNA stability. Additionally, expression of several transcription factors (SOX2, GATA2, NKX6.1, LHX1/5, EN1, PAX2, PAX7) in $Pitx2^{-/-}$ ventral r1 appeared normal as compared to $Pitx2^{+/-}$ littermate (Fig. 6) or wild type (Fig. 4) embryos. These data suggest that PITX2 is not required for the proper early migration of *Pitx2*-expressing cells or early patterning of ventral r1.

By E18.5, most neurons in the hindbrain have undergone initial stages of differentiation and many of the early transcription factors are no longer expressed. To assay *Pitx2*-lineage neuronal fates, we crossed $Pitx2^{Cre/+}$ mice (Liu et al., 2002; Skidmore et al., 2008) with a *ZsGrn Cre* reporter strain. Interestingly, at E18.5 PITX2-positive neurons comprised two contiguous regions in ventral r1. Transverse sections revealed a deep PITX2-positive population with larger cell bodies and a more superficial population of smaller cells (Fig. 7B). ZsGrn staining showed short neurites in $Pitx2^{Cre/+}$*;ZsGrn* embryos, further suggesting these cells may be interneurons. Analysis of sagittal sections of $Pitx2^{Cre/+}$*;ZsGrn* also highlighted the cell localization and size differences between the superficial and deep populations of PITX2-positive cells in ventral r1 (Fig. 7D–H).

Previous studies in the spinal cord identified a requirement for LHX1 and LHX5 in the maintenance of inhibitory interneuron identity (Pillai et al., 2007). We observed LHX1/5-positive cells distributed throughout the rostral hindbrain and both PITX2-positive populations in r1 contained many LHX1/5-positive cells (Fig. 7I–I”). Interestingly, the more superficial population of PITX2-positive cells was mostly SOX2-positive, whereas only a few cells in the deep population expressed SOX2 (Fig. 7J–J”). Although PITX2-positive cells appear to constitute two separate r1 populations based on localization and morphology, they are both GABAergic at E18.5 (Fig. 7K–K’). PITX2-lineage E18.5 neurons did not express the transcription factor EN1 (Fig. 7L) similar to results at E12.5 (Fig. 3D–F). Interestingly, neither PITX2-positive r1 population was PAX2-positive at E18.5, in contrast to E12.5, when some PITX2-positive cells co-labeled with PAX2 (Fig. 4O and Fig. 7M). Additionally, PITX2-positive cells were negative for GATA2, which marks serotonergic neurons in r1 (Gavalas et al., 2003), and for choline acetyltransferase (ChAT), an enzyme produced in cholinergic populations (Fig. 7N, O). A few ChAT-positive cells were observed caudal to *Pitx2*-positive neurons but appeared to constitute distinct neuronal groups, such as the pedunculopontine, parabrachial, and microcellular tegmental nuclei (Machold and Fishell, 2005; Mizukawa et al., 1986).

To determine whether loss of *Pitx2* affects hindbrain neuronal fate specification, we analyzed E18.5 *Nestin-Cre* conditional *Pitx2*-knockout embryos for neurotransmitter identity in r1. *Pitx2*-positive cells in *Nestin-Cre;*$Pitx2^{flox/+}$ embryos occupied a region that highly expresses *Gad1* but not *Vglut2* or 5-HT (Fig. 8B–D), consistent with GABAergic but not glutamatergic or serotonergic fates. Loss of *Pitx2* in *Nestin-Cre;*$Pitx2^{flox/tlz}$ embryos did not disrupt *Gad1*, *Vglut2*, or 5-HT, suggesting that reduced *Pitx2* dosage does not alter the GABAergic fate of PITX2-positive hindbrain neurons.

4. Discussion

Here we show that PITX2, a paired-like homeodomain transcription factor, is expressed in GABAergic neurons in the ventral aspect of mouse embryonic r1. These PITX2-positive GABAergic neurons may comprise a population of inhibitory interneurons, based on their (a) co-expression with LHX1/5 and PAX2 (Pillai et al., 2007), (b) location in ventral r1, and (c) lack of co-expression with markers of glutamatergic, serotonergic, noradrenergic, and cholinergic neurons. We also show that subsets of GABAergic PITX2-positive neurons

express the transcription factors SOX2, NKX6.1, and NKX6.2 at E12.5. Interestingly, *Pitx2* loss of function does not disrupt the production or specification of these GABAergic neurons, suggesting compensatory mechanisms likely exist.

A summary of our marker analysis in wild type embryos is depicted in Fig. 9. PITX2-positive cells mark a distinct, bilaterally symmetric region of ventromedial r1 containing some cells that also express LHX1/5, NKX6.1, NKX6.2, PAX2, and SOX2 (Fig. 9). The EN1-positive domain spans this PITX2-positive region but extends further toward the ventricle than PITX2-positive cells. LHX1/5-positive cells comprise two separate populations in ventral r1, a medial population that contains PITX2-positive cells and a separate, more lateral population that is PITX2-negative.

4.1 Axial level and context determine PITX2-positive neuronal identity

PITX2-positive neuronal fate (as defined by neurotransmitter phenotype) appears to depend on rostro-caudal and dorso-ventral location along the neural tube. At rostral axial levels in the hypothalamus, PITX2-lineage neurons contribute to the glutamatergic subthalamic nucleus, where it is required for neuronal migration (Martin et al., 2004). In the midbrain, PITX2-positive superior colliculus GABAergic neurons also require *Pitx2* for proper migration and differentiation (Martin et al., 2004; Waite et al., 2011). At more caudal levels in the spinal cord, PITX2-positive interneurons of the $V0_C$ subclass are cholinergic, whereas those of the $V0_G$ class are glutamatergic (Zagoraiou et al., 2009). These subclasses of PITX2-positive V0 interneurons are unevenly distributed along the rostro-caudal axis of the spinal cord, wherein cholinergic neurons occupy rostral lumbar levels while glutamatergic neurons occupy caudal lumbar areas (Zagoraiou et al., 2009). The axial dependence of PITX2-positive neuronal identity suggests that neurotransmitter fate specification is not likely to be determined by PITX2-mediated transcriptional regulation.

Previous studies have attempted to relate the dorsal-ventral developmental patterning in spinal cord to hindbrain patterning (Lebel et al., 2007). Our data suggest that both similarities and differences exist between ventral r1 and spinal cord development. The observation that a subpopulation of PITX2-positive cells in ventral r1 is PAX2/LHX1/5-positive, EN1-negative, and derives from *Dbx1*-positive progenitors suggests that these cells are homologous to V0 interneurons like their PITX2-positive spinal cord counterparts (Gray, 2008; Lanuza et al., 2004). V0 PITX2-positive cells in r1 and the V0 domain in spinal cord differentiate into several different types of neurons (cholinergic and glutamatergic in spinal cord, GABAergic in r1), suggesting that neural progenitors destined to express PITX2 are not necessarily pre-specified with respect to neurotransmitter fate but influenced by local factors dependent on axial level. We also show that a subpopulation of PITX2-positive cells was NKX6.1-positive and PAX2/LMX1B-negative, suggesting that some of the PITX2-positive r1 cells might be homologous to a spinal V2 interneuron population (Gray, 2008; Lebel et al., 2007; Sander et al., 2000). Thus, PITX2 expression in r1 may be progenitor derived lineage-independent and instead be regulated by planar positioning within the developing hindbrain.

Through comparison of the identities of r1 PITX2-positive cells to previously published spinal cord patterning maps, we found differences between cell populations in r1 versus spinal cord. In spinal cord, V2 neurons are GATA2-positive, whereas r1 PITX2-positive V2 neurons (based on *Nkx6.1*-positive and *Lmx1b*-negative expression similar to spinal V2 interneurons), did not express *Gata2*. Because our studies did not include GATA2-lineage tracing, we cannot distinguish between transient and absent expression of GATA2 by r1 PITX2-positive cells. It is also possible that V2 neurons have different molecular signatures in r1 and spinal cord, as is true for several of the dorsal populations, the pMNvs, and the V3 populations (Gray, 2008). Alternatively, previous studies have shown that several D–V

populations in r1 may not exist in the developing spinal cord (such as the DA4 and DB2 populations), or may exist in developing spinal cord and caudal rhombomeres but not r1 (such as the DI2, DI3, DI5, and DI6) and that some populations have unique expression patterns between the hindbrain and spinal cord (such as DI1–DI3, pMN, and V3 populations) (Gray, 2008).

4.2 Transcriptional mechanisms of GABAergic neuronal differentiation

Throughout the central nervous system, distinct neuronal populations are distinguished by the complement of transcription factors they express as well as by characteristics such as neurotransmitter fate and unique projection patterns. Here we provide a description of several early r1 transcription factors and their mapping with respect to PITX2-positive populations. There is substantial overlap between PITX2 and LHX1/5 expression in GABAergic neurons, and some PITX2-positive cells also express PAX2 in ventral r1 at E12.5. PAX2 and LHX1/5, along with PAX5 and PAX8, function to regulate formation of spinal cord GABAergic inhibitory interneurons (Pillai et al., 2007). Further genetic studies are necessary to identify the factors that regulate formation of PITX2-positive GABAergic inhibitory neurons in r1.

In addition to PAX and LHX genes, several other transcription factor genes have been shown to regulate GABAergic neuronal differentiation. Interestingly, mutations of many of these transcription factor genes also disrupt (or augment) glutamatergic differentiation, although findings vary by neuronal population and rostro-caudal axial level (Cheng et al., 2005; Pillai et al., 2007). The basic helix-loop-helix (bHLH) transcription factor Ptf1a is required for proper formation of dorsal spinal cord, cerebellar, and retinal GABAergic inhibitory interneurons and loss of *Ptf1a* leads to an expansion of spinal cord glutamatergic neurons (Glasgow et al., 2005). The homeobox gene *Lbx1* is also necessary (and sufficient) for spinal cord GABAergic neuronal differentiation (Cheng et al., 2005), but *Lbx1* activity can be modified by the homeobox gene *Tlx3*, itself an important regulatory of GABAergic vs. glutamatergic spinal cord neuronal differentiation (Cheng et al., 2005). While *Pitx2* does not appear critical for GABAergic fate specification, it may regulate expression of genes that modulate other, as yet unidentified aspects of GABAergic neuronal function or maintenance.

4.3 Potential roles for PITX2-positive GABAergic r1 neurons

There are several important caveats to consider in our interpretation of the functional and molecular identities of r1 PITX2-positive GABAergic interneurons. First, the molecular characteristics of r1 neurons are incompletely described in the literature, forcing us to rely on comparisons with spinal cord or other axial levels. For example, studies showing that *Dbx1*-lineage cells in more caudal rhombomeres are responsible for regulating breathing have excluded r1 from their analysis (Borday et al., 2006). Second, r1 is unique among rhombomeres in that it develops through signaling from the isthmic organizer, does not express any *Hox* genes, and has unique requirements for *Shh* signaling (Blaess et al., 2006; Irving and Mason, 2000; Lebel et al., 2007). Nonetheless, the potential V0 and V2 GABAergic neurotransmitter identities of PITX2-positive cells and their transcription factor profiles are important for future studies exploring the functions of PITX2-positive cells in r1.

Ventral r1 contains many neurons that participate in critical life processes such as control of respiration (Gray, 2008). Locomotion is also controlled in part by the activities of ventral hindbrain interneuron populations (Grossmann et al., 2010). Our studies provide evidence that r1 PITX2-positive GABAergic neurons are distinct from neurons of the trochlear motor nucleus, serotonergic neurons of the dorsal raphe, *Lmx1a*-positive parabrachial neurons,

Phox2a/b-positive visceral motor neurons, locus coeruleus neurons, and trigeminal neurons. Several nuclei in ventral r1 are known to contain GABAergic neurons including the dorsal raphe, laterodorsal tegmental nucleus, and pedunculopontine nucleus (Mena-Segovia et al., 2009). In the ventral r1 field, the dorsal raphe is medial, the dorsal tegmental nucleus is in deep ventral r1 near the ventricle, while the pedunculopontine tegmental nucleus is more lateral and superficially localized (Martin, 2003; Schambra et al., 1992). Based on GABAergic identity and localization, we predict that PITX2-positive cells contribute to the pedunculopontine tegmental nucleus which is thought to be involved in local inhibition controlling locomotion, REM, alertness, and respiratory patterns (Datta et al., 2001; Kozak et al., 2005; Saponjic et al., 2005; Tsang et al., 2010). A modulatory role for PITX2-positive GABAergic neurons in respiration, alertness, or other important autonomic functions could help explain why *Nestin-Cre* conditional *Pitx2* mutants fail to survive beyond the immediate postnatal period (Sclafani et al., 2006). Further studies should help clarify the physiological roles of PITX2-positive hindbrain neurons and their potential contributions to control of locomotion, respiration, or other autonomic functions.

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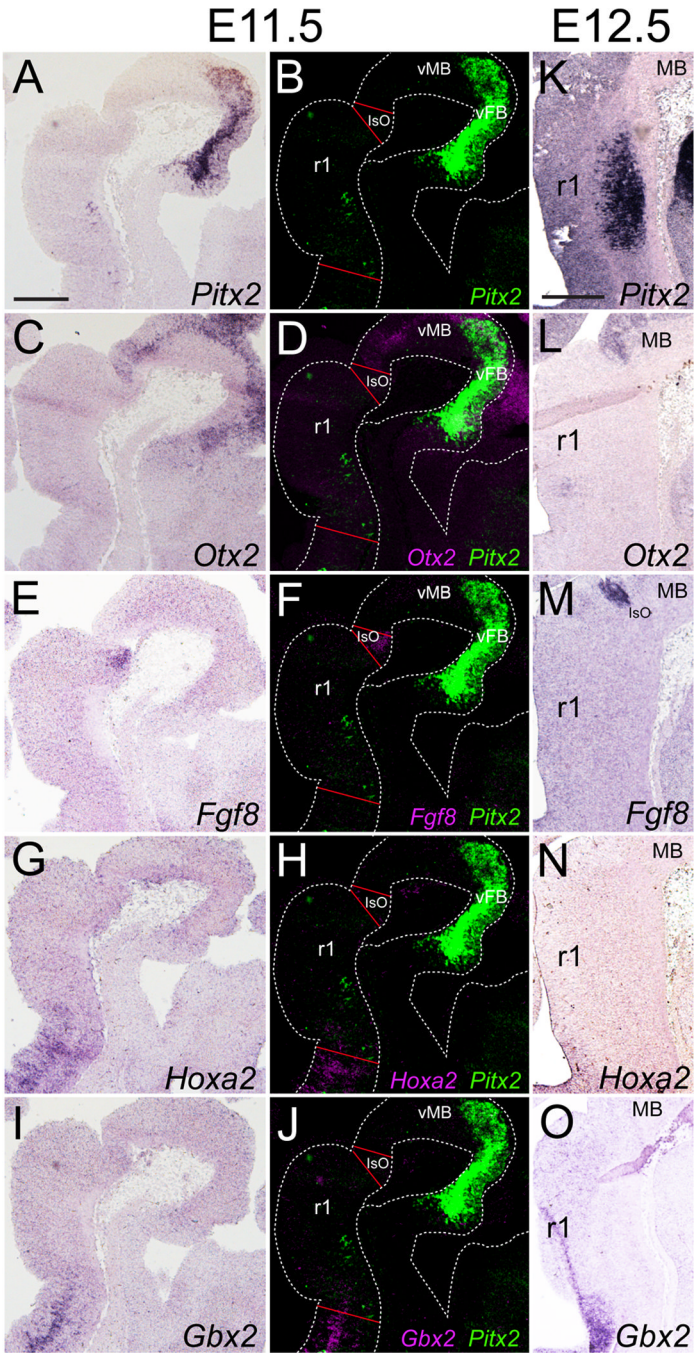

Figure 1. *Pitx2*-expressing cells localize to rhombomere 1

E11.5 (A–J) or E12.5 (K–O) sagittal sections processed for (A, K) *Pitx2*, (C, L) *Otx2*, (E, M) *Fgf8*, (G, N) *Hoxa2*, *or* (I, O) *Gbx2* single *in situ* hybridization. Pseudocolored and merged images of neighboring slides processed for *in situ* hybridization to detect mRNA for *Pitx2* and either *Otx2* (D), *Fgf8* (F), *Hoxa2* (H), *or Gbx2* (J). Scale bars in A and K are 250 μm and apply to panels A–J and K–O, respectively.

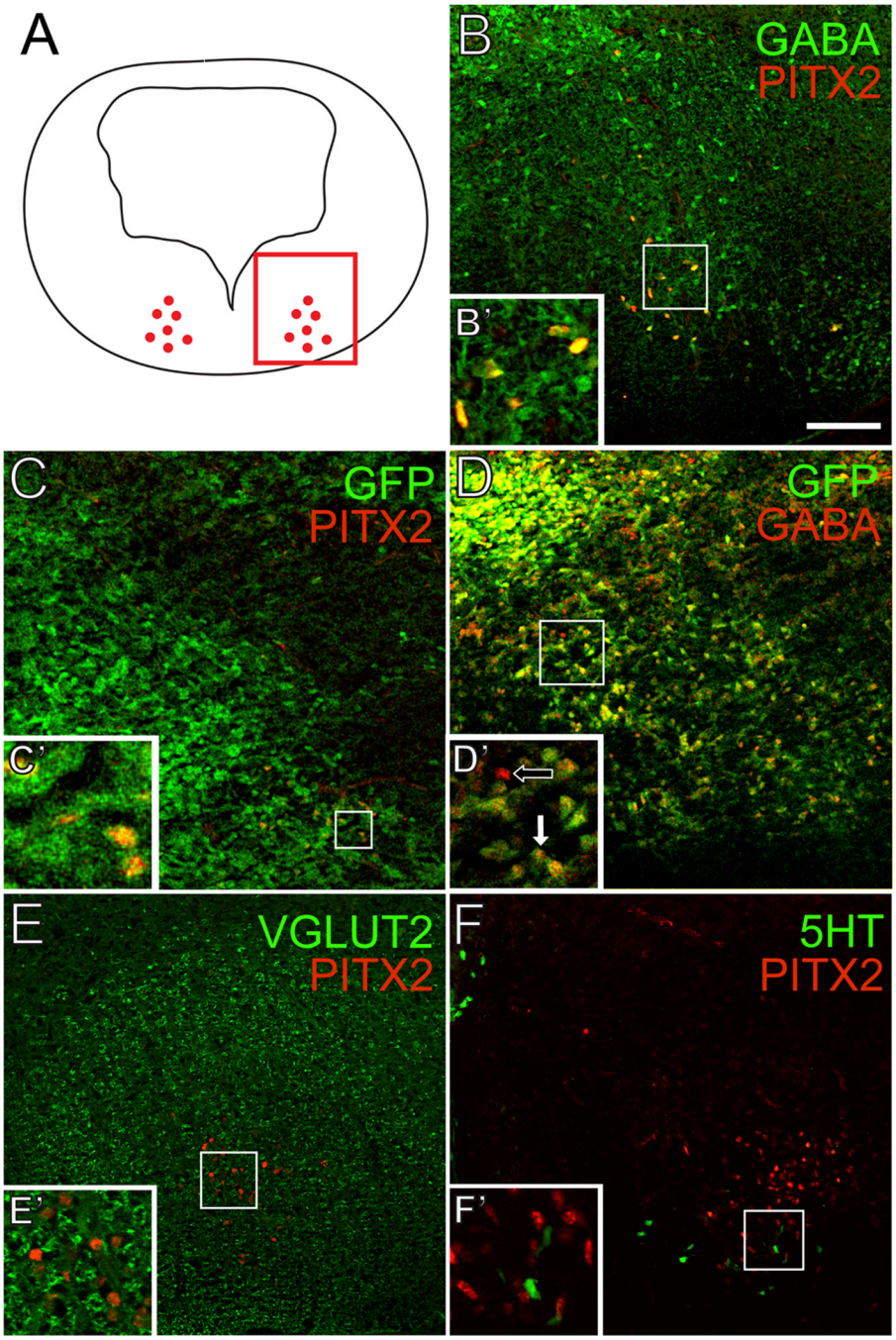

Figure 2. PITX2-positive GABAergic neurons in r1 are distinct from serotonergic, glutamatergic, and cholinergic neurons

Double immunofluorescence of E14.5 mouse brain tissues sectioned transversely at the level of r1. Schematic in A indicates transverse orientation for panels B–F. Square inset in A shows the area represented in B–F. PITX2 co-localizes with GABA (B–B') and GAD67-GFP (C–C'). Most GFP-positive cells are also GABA-positive (D–D'). PITX2-positive cells are negative for VGLUT2 (E–E') and 5-HT (F–F'). Scale bar in B is 100 μm and applies to panels B–F. All images were taken using confocal microscopy.

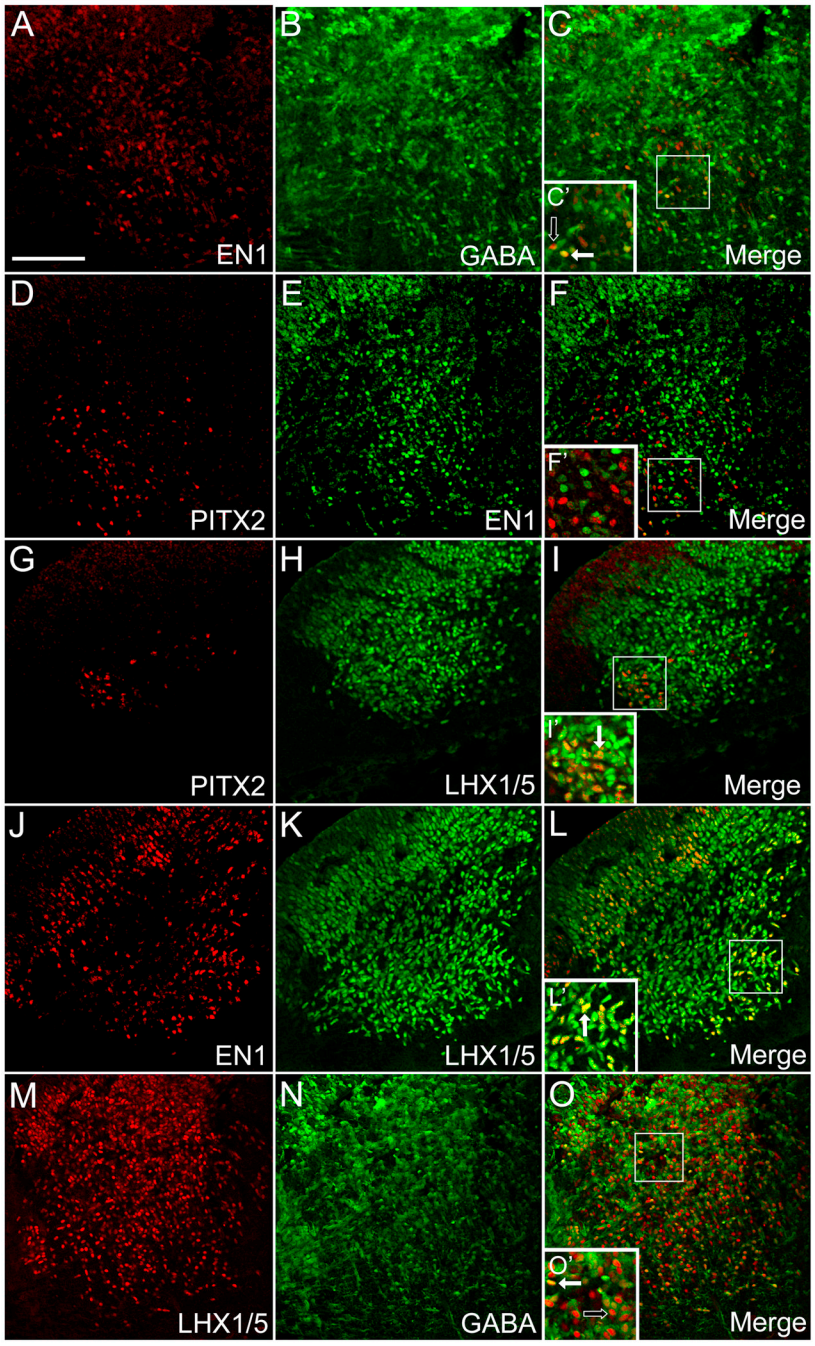

Figure 3. GABAergic r1 neurons express PITX2, EN1, and LHX1/5

Double immunolabeling of E12.5 mouse brain tissues sectioned transversely at the level of r1 (orientation as in Figure 2A) reveals distinct patterns of overlap among GABA and several transcription factors. Insets in C–O are enlarged in C'–O' and show double (solid arrow) or single (open arrow) labeled cells. Scale bar in A is 100 μm and applies to panels A–O. All images were taken using confocal microscopy.

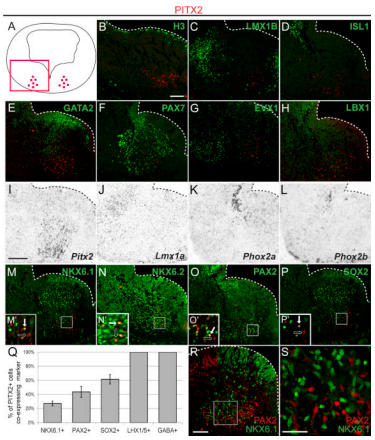

Figure 4. PITX2-positive GABAergic neurons occupy distinct regions of the ventral hindbrain

Double immunolabeling (A–H, M–P, R, S) or *in situ* hybridization (I–L) of E12.5 mouse brain tissues sectioned transversely at the level of r1 (orientation as in panel A) reveals distinct patterns of overlap between PITX2 and several transcription factors. Insets in M–P are enlarged in M'–P', and show double (solid arrow) or single (open arrow) labeled PITX2-positive cells. Graph in Q indicates the percentage of PITX2-positive cells which co-express the marker indicated (NKX6.1, PAX2, SOX2, and LHX1/5 are from E12.5 embryos; GABA is from E14.5 embryos). Error bars are +/− standard error of the mean of cell counts from $N \geq 3$ sections. (R, S) PAX2 and NKX6.1 mark separate cell populations in r1. Cells in the intermediate layer of panel R are enlarged in S. Scale bar in B is 100 μm and applies to panels B–H and M–P. Scale bar in I is 100 μm and applies to panels I–L. Scale bars in R and S are 75 μm and 30 μm, respectively. All immunofluorescent images were taken using confocal microscopy.

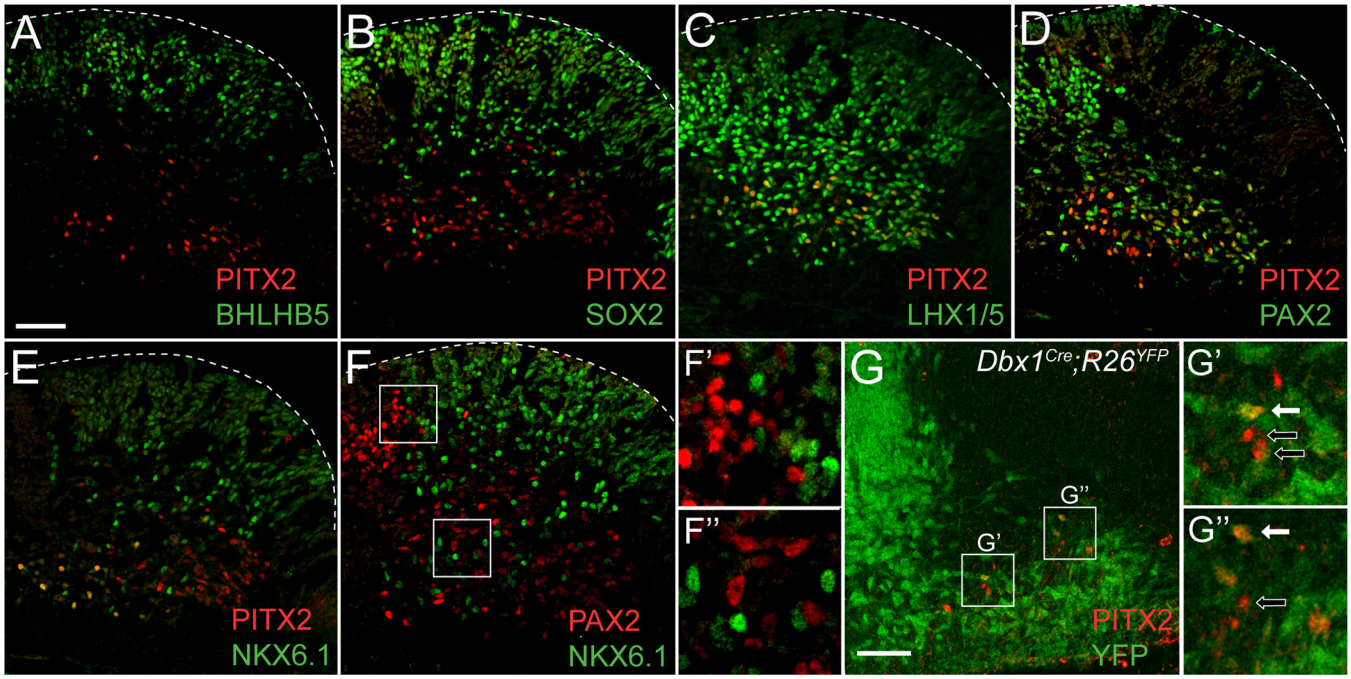

Figure 5. Early expression of PITX2 is similar to E12.5 patterning and includes *Dbx1*-lineage cells

Immunolabeling of E11.5 (A–G") *Pitx2*$^{+/+}$ or *Dbx1*^{Cre};*R26*^{YFP} embryos sectioned transversely at the level of r1 (orientated as in Figure 4A). (A–E) E11.5 PITX2-positive cells in r1 are negative for BHLHB5, some are SOX2-positive, and many are positive for LHX1/5, PAX2, and NKX6.1. (F–F") In the E11.5 r1, PAX2 and NKX6.1 mark separate cell populations. (G–G") Many PITX2-positive cells are YFP-positive, although some are negative. Insets in F and G are enlarged in F'/F" and G'/G", respectively. G' and G" show double (solid arrow) or single (open arrow) labeled cells. Scale bar in A is 50 μm and applies to panels A–F. Scale bar in G is 50 μm. All immunofluorescent images were taken using confocal microscopy.

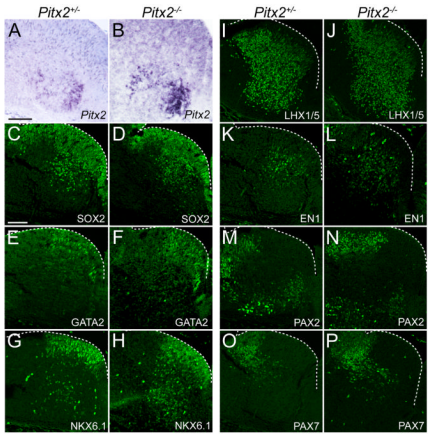

Figure 6. PITX2 is dispensable for early neuronal migration and ventral r1 patterning

In situ hybridization (A–B) or immunolabeling (C–P) of E12.5 *Pitx2*$^{+/-}$ and *Pitx2*$^{-/-}$ mouse brain tissues sectioned transversely at the level of r1 (orientation as in Figure 4A) reveals normal transcription factor patterning. Scale bars in A and C are 100 μm and apply to panels A–B and C–P, respectively. All immunofluorescent images were taken using confocal microscopy.

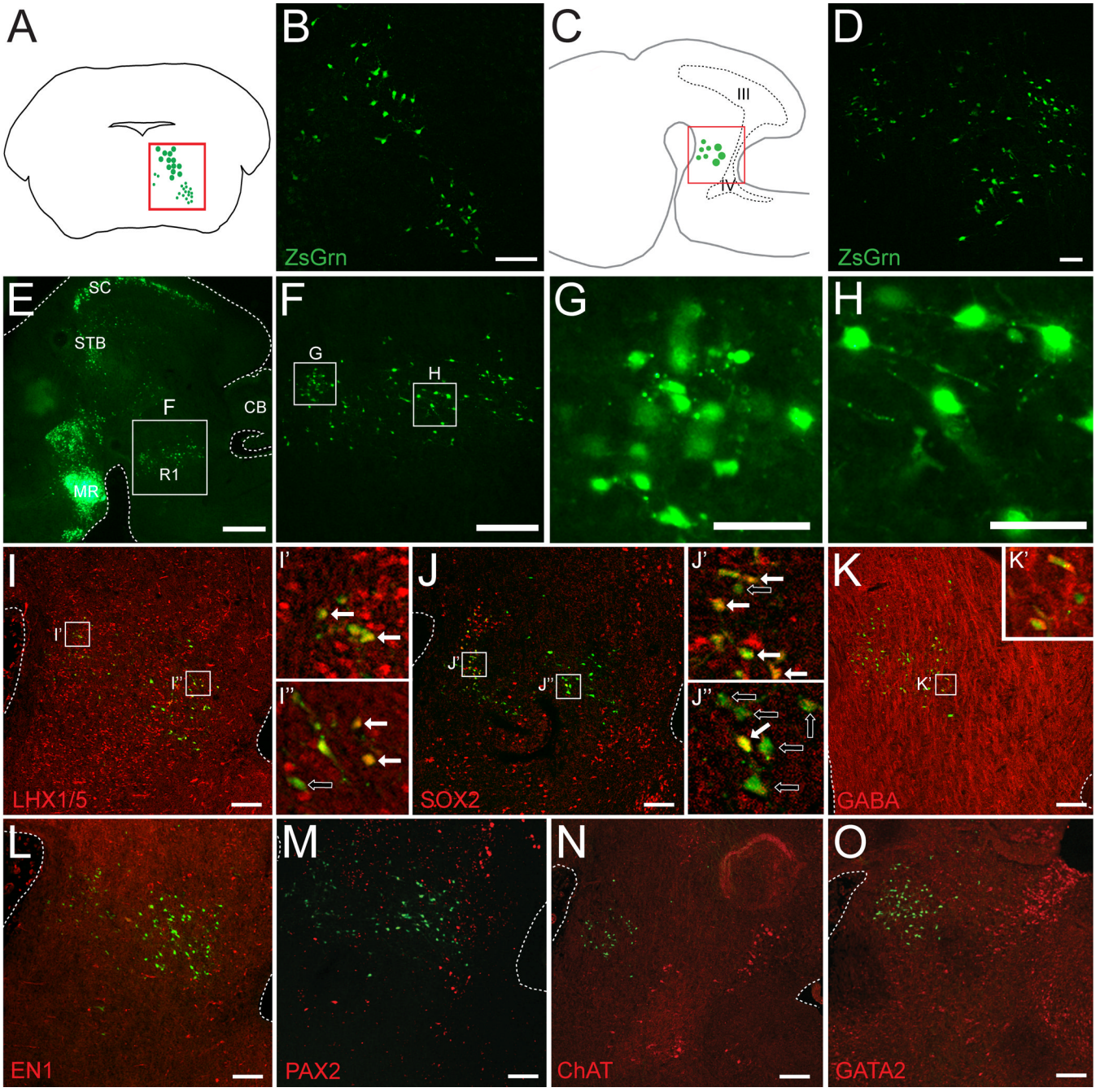

Figure 7. Two PITX2-positive populations span ventral r1

E18.5 *Pitx2*$^{Cre/+}$*;ZsGrn* transverse (B) and sagittal (D–O) sections processed for immunofluorescence with antibodies against transcription factors, ChAT, and GABA in the hindbrain. (A) Schematic of a transverse section identifying the region pictured in panel B. (C) Schematic of a sagittal section identifying the region pictured in panels D–O. Boxes in I–K are enlarged in I'–K" and show double (solid arrow) or single (open arrow) labeled PITX2-lineage cells. Panels are arranged medial (I, L) to lateral (K, O). Scale bar in B is 100 μm, in E is 500 μm, in F is 200 μm, and in G–H is 50 μm. Scale bars in D and I–O are 100 μm. All immunofluorescence images were taken using confocal microscopy. Abbreviations:

III, third ventricle; IV, fourth ventricle; CB, cerebellum; MR, mammillary region; SC, superior colliculus; STB, subtectal band.

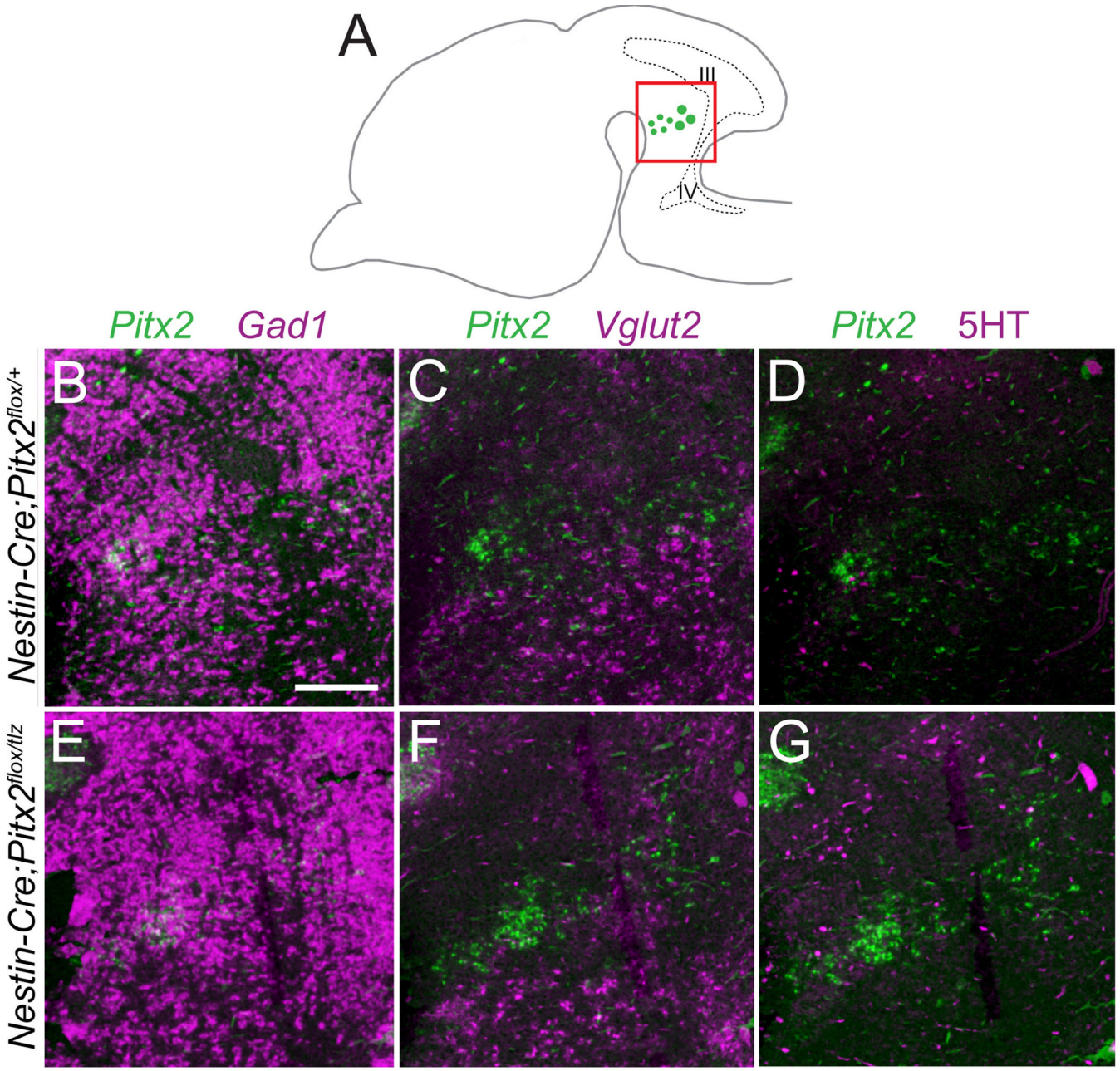

Figure 8. Ventral r1 GABAergic identity is PITX2-independent

Sagittal E18.5 *Nestin-Cre;Pitx2*$^{flox/+}$ and *Nestin-Cre;Pitx2*$^{flox/tlz}$ littermate brains processed for single *in situ* hybridization for *Pitx2*, *Gad1*, or *Vglut2* or immunohistochemistry with antibodies against 5-HT. (A) Cartoon of a sagittal section showing the orientation of panels B–G. (B–G) Merged images of neighboring sections processed for *in situ* hybridization for *Pitx2* and either *Gad1* (B, E), *Vglut2* (C, F), or 5-HT (D, G) immunohistochemistry and pseudocolored. Scale bar in B is 200 μm and applies to panels B–G.

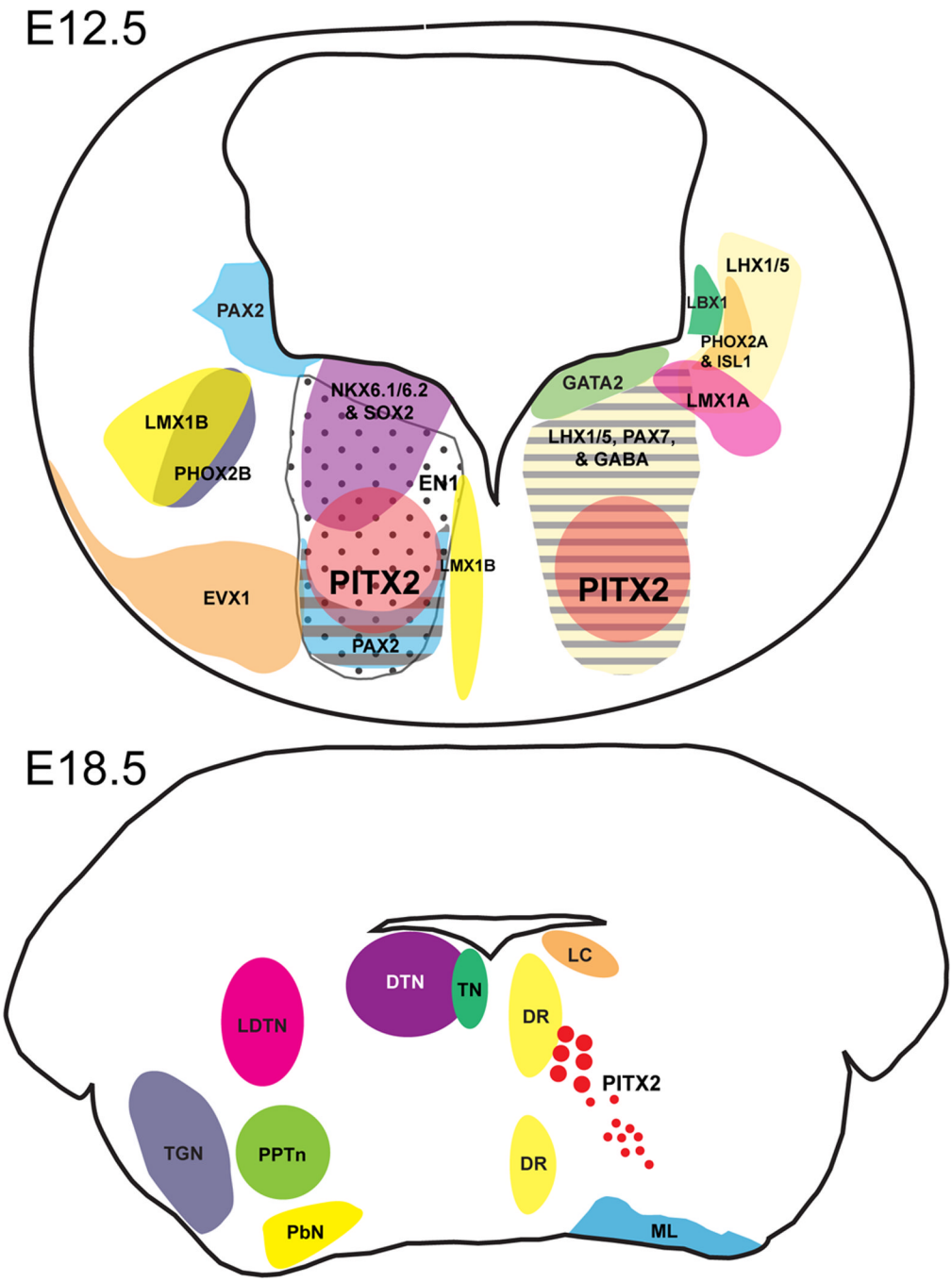

Figure 9. Schematic of transverse sections through the developing mouse brain at the level of rhombomere 1

(A) E12.5 transverse section through r1 showing transcription factor and GABA patterning in relation to PITX2. (B) E18.5 transverse section through r1 showing nuclei patterning based on “Atlas of the Prenatal Mouse Brain” (Schambra et al., 1992) in relation to PITX2 (red circles). Abbreviations: DR, dorsal raphe nucleus; DTN, dorsal tegmental nucleus; LC, locus coeruleus; LDTN, laterodorsal tegmental nucleus; ML, medial lemniscus; PbN, parabrachial nucleus; PPTn, pedunculopontine nucleus; TN, trochlear nucleus; TGN, trigeminal motor nucleus.