PLEIOTROPIC AND ISOFORM-SPECIFIC FUNCTIONS OF PITX2 IN BRAIN DEVELOPMENT

by

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Yesterday is but a dream, tomorrow but a vision. But today well lived makes every yesterday a dream of happiness, and every tomorrow a vision of hope. Look well, therefore, to This Day.

-Sanskrit Proverb

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ABSTRACT

Pleiotropic and isoform-specific functions of PITX2 in brain development

by

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Neuronal diversification in the developing brain is a coordinated process requiring complex genetic regulation. Transcriptional control of gene expression is known to regulate proliferation, migration, differentiation, and survival of developing neurons, although the precise mechanisms underlying these processes are not fully understood. One transcription factor, *pituitary homeobox 2* (*Pitx2*), is expressed in the developing and adult mammalian brain, eye, and pituitary, and in asymmetric organs such as the heart, lungs, and gut. In humans, mutations in *PITX2* result in the autosomal dominant disorder Rieger syndrome which is characterized by defects in the eyes, umbilicus, and teeth along with variable abnormalities in the heart, pituitary, and brain. *Pitx2* encodes a paired-like homeodomain transcription factor expressed in highly restricted domains of the mouse brain beginning at E9.25 and continuing through adulthood. Alternative splicing of *Pitx2* in the mouse results in three isoforms (*Pitx2a, Pitx2b,* and *Pitx2c*) which are expressed symmetrically in the brain throughout development. The contribution of each isoform to brain development is not known. We have identified three *Pitx2*-positive neuronal populations in the midbrain and at least two populations in rhombomere 1 (r1). In the dorsal midbrain and r1, *Pitx2*-positive neurons are GABAergic, whereas in the ventral midbrain *Pitx2*-positive neurons are glutamatergic, consistent with the idea that *Pitx2*-

positive cell fate depends on anatomic context and axial level. Loss of *Pitx2* in the dorsal midbrain results in failure of cells to fully migrate and undergo GABAergic differentiation. Chimera analyses suggest that dorsal midbrain *Pitx2* regulates migration through cell autonomous mechanisms. Isoform-specific knockout studies show unique dosage-specific requirements for *Pitx2* isoforms in midbrain neuronal migration, GABAergic differentiation, and mammillothalamic tract formation. Loss of *Pitx2* in the ventral midbrain and r1 does not affect neuronal migration per se, indicating region-specific requirements for *Pitx2*. Collectively, these studies expand our knowledge about region- and isoform-specific requirements for transcription factors in the developing brain, which may prove critical for developing future stem cell and other therapies in neurological disorders.

Chapter I
Introduction

Brain development is an extremely complex process that requires regional proliferation, specification, neuronal migration, cell fate determination, axonal outgrowth, and formation of complex neural networks. In order to ensure proper development, each of these steps must be tightly regulated by molecular and cellular mechanisms including morphogen and growth factor signaling, transcription factor gene expression, expression of axon guidance genes, and synaptic activity. Incorrect signaling in any of these regulatory steps can result in abnormal brain development and disorders with resultant lethality, disability, or mental deficiencies and lifelong impacts on social, behavioral, and intellectual pursuits. Studies on elucidation of the molecular mechanisms underlying brain development should suggest gene targets to improve methods of prevention or treatment for brain disorders.

An early step in brain development is the segmentation of the neuroepithelium along the rostral-caudal and dorsal-ventral axes into discrete regions with similar developmental programs and neuronal populations. This division among brain regions occurs through the use of morphogen and growth factor gradients secreted from signaling centers, which control brain patterning (Kutejova et al., 2009; Ulloa and Briscoe, 2007). In turn, this patterning influences cell-fate choices by inducing the expression of transcription factors that are capable of repressing or activating target gene transcription in a cell autonomous manner. The evolutionarily conserved homeodomain transcription factors contain a DNA-binding homeobox domain which is necessary for direct binding to the promoters of target genes. Homeodomain proteins are critical throughout embryonic development to regulate genes involved in patterning, proliferation, migration, and cellular differentiation (Wigle and Eisenstat, 2008). By acting in concert with bHLH

transcription factors and other local proteins, homeodomain proteins are necessary for cell-fate decisions and neural differentiation (Guillemot, 2007).

The Pitx family in development

Members of the *Pitx* family are bicoid-like homeodomain transcription factors required for proper development of embryonic tissues and viability (Gage et al., 1999a; Szeto et al., 1999). The *Pitx* family consists of the three paralogous genes, *Pitx1, Pitx2,* and *Pitx3* (Fig. 1.1). All three paralogues have been identified in human, rodents, chick, zebrafish, *Xenopus*, and *Ciona*, whereas *Drosophila* and *C. elegans* express only *Pitx1* (Christiaen et al., 2005; Vorbruggen et al., 1997). PITX proteins have unique N-termini, but similar C-termini consisting of a well-conserved 60 amino acid homeodomain that is critical for DNA binding and an aristaless domain that is important for protein-protein interactions (Amendt et al., 1999). The *Pitx* homeodomains are "bicoid-like" in that they contain a lysine at position 50, which confers binding specificity to the bicoid TAA(T/G)CC motif in target gene promoters (Gage et al., 1999a). The *PITX* homeodomain is well conserved between family members; the *PITX2* homeodomain has 97% and 100% similarity to the homeodomains in *PITX1* and *PITX3*, respectively, and there is 100% *Pitx2* homeodomain homology between human, mouse, chick, zebrafish, and *Xenopus* (Campione et al., 1999). As such, all *Pitx* family members have homeodomains with similar form and function.

The *Pitx2* homeodomain forms three alpha helices which are held in place by a tightly packed core of hydrophobic, highly conserved/invariant amino acids (Fig. 1.1B) (Chaney et al., 2005; Gehring et al., 1994). Helix 1 (amino acids 10-20 of the homeodomain) is followed by a loop region and by helix 2 (amino acids 28-37), which runs anti-parallel to helix 1 (Fig. 1.1C). Helix 2 and helix 3 form a helix-turn-helix motif, whereby helix 3 ends up perpendicular to helices 1 and 2 to fit into the major groove of DNA. The target sequence specificity of homeodomain transcription factors is largely determined by specific amino acids in helix 3. Among homeodomain proteins, residues 47 and 51 in helix 3 of the homeodomain recognize the core 5'-TAAT-3' motif and the amino acid at residue 50 is crucial for further DNA binding specificity (Gehring et al., 1994; Tucker-Kellogg et al., 1997). For example, bicoid-like homeodomain transcription

factors bind 5'-TAATCC-3' due to K50 recognition of a 5'-CC/G-3', whereas Q50 class homeodomains prefer the 5'-TAATTA-'3 sequence (Ades and Sauer, 1994; Treisman et al., 1989). Additional DNA binding specificity is created by contacts between the flexible amino-terminal arm of the homeodomain and the minor groove and contacts between the DNA backbone with the loop between helices 1/2 and with the initial residues of helix 2 groove (Percival-Smith et al., 1990). These homeodomain-DNA interactions are necessary for regulation of gene expression by homeodomain transcription factors, and thereby critical for proper embryonic development.

Pitx1

Each *Pitx* family member has a distinctive expression pattern and performs unique functions during embryonic development. *Pitx1* is required for viability and is expressed very early in mouse development (E8), beginning in the stomodeum and later in its derivatives: the pituitary anlage, Rathke's pouch, and oral, dental and olfactory epithelia (Gage et al., 1999a; Marcil et al., 2003; Szeto et al., 1999). *Pitx1* is also required for development of the pituitary, hindlimb, and craniofacial regions (Lanctot et al., 1999; Szeto et al., 1999). In humans, *PITX1* mutations can result in limb malformations including clubfoot (Alvarado et al., 2011; Klopocki et al., 2012), and *PITX1* polymorphisms have been associated with autism (Philippi et al., 2007).

Pitx2

Like *Pitx1, Pitx2* is expressed very early in mouse development (E8) in the stomodeum and is required for viability past E14.5 (Gage et al., 1999a; Marcil et al., 2003). *Pitx2* has a broader range of expression than other *Pitx* family members and is required during mouse development in the eyes, teeth, heart, lungs, gut, umbilicus, and central nervous system (CNS) (Fig. 1.2) (Gage et al., 1999a; Hjalt et al., 2000; Kitamura et al., 1999; Lin et al., 1999; Martin et al., 2004; Ryan et al., 1998; Suh et al., 2002). In the CNS, *Pitx2* is expressed in the subthalamic nucleus, mammillary region, zona limitans intrathalamica, superior colliculus, red nucleus, ventral rhombomere 1, and in the spinal cord (Lindberg et al., 1998; Martin et al., 2002b; Mucchielli et al., 1997; Waite et al., 2012; Waite et al., 2011; Zagoraiou et al., 2009). *PITX2* haploinsufficiency in

humans results in the autosomal dominant disorder, Axenfeld-Rieger Syndrome, which occurs in approximately 1 in 200,000 births (Suri, 2004).

Axenfeld-Rieger Syndrome was first described in 1883 by Vossius, who noted bilateral iris defects and dental abnormalities in a 9 year-old patient (Vossius, 1883). In 1920, Theodor Axenfeld described a patient with mild iris stromal hypoplasia and a white line in the peripheral cornea. He called this condition "embryotoxon cornea posterius" (Axenfeld, 1920). In 1934, Herwig Rieger described two of his patients with mild iris hypoplasia and other eye and systemic defects (Rieger, 1934). At an annual Ophthalmology Society meeting in 1935, Rieger presented his work on a family with hypoplasia of the anterior leaf of the iris stroma, a phenotype he called "dysgenesis mesodermalis corneae et iridis" (Rieger, 1935b). He suggested the syndrome had dominant inheritance and was caused by improper development early in gestation. Later, dental abnormalities were identified in these patients. During this time, other articles were published describing additional phenotypes including craniofacial and dental abnormalities (Mathis, 1936; Rossano, 1934), although the underlying mechanisms remained unidentified.

The genes involved in Axenfeld-Rieger Syndrome were unknown for many years. The first studies on the genetic cause of the syndrome suggested a link with chromosome 4q23-26 (Mitchell et al., 1981; Serville and Broustet, 1977; Vaux et al., 1992). In 1992, a linkage analysis study was performed on three small families where at least one family member had the cardinal indicators of Rieger Syndrome, including anterior segment ocular disorder, hypodontia, and abnormal periumbilical skin (Murray et al., 1992). This analysis identified genetic linkage between Rieger Syndrome and a narrow interval on chromosome 4q. A causative gene was finally identified in 1996 – over 100 years since Rieger Syndrome was first described. The gene was identified using positional cloning and named *RIEG* (*PITX2*) (Semina et al., 1996). These studies also identified the expression pattern of *Rieg* in the embryonic mouse, leading to tissue-specific studies on *Pitx2* function and a greater understanding of Axenfeld-Rieger syndrome in humans.

Rieger Syndrome is a highly variable, multiple congenital anomaly syndrome resulting from heterozygous mutations in the *PITX2* (4q25), *RIEG2* (13q14), or *FOXC1* (6p25) genes (Nishimura et al., 1998; Phillips et al., 1996; Semina et al., 1996). Rieger

Syndrome is characterized by dental malformations/hypodontia, mild craniofacial abnormalities, ocular defects, and periumbilical involution failure (Childers and Wright, 1986; Jorgenson et al., 1978; Shields, 1983). Some Rieger patients have additional developmental abnormalities including intestinal protrusions, glaucoma, and rare structural brain defects that include cerebellar hypoplasia, enlargement of the cisterna magna, hydrocephalus, impaired cognitive function, mild psychomotor defects, and sensorineural hearing loss (Cunningham et al., 1998; De Hauwere et al., 1973; Idrees et al., 2006; Moog et al., 1998; Rieger, 1935a; Shields, 1983).

Making direct correlations between specific phenotypes and *PITX2* gene mutations have been challenging. Several types of mutations in the *PITX2* gene can result in Rieger Syndrome, including missense mutations in the homeodomain (Priston et al., 2001; Semina et al., 1996; Vieira et al., 2006), occasional splice-site or nonsense mutations leading to protein truncation (Perveen et al., 2000; Semina et al., 1996), and microdeletions (de la Houssaye et al., 2006; Engenheiro et al., 2007). Because the homeodomain is key for DNA binding and specificity, mutations affecting the homeodomain result in decreased protein stability, reduced DNA binding specificity, and weakened gene transactivation (Amendt et al., 1998; Footz et al., 2009). Consistent with this, most Rieger Syndrome mutations occur in the *PITX2* homeodomain and are especially prevalent within the sequences defining the three helices (Footz et al., 2009).

PITX2 dysfunction can also lead to other disorders such as heart disease and cancer. In humans and mice, reduced *PITX2* expression in cardiac tissue predisposes the heart to atrial fibrillation, which is caused by abnormal cardiac electrical impulses (Chinchilla et al., 2011; Wang et al., 2010). Because *Pitx2* is a downstream effector of *Wnt/β-catenin* signaling and aberrant signaling in these pathways is common in human cancers, it is unsurprising that incorrect regulation of *PITX2* has also been linked to the incidence of cancer. Hypermethylation of the *PITX2* promoter, resulting in decreased *PITX2* gene expression, is strongly associated with prostate cancer (Vinarskaja et al., 2011). Follicular thyroid cell-derived cancers exhibit increased *PITX2* expression and its downstream cell-cycle effectors *CyclinD1* and *CyclinD2*, whereas *PITX2* knockdown results in decreased tumor proliferation and colony formation (Huang et al., 2010). In colorectal cancer cell lines, *PITX2* expression is higher than in control lines (Hirose et al.,

2011), and the methylation status of *PITX2* can be used to predict outcomes of patients with breast cancers (Gu et al., 2010; Martens et al., 2009).

Pitx3

Pitx3 expression is much more restricted than *Pitx2* and is found in the lens of the eye, skeletal muscle, and dopaminergic neurons of the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of the ventral midbrain (L'Honore et al., 2007; Semina et al., 1997; Smidt et al., 1997). Loss of *PITX3* in humans results in eye defects such as mesenchymal dysgenesis and dominant cataracts (Addison et al., 2005; Semina et al., 1998). In mice, *Pitx3* mutations result in dopaminergic neuronal differentiation defects and survival defects in the midbrain (Maxwell et al., 2005).

In humans, loss of dopaminergic neurons from the substantia nigra is a hallmark of Parkinson’s disease, which is characterized by both movement and cognitive impairments. This suggests that *PITX3* may be involved in disease development and could therefore be a therapeutic target. Indeed, recent studies suggest that specific polymorphisms within the *PITX3* gene are associated with higher incidence of Early-Onset Parkinson’s disease (Bergman et al., 2010; Guo et al., 2011; Le et al., 2011), although these data are controversial (Cai et al., 2011). *Pitx3* is a key determinant of dopaminergic differentiation and can be used to efficiently differentiate stem cells into dopaminergic neurons for engraftment therapies (Chung et al., 2005; O'Keeffe et al., 2008). Transplantation of these ES-cell generated dopaminergic neurons into the midbrains of rodent models of Parkinson’s disease has induced recovery of some motor functions (O'Keeffe et al., 2008). However, the effects of targeting human *PITX3* early in Parkinson’s disease progression are unknown.

Pitx2 isoform expression and requirements

In humans, the *PITX2* gene comprises six exons which utilize alternative promoters and splicing to generate four unique isoforms (*PITX2A, PITX2B, PITX2C,* and *PITX2D*) (Fig. 1.3A). *PITX2A* and *PITX2B* utilize a promoter upstream of exon 1 and comprise unique combinations of exons 1-3 and 5-6 (Fig. 1.3B) (Liu et al., 2003; Shiratori et al., 2001). *PITX2C* and *PITX2D* use a promoter upstream of exon 4 and

employ different combinations of exons 4-6. *PITX2D* is unusual in that it has only been identified in human craniofacial tissue and contains a truncated homeodomain sequence, suggesting that it may not directly regulate gene transcription (Cox et al., 2002). Consistent with this, PITX2D is thought to repress the transactivation functions of other PITX2 isoforms by forming repressive heterodimers.

Although *PITX2D* only been identified in humans, *Pitx2a, b,* and *c* are expressed in various combinations in other species. For example, the mouse expresses all three isoforms (Liu et al., 2001; Schweickert et al., 2000; Smidt et al., 2000), whereas only the *Pitx2a* and *Pitx2c* isoforms have been identified in the chick (Yu et al., 2001). In mouse, all three isoforms are expressed in the developing craniofacial tissue, brain, hematopoietic stem cells in the liver, pituitary, eyes, and teeth (Ai et al., 2007; Gage and Camper, 1997; Kieusseian et al., 2006; Liu et al., 2001; Liu et al., 2003; Smidt et al., 2000; Waite et al., unpublished), whereas only *Pitx2c* is expressed in the lateral plate mesoderm, heart, lungs, and gut (Kitamura et al., 1999; Schweickert et al., 2000; Yu et al., 2001). In the developing mouse brain, *Pitx2ab* and *Pitx2c* isoform promoters are active early, beginning at E9.25-E9.5 and continue to be expressed through adulthood (Smidt et al., 2000; Waite et al., unpublished). *Pitx2* isoform expression patterns in the rodent brain are identical and symmetric with respect to the midline. In contrast, *Pitx2c* is expressed asymmetrically in the developing zebrafish brain where *Pitx2a* and *Pitx2b* are not present (Essner et al., 2000), suggesting that the requirements and roles for *Pitx2* isoforms are species-specific.

All three PITX2 isoforms have similar C-termini containing the homeodomain, encoded by exons 5 and 6; however, the N-terminus of each isoform is unique (Fig. 1.3B). PITX2A and PITX2B differ by a single N-terminal exon (exon 2), whereas the N-terminus of PITX2C is completely unique (Lamba et al., 2008). The differences between PITX2 N-termini are important for isoform-specific gene transactivation, as the unique N-termini have different abilities to synergize with co-regulator proteins (Amendt et al., 1998; Kioussi et al., 2002; Simard et al., 2009). This creates isoform-specific promoter sequence binding potentials, consistent with cell and tissue-dependent gene regulation capabilities unique to each isoform (Cox et al., 2002; Ganga et al., 2003). Although the

requirements for *Pitx2* are well studied, the tissue-specific requirements for each isoform are still under investigation.

Pleiotropic roles for Pitx2 in non-CNS tissues during development

Establishment of asymmetry

In embryonic tissues, *Pitx2* is necessary for proper development by regulating patterning, progenitor proliferation, cell migration, differentiation, and survival. *Pitx2* is critical for maintaining the left-right patterning of the embryo by acting downstream of a left-sided signaling cascade beginning in the lateral plate mesoderm (LPM) and ending with left-sided morphogenesis of organs such as the heart, lungs, and gut (Kitamura et al., 1999; Lin et al., 1999; Ryan et al., 1998; Shiratori et al., 2001). In the LPM, SHH induces *Nodal* expression at E8.0, where it is exclusively and temporarily expressed (Fig. 1.4A) (Pagan-Westphal and Tabin, 1998; Shiratori and Hamada, 2006). *Nodal* induces *Pitx2* expression in the LPM and is necessary for later *Pitx2* expression in left-sided organs (Brennan et al., 2002; Campione et al., 1999), although the mechanism by which *Nodal* in the LPM influences *Pitx2* expression during organ development is not known (King and Brown, 1997). Interestingly, the only *Pitx2* isoform expressed in left-sided organs is *Pitx2c*, which is required for left-sided morphogenesis of the developing heart, lungs, and foregut (Liu et al., 2001; Liu et al., 2002). Loss of *Pitx2c* in these organs results in right isomerization of the heart, right pulmonary isomerism, right isomerism of the lungs, and duodenal rotation defects (Liu et al., 2001). Therefore, *Pitx2a* and *Pitx2b* are dispensable whereas *Pitx2c* is necessary to mediate *Nodal* signaling from the LPM into proper morphogenic patterning of sided organs.

Proliferation

Pitx2 is necessary for progenitor proliferation in the pituitary, cardiac neural crest, liver, muscle, and gonads, and loss of *Pitx2* results in tissue hypoplasia (Baek et al., 2003; Kioussi et al., 2002; Rodriguez-Leon et al., 2008; Suh et al., 2002; Zhang et al., 2006). Conversely, overexpression of *Pitx2* causes pituitary hyperplasia (Kioussi et al., 2002). Of the three *Pitx2* isoforms, only *Pitx2c* has been implicated in proliferation. Loss of *Pitx2c* results in liver hypoplasia and *Pitx2c* overexpression in proliferating myoblasts

promotes an undifferentiated state and greater proliferative capacity by promoting expression of the cell cycle regulators *CyclinD1, CyclinD2, and c-myc* (Martinez-Fernandez et al., 2006; Wilting and Hagedorn, 2011; Zhang et al., 2006).

The canonical *Wnt* signaling cascade has been implicated in regulating *Pitx2*-mediated control of proliferation in myoblasts (Fig. 1.4B). Extracellular *Wnt* signaling induces activation of Dishevelled (DVL), which rescues β-Catenin from GSK-3β/Axin/APC complex-mediated proteasomal degradation (Pagan-Westphal and Tabin, 1998). β-Catenin is then free to translocate into the nucleus and associate with transcription factors. TCF/LEF targets, such as *Pitx2*, are then transactivated by removal of transcriptional inhibitors, such as histone deacetylases (HDACs), from the promoter (Briata et al., 2003; Kioussi et al., 2002). *Pitx2* then regulates cell proliferation by activating transcription of cell cycle genes (Baek et al., 2003; Kioussi et al., 2002). Additionally, *Pitx2* can bind the HuR protein to stabilize *Pitx2* and other target RNA transcripts, thereby creating a positive *Pitx2* feedback loop while promoting expression levels of target genes (Briata et al., 2003).

Migration

Pitx2 is also capable of regulating cellular migration during development. *Pitx2* is necessary for the migration of neural crest cells to the cardiac truncus (Kioussi et al., 2002), and is required in a dosage-dependent manner for migration of craniofacial and oral cellul populations (Kioussi et al., 2002; Liu et al., 2003). *In vitro, Pitx2* has been shown to control migration of HeLa cells through activation of Rho GTPase family members RAC1 and RHOA (Wei and Adelstein, 2002). PITX2A upregulates the guanine nucleotide exchange factor, *Trio,* which activates RAC1 and RhoA (Fig. 1.4C). Activated RAC1 and RHOA regulate actin-myosin cytoskeletal remodeling which influences cell spreading, cell-cell contacts, and migration (Logan et al., 1998; Shiratori and Hamada, 2006; Wei and Adelstein, 2002). However, the *in vivo* mechanisms of *Pitx2* migrational regulation have not been identified.

Differentiation

During and after migration, cells make terminal fate choices and undergo differentiation. *Pitx2* is implicated in the differentiation of several cell populations including gonadal cortical cells, follicular outer root sheath cells, and several types of muscle cells (Dong et al., 2006; L'Honore et al., 2010; Rodriguez-Leon et al., 2008; Shang et al., 2008; Sohn et al., 2009). In smooth muscle cells, *Pitx2* regulates cellular differentiation by promoting expression of a subset of muscle differentiation markers such as *SM-actin, SM22*, and *h1-calponin* by inducing promoter acetylation (Shang et al., 2008). Conversely, *Pitx2* is also capable of inhibiting differentiation, as *Pitx2c* expression keeps proliferating myoblasts in an undifferentiated, proliferative state (Martinez-Fernandez et al., 2006).

Maintenance and Survival

Pitx2 not only regulates differentiation, but is required to maintain cell fate choice and cell survival. In muscle cells, *Pitx2* is necessary to both promote muscle differentiation and maintain inhibition of bone differentiation programs by inhibiting *Osterix*, a bone-specific differentiation factor (Hayashi et al., 2008). Identification of requirements for *Pitx2* in cellular survival is complicated, as survival depends on proper cell autonomous and non-cell autonomous cues, developmental patterning, proliferation, migration, and differentiation, and loss of *Pitx2* may negatively affect one or more of these processes. For example, loss of *Pitx2* may simply disrupt local developmental signals, resulting in cell death. Thus, any role of *Pitx2* in cell survival may be coupled with its other developmental functions. In the extraocular muscles of the eye, *Pitx2* is required during a brief window (E10.5) for cell survival (Zacharias et al., 2011). Similarly, pituitary and craniofacial cells require *Pitx2* from E10.5-E11.5 for survival (Charles et al., 2005). Interestingly, *Pitx2* is not just important for cell maintenance, but may promote survival during times of cellular stress, as reduced *Pitx2* results in increased cell death in human trabecular meshwork cells exposed to the stressor hydrogen peroxide (Strungaru et al., 2011).

Combined, these studies indicate that *Pitx2* is required for left-right asymmetry, cellular proliferation, migration, differentiation, and survival during embryonic

development. Because a single gene (*Pitx2*) is capable of performing a variety of functions in a tissue- and time-dependent manner, this suggests *Pitx2* gene expression and PITX2 protein function are regulated by local tissue signals.

Roles for Pitx genes in the developing CNS

Patterning of the mouse midbrain and rhombomere 1 during development

The formation of the adult brain from the neural tube is extremely complex and requires intricate gene regulation. At E8.5, the neural epithelium folds to form the neural tube, which consists of a cell layer surrounding a lumenal space known as the ventricle. Neural progenitors lining the ventricle make up the ventricular zone. Early in mouse development (around E8-E8.5), neural progenitors undergo rapid symmetrical proliferation and at later stages (E10.5-E18) undergo neurogenesis, creating one progenitor and one immature neuron (Gotz and Huttner, 2005; Yingling et al., 2008). Newly born neurons begin to specialize as precursors and migrate away from the proliferative zone to more mature positions where they undergo differentiation.

The developing neural tube can be subdivided along the rostral-caudal axis into separate regions based on morphology and gene expression: the prosencephalon (forebrain), mesencephalon (midbrain), rhombencephalon (hindbrain), and spinal cord (Fig. 1.5). The hindbrain can be subdivided into rhombomeres (r1-r8), each distinguishable by gene patterning and morphology (Lumsden and Keynes, 1989). Each brain region is responsible for multiple processes, but overall the forebrain is involved in sensory/associative information processing and visceral functions, the midbrain acts a sensory/motor relay system, and the hindbrain is responsible for coordinating motor control and autonomic processes (Meredith and Stein, 1986; Saper, 2000; Wickelgren, 1971).

At the midbrain/hindbrain boundary is the isthmic organizer (IsO), a narrow ring of neuroepithelium that acts as a local organizing center to induce development and polarity of the midbrain and hindbrain through morphogen secretion. Before IsO establishment, the homeobox genes *Otx2* and *Gbx2* are expressed in the rostral and caudal neuroepithilia, respectively. *Otx2* and Gbx2 signaling is cross-repressive, thereby defining a sharp border between midbrain/hindbrain regions (Broccoli et al., 1999;

Katahira et al., 2000; Liu et al., 1999; Millet et al., 1999), whereby *Otx2* delineates the forebrain/midbrain and *Gbx2* identifies the hindbrain (Acampora et al., 1995; Burroughs-Garcia et al., 2011; Rhinn et al., 1998). The small region of overlapping *Otx2/Gbx2* expression is necessary for early midbrain/hindbrain identity and IsO localization/establishment (Li and Joyner, 2001). After IsO establishment, the IsO secretes WNT1 and FGF8 which induce gradients of gene expression and identity. FGF8 is capable of regulating the expression of mid/hindbrain patterning genes such as *Gbx2* and *Otx2* and is necessary and sufficient for inducing midbrain and r1 development in the neuroepithelium (Chi et al., 2003; Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999). Loss of the IsO or *Fgf8* signaling results in lack of midbrain/hindbrain development as well as increased cell death (Chi et al., 2003) (Meyers et al., 1998; Reifers et al., 1998). Thus, the IsO and its proper signaling are necessary for rostral-caudal patterning of the midbrain and hindbrain, as well as neuronal survival and maintenance.

Patterning of the neuroepithelium also occurs in a dorsal-ventral manner, whereby dorsal-ventral coordinates determine development and function of a given neuron. Very early signals for dorsal identity come from the epidermis (*Bmp4/7*), whereas ventral signals (*Shh*) are provided by the notochord (Echelard, 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994; Liem et al., 1995). The dorsal and ventral midbrain undergo two very different developmental programs. The dorsal midbrain, also known as the superior colliculus in mammals, develops into a layered structure, whereby newborn neurons migrate radially from the ventricular zone and then undergo tangential migration within their fated layer (Tan et al., 2002). The dorsal midbrain layers are important for processing different multisensory inputs from the retina, cortex, and spinothalamic pathways to promote saccades and head/limb movement (Garey et al., 1968; Lunenburger et al., 2001; Mehler et al., 1960; Sparks and Mays, 1990; Valverde, 1973).

The ventral midbrain controls limb movement, mediates locomotor coordination, and manages reward/stress responses (Feenstra et al., 1992; Le Moal and Simon, 1991; Sinkjaer et al., 1995). Whereas the dorsal midbrain relies on WNT signaling for dorsal identity and patterning, the ventral midbrain relies on SHH signaling from the floor plate to induce ventral patterning and repress dorsal identity (Dessaud et al., 2007; Placzek and

Briscoe, 2005; Watanabe and Nakamura, 2000). In the ventral midbrain, neurons migrate away from the ventricular zone in arcs (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Sanders et al., 2002). The arcs are patterned by a gradient of basal plate SHH expression which translates into unique combinations of transcription factor expression in each arc.

Much like the midbrain, r1 can be divided into dorsal and ventral regions. Dorsal r1 develops into the cerebellum, which is important for motor control and motor learning (Voogd and Glickstein, 1998). Ventral r1, which is part of the anterior pons, forms distinct populations of nuclei including the dorsal raphe nucleus, dorsal tegmental nucleus, locus coeruleus, laterodorsal tegmental nucleus, medial lemniscus, parabrachial nucleus, pedunculopontine nucleus, trochlear nucleus, and trigeminal nucleus (Waite et al., 2012). These nuclei are involved in various physiological functions including control of respiration, eye movement, head movement, and alertness (Berridge et al., 2012; Buttner-Ennever, 2006; Gray, 2008; Taube, 2007).

Whereas *Pitx3* is only expressed in the ventral midbrain, *Pitx2* is expressed in distinct populations throughout the CNS including the hypothalamus, superior colliculus and red nucleus in the midbrain, ventral r1, and in a subpopulation of V0 interneurons (the dorsal-most ventral population) in the spinal cord (Lindberg et al., 1998; Martin et al., 2004; Smidt et al., 2000; Waite et al., 2012; Waite et al., 2011; Zagoraiou et al., 2009). While *Pitx2* family members do not appear to be involved in rostral-caudal or dorsal-ventral patterning, *Pitx2* and *Pitx3* are required for other developmental processes in the developing CNS including proliferation and neuronal migration, differentiation, and axon tract formation.

Proliferation and Migration

In the developing CNS, both *Pitx2* and *Pitx3* are expressed in post-mitotic neurons (Martin et al., 2002b; Smidt et al., 1997). As such, they have no known cell autonomous roles in neural progenitor proliferation. However, early global knockout of *Pitx2* results in significantly reduced numbers of phosphohistone H3 (H3)-positive and BrdU-positive neurons in the midbrain (Waite et al., unpublished). This loss of H3 and BrdU-positive neurons may be attributable to non-tissue autonomous defects in the heart

and liver and hematopoietic dysfunction (Degar et al., 2001; Gage et al., 1999b; Kieusseian et al., 2006; Kitamura et al., 1999; Zhang et al., 2006). Unlike *Pitx2,* global loss of *Pitx3* does not appear to affect CNS populations beyond dopaminergic neurons of the substantia nigra pars compacta (SNpc), as *Pitx3* is not required for the development of vital organs and *Pitx3* null mice are viable (Lebel et al., 2001; Zagoraiou et al., 2009). Global or neural-specific loss of *Pitx2* results in mismigration of neurons in the subthalamic nucleus and midbrain, indicating that *Pitx2* is required for neuronal migration in a cell autonomous manner (Martin et al., 2004; Skidmore et al., 2008; Waite et al., unpublished).

Differentiation

In the dorsal midbrain, *Pitx2* is expressed late in a cascade of GABAergic differentiation factors around E12.5 (Fig. 1.4D). This cascade begins with *Helt,* a bHLH transcription factor expressed early in GABAergic midbrain progenitors that is required for GABAergic differentiation of midbrain neurons (Cazorla et al., 2000; Miyoshi et al., 2004; Nakatani et al., 2004). *Helt* cooperates with *Ascl1* to promote differentiation, and is required for the expression of the transcription factor *Gata2* in cells exiting the cell cycle (Kala et al., 2009). In turn, *Gata2* is necessary in neuronal GABAergic precursors to promote GABAergic over glutamatergic fates and for downstream *Pitx2* expression. *Pitx2*-null mice exhibit loss of GABA in midbrain *Pitx2*-expressing neurons, suggesting that *Pitx2* is required for GABAergic differentiation of a midbrain neuronal subpopulation (Waite et al., 2011). *Pitx2* is known to activate the *Gad1* promoter (Chen et al., 2011; Westmoreland et al., 2001) and may function as a GABAergic terminal differentiation factor in the midbrain *Helt* cascade.

Similar to *Pitx2, Pitx3* also acts as a neuronal terminal differentiation factor in a differentiation cascade downstream of *Wnt1, Lmx1a,* and possibly *Nurr1* (Chung et al., 2009; Friling et al., 2009; Volpicelli et al., 2012). PITX3 is required for the expression of *tyrosine hydroxylase* (*TH*)*, vesicular monoamine transporter 2* (*Vmat2)*, and *dopamine active transporter* (*DAT)*, which are responsible for dopamine synthesis, storage, and uptake, respectively (Alavian et al., 2008; Cazorla et al., 2000; Hwang et al., 2009; Lebel et al., 2001). Loss of *Pitx3*, such as in the *Aphakia* mouse, results in loss of dopaminergic

(TH-positive) neurons in the SNpc, although dopaminergic neurons in the ventral tegmental area and retrorubral field remain intact (Nunes et al., 2003; Smidt et al., 2004). Therefore, both *Pitx2* and *Pitx3* act as terminal differentiation factors in different neuronal subpopulations of the developing midbrain.

Axon Formation

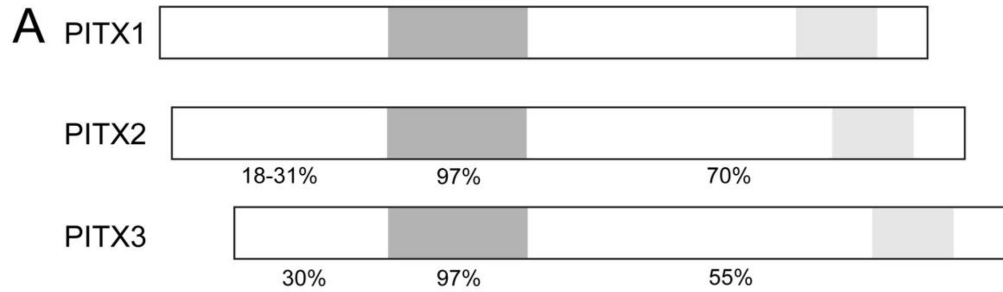
Pitx3 null mice have decreased dopaminergic projections from the substantia nigra to the striatum (nigrostriatal pathway) (Smidt et al., 2004). However, loss of dopaminergic projections is likely a by-product of deficient dopaminergic neuron development, as no studies have implicated *Pitx3* in axon formation. In contrast to *Pitx3, Pitx2* has been shown to regulate axon tract formation of the mammillothalamic tract (MTT) which is involved in self-movement cue processing and spatial memory (Kim et al., 2009; Vann and Aggleton, 2004; Vann et al., 2003; Winter et al., 2011). Normally, the MTT is visible at E18.0 as it branches rostrally from the principal mammillary tract (PMT) (Skidmore et al., 2012; Valverde et al., 2000). However, embryos with *Nestin-Cre* conditional knockout of *Pitx2* have a normal PMT but are missing the MTT (Skidmore et al., 2012).

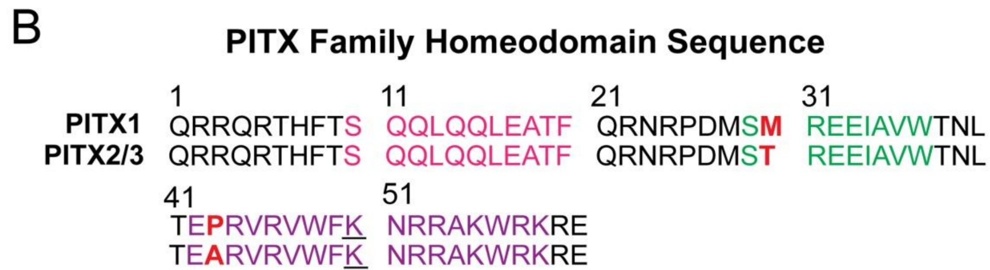
Survival/Maintenance

Pitx2 and *Pitx3* are expressed in the brain from embryonic development through adulthood, suggesting important roles in neuronal maintenance (Katunar et al., 2009; Peng et al., 2011; Smidt et al., 2000). However, *Pitx2* is not required for survival, as loss of *Pitx2* does not result in increased neuronal death (Martin et al., 2004; Waite et al., unpublished). It is unknown whether *Pitx2* is required for maintenance of mature neuronal identity. In contrast to *Pitx2, Pitx3* is required for survival of neurons in the SNpc. In *Pitx3* null mice, SNpc neurons begin to progressively die off after failing to terminally differentiate (Maxwell et al., 2005). This progressive loss of neurons continues through adulthood. Thus, even though *Pitx2* and *Pitx3* are both expressed through adulthood, they may have unique roles in adult neuronal maintenance.

Summary

The *Pitx* family is critical for proper embryonic development and viability. PITX proteins are required in a tissue- and temporal-specific manner and mutations in human *PITX* genes result in human genetic disorders. In the CNS, *Pitx2* is expressed in distinct neuronal populations in the forebrain, midbrain, hindbrain, and spinal cord. *Pitx2* is required in an isoform-specific manner for formation of the MTT in the forebrain and migration and differentiation of a midbrain GABAergic subpopulation. The requirements for *Pitx2* in the hindbrain and spinal cord have not yet been identified. Further characterization of brain region-specific requirements for *Pitx2* and the mechanisms by which *Pitx2* functions could greatly enhance our understanding of basic brain development at the molecular level. Additionally, identifying the consequences of *Pitx2* mutations in the brain could improve our knowledge of brain disorders, thereby improving diagnoses and therapies.





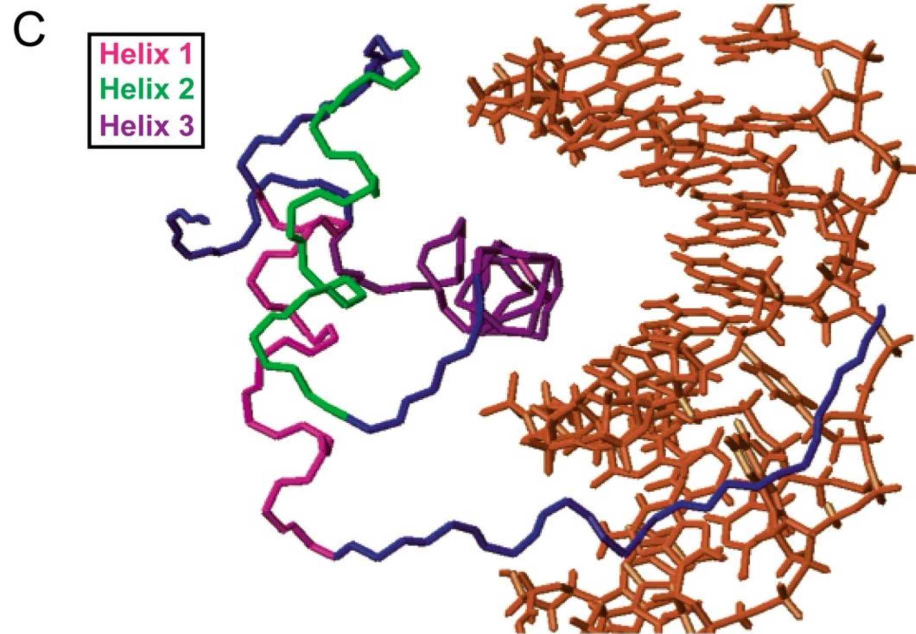


Figure 1.1. PITX transcription factor family. (A) Schema of PITX proteins comparing the percent amino acid sequence similarity of PITX2 and PITX3 to PITX1. Dark gray area represents the homeodomain, whereas light gray area represents the aristaless domain. Adapted from Gage et al., 1999a. (B) Alignment of amino acid sequences of PITX1 versus PITX2/3. Amino acids in pink, green, and purple font represent residues in helix 1, helix 2, and helix 3, respectively. Diverging amino acids are in red. Residue 50, which is responsible for DNA target recognition, is underlined. Numbers indicate the residue position within the helix. Adapted from Chaney et al., 2005. (C) Structure of the PITX2 homeodomain in complex with DNA. Helices in pink, green, and purple represent helix 1, helix 2, and helix 3, respectively. Amino acids not contributing to helices are shown in blue and the sample DNA is shown in orange. Adapted from Chaney et al., 2005.

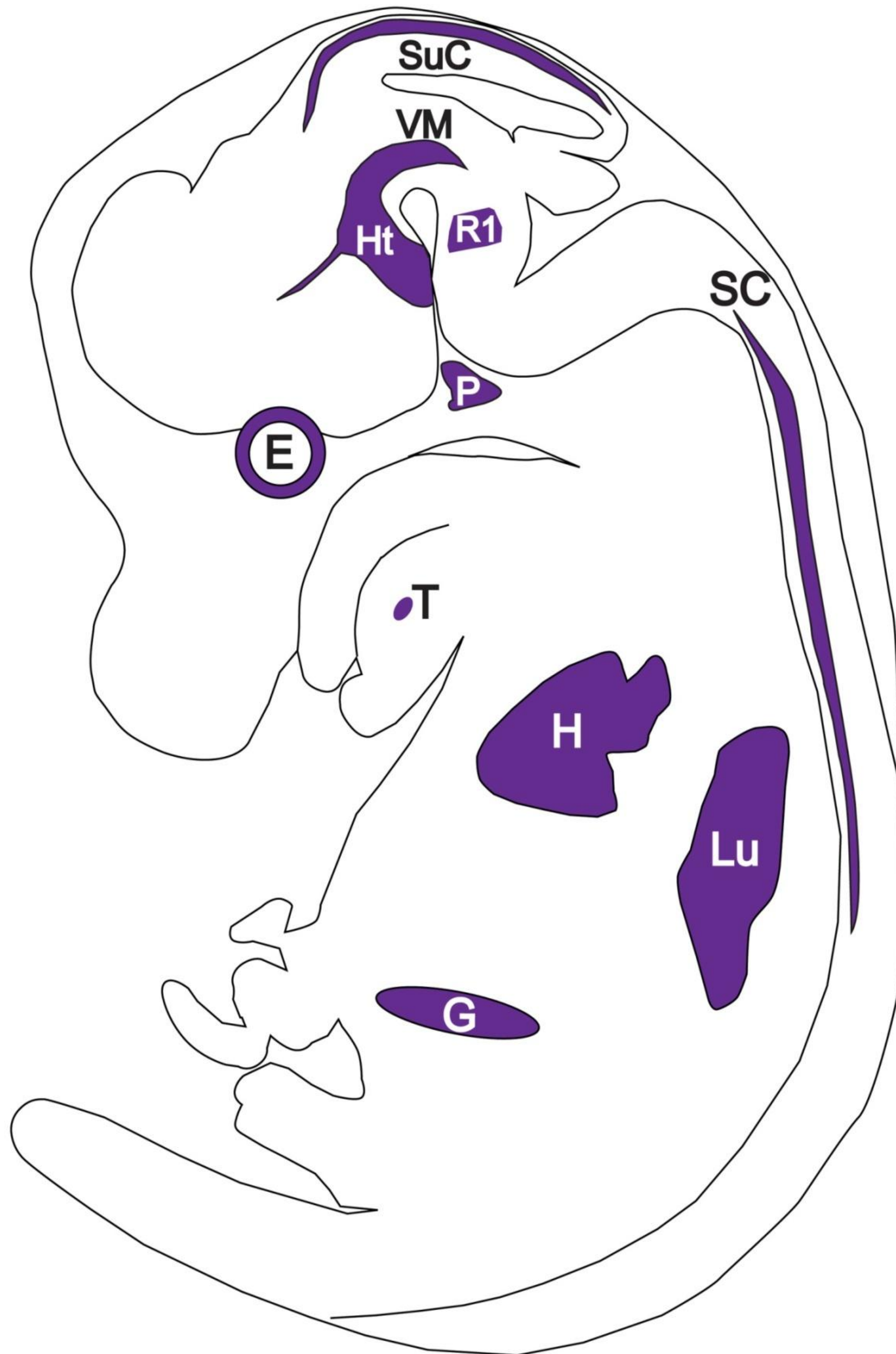


Figure 1.2. Map of *Pitx2* expression during mouse embryonic development. Cartoon of a sagittally-oriented mouse embryo with organs expressing *Pitx2* labeled in purple. Abbreviations: E, eye; G, gut; H, heart; Ht, hypothalamus; Lu, lungs; R1, rhombomere 1; SC, spinal cord; P, pituitary; SuC, superior colliculus; T, teeth; VM, ventral midbrain.

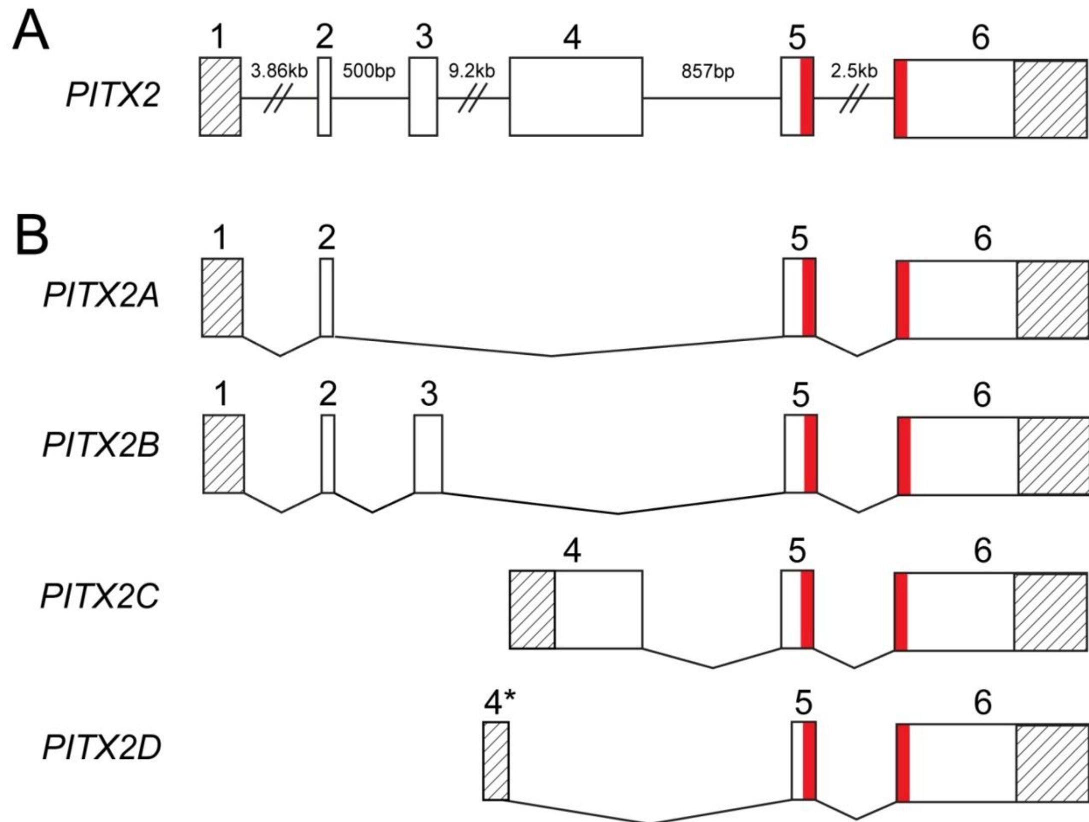


Figure 1.3. ***PITX2*** **gene and map of alternative splicing**. (A) Schematic of the *PITX2* gene showing introns and exons 1-6. Arrows indicate alternative start sites. Gray shaded boxes represent untranslated regions (UTRs) and red shading indicates the homeodomain. (B) Map of the exons utilized by different *PITX2* splice variants, *PITX2A, PITX2B, PITX2C,* and *PITX2D.* Exon 4* indicates upstream, alternative use of exon 4. Adapted from Cox et al., 2002; Liu et al., 2003.

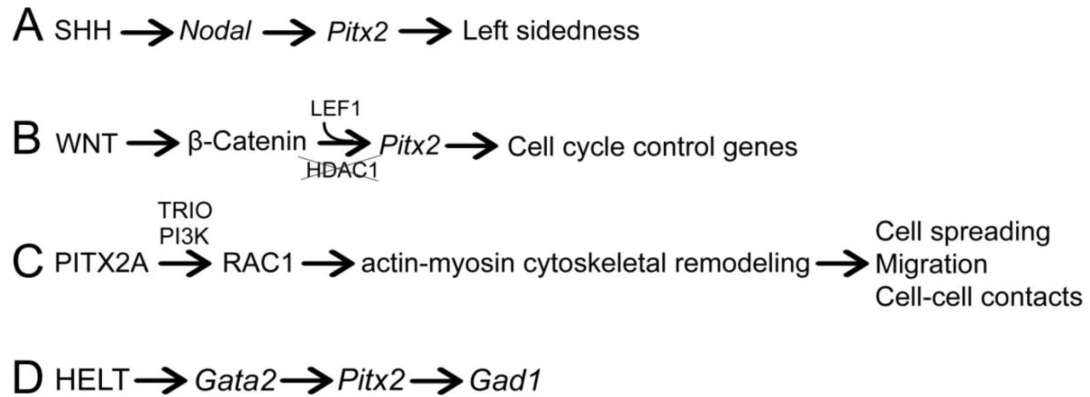


Figure 1.4. *Pitx2* in developmental pathways. (A) The SHH pathway induces *Nodal* expression, which induces *Pitx2* expression necessary for translation of left-sided patterning signals. (B) WNT induces stabilization of β-Catenin, thereby inducing expression *Pitx2* by removing HDAC1 from the *Pitx2* promoter and recruiting LEF1. PITX2 goes on to induce cell cycle control genes. Adapted from Kioussi et al., 2002. (C) PITX2A induces expression of *Trio* and activates the PI3K pathway, which induce RAC1 and RHOA activation, thereby inducing actin-myosin cytoskeletal remodeling leading to cell spreading, migration, and increased cell-cell contacts. (D) HELT induces expression of *Gata2*, which induces *Pitx2* expression, which can activate the *Gad1* promoter.

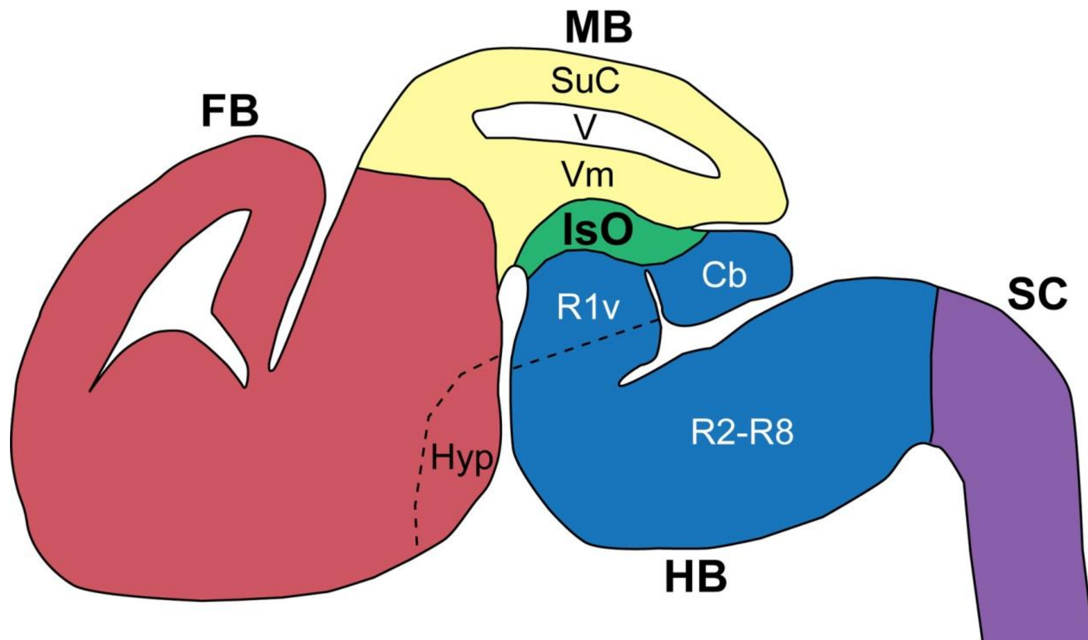


Figure 1.5. Sagittal E14.5 mouse CNS map. Schematic of a sagittally-oriented mouse embryonic CNS with major divisions marked in bold. Labeled areas mark regions pertinent to *Pitx2* in the CNS. Abbreviations: Cb, cerebellum; FB, forebrain; HB, hindbrain; Hyp, hypothalamus; IsO, isthmic organizer; MB, midbrain; R1v, ventral rhombomere 1; R2-R7, rhombomeres 2-8; SC, spinal cord; SuC, superior colliculus; V, ventricle; Vm, ventral midbrain. Adapted from (Allen Institute for Brain Science, 2009).

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Chapter II

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

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.

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.

Materials and Methods

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* (Skidmore et al., 2012). *Nestin-Cre;Pitx2*$^{tlz/+}$ mice were then bred to *Pitx2*$^{flox/flox}$ (Gage et al., 1999) mice to generate embryos for analysis.

Tissue Preparation

Timed pregnancies were established with the morning of vaginal 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.

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*.

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 under brightfield illumination. 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.

Results

Pitx2 is expressed in ventral r1 GABAergic neurons

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. 2.1C,D) and *Fgf8*-positive isthmic organizer (Fig. 2.1E,F) and caudally by *Hoxa2*-expressing r2 (Fig. 2.1G-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. 2.1A). By E12.5, all *Pitx2*-positive cells were in a single population in mid-r1 (Fig. 2.1K). At this timepoint, r1 continued to be bordered rostrally by the *Otx2*-positive midbrain and *Fgf8*-positive isthmic organizer (Fig. 2.1L,M) and caudally by the *Hoxa2/Gbx2*-positive r2 (Fig. 2.1N,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. 2.2B-C'). Importantly, most GAD67-GFP-positive neurons in r1 were also positive for GABA immunoreactivity (Fig. 2.2D,D'), confirming that GABA is an accurate marker of GABAergic neurons in this tissue. PITX2-positive cells were negative for VGLUT2 immunofluorescence (Fig. 2.2E,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. 2.2F), 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. 2.2F) (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. 2.3 and 2.4). 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. 2.3A-C'). At E12.5 there was no significant co-expression of PITX2 and EN1 in ventral r1 cells (Fig. 2.3D-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. 2.3G-I' and Fig. 2.4Q), 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. 2.3J-L') and some were also positive for GABA (Fig. 2.3A-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. 2.3M-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. 2.4B). 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. 2.4C). The PITX2-positive neurons were located between these two groups of LMX1B-positive cells (Fig. 2.4C).

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. 2.4D). The lack of ISL1 co-localization with

PITX2 suggests that PITX2-positive cells do not contribute to the r1-derived trochlear nucleus (see Fig. 2.9).

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. 2.4E). PAX7, a paired-like transcription factor, is widely expressed in ventral r1, but did not mark PITX2-positive cells (Fig. 2.4F). 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. 2.4G). LBX1 marked a distinct population of neurons dorsal to the PITX2-expressing population (Fig. 2.4H). 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. 2.4I-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. 2.4I-J and Fig. 2.3H,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. 2.4M,N). Quantitative analysis showed that 27.0% (+/-3.3%) of PITX2-positive cells were NKX6.1-positive (Fig. 2.4Q). 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. 2.4O). We also observed 61.6% (+/-6.8%) overlap between PITX2-positive cells and SOX2 (Fig. 2.4P). 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. 2.4R,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. 2.5A,B). Like the E12.5 PITX2-positive population, PITX2- positive E11.5 cells were also positive for LHX1/5, PAX2, and NKX6.1 (Fig. 2.5C-E). Again, PAX2 and NKX6.1 marked separate r1 populations with PAX2-positive cells medially bordering the NKX6.1-positive population (Fig. 2.5F-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. 2.5G-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.

Pitx2 is not required for early patterning or differentiation

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. 2.6A,B) as determined by *in situ* hybridization using a cRNA probe that is expressed from both the wild type and null alleles (Suh et al., 2002). 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. 2.6) or wild type (Fig. 2.4) 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. 2.7B). 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. 2.7D-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. 2.7I-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. 2.7J-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. 2.7K-K'). PITX2-lineage E18.5 neurons did not express the transcription factor EN1 (Fig. 2.7L) similar to results at E12.5 (Fig. 2.3D-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. 2.4O and Fig. 2.7M). 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. 2.7N,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. 2.8B-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.

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. 2.9. 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. 2.9). 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.

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).

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.

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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Chapter II Notes

[1]A revised version of Chapter II has been published as Waite, M.R., Skaggs, K., Kaviany, P., Skidmore, J.M., Causeret, F., Martin, J.F., and Martin, D.M. (2012). Distinct populations of GABAergic neurons in mouse rhombomere 1 express but do not require the homeodomain transcription factor PITX2. *Molecular and Cellular Neuroscience* Jan;49(1):32-43.

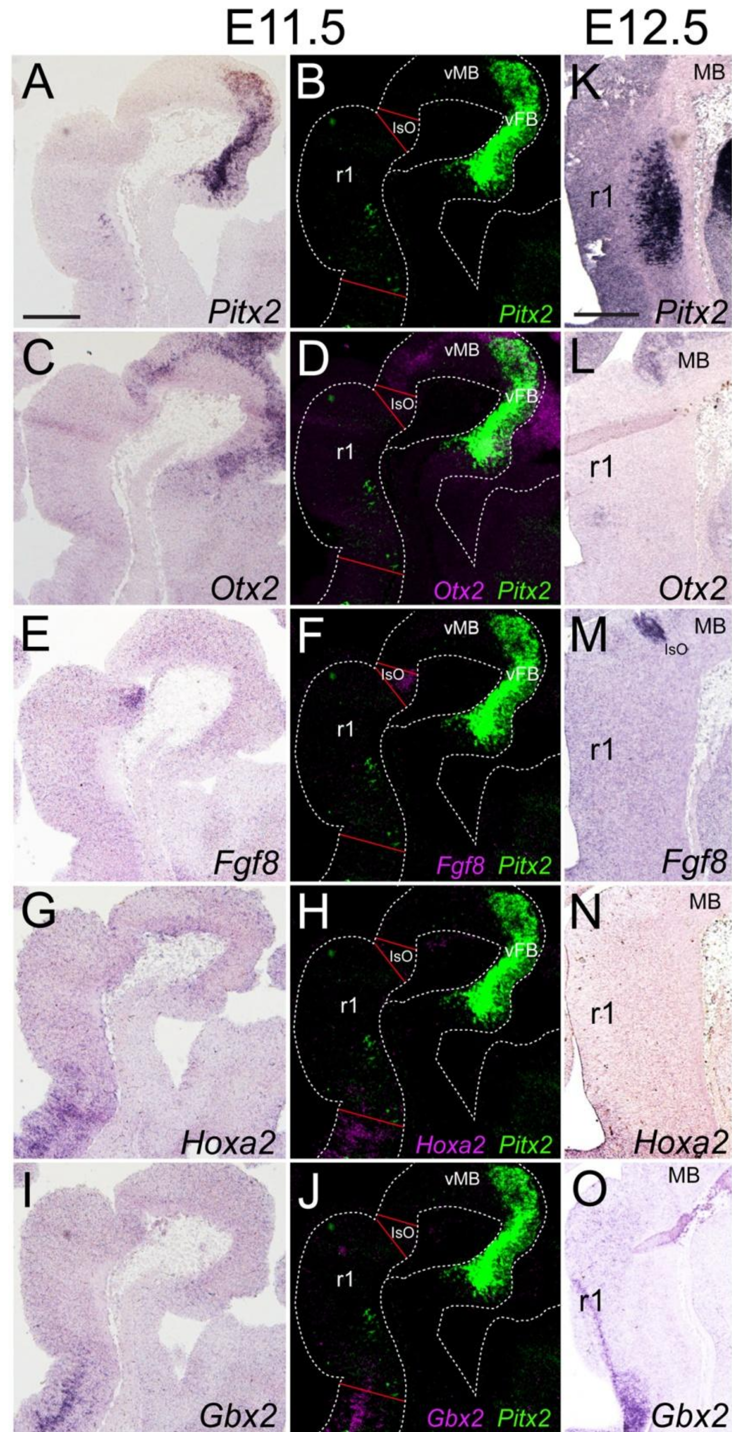


Figure 2.1. ***Pitx2*-expressing cells localize to rhombomere 1.** E11.5 (A-J) or E12.5 (K-O) sagittal sections processed for (A,K) *Pitx2,* (C,L) *Otx2,* (E,M) *Fgf8,* (G,N) *Hoxa2, or* (I,O) *Gbx2* single *in situ* hybridization. Pseudocolored and merged images of neighboring slides processed for *in 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.

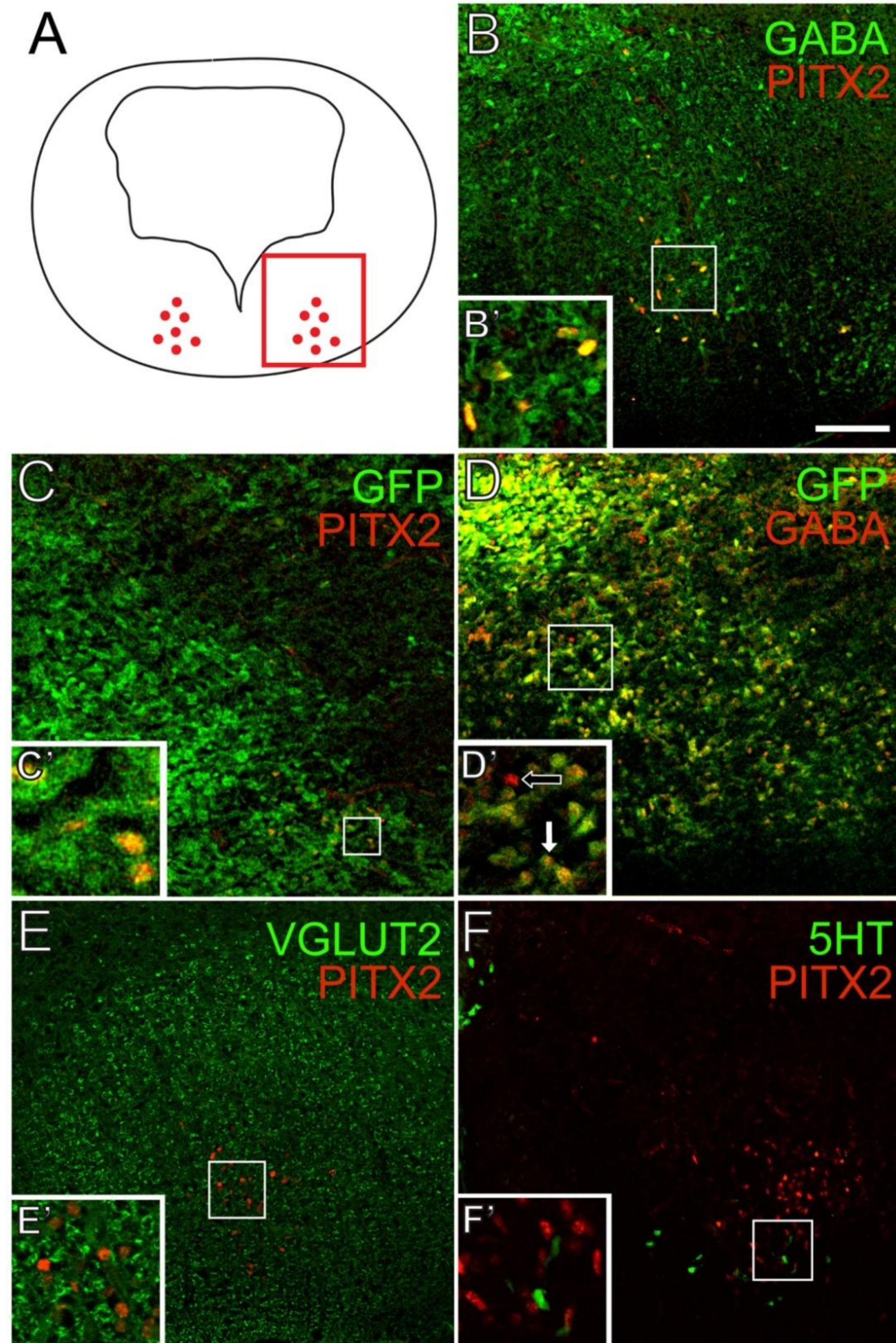


Figure 2.2. PITX2-positive GABAergic neurons in r1 are distinct from serotonergic, glutamatergic, and cholinergic neurons. Double immunofluorescence of E14.5 mouse brain tissues sectioned transversely at the level of r1. Schematic in A indicates transverse orientation for panels B-F. Square inset in 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.

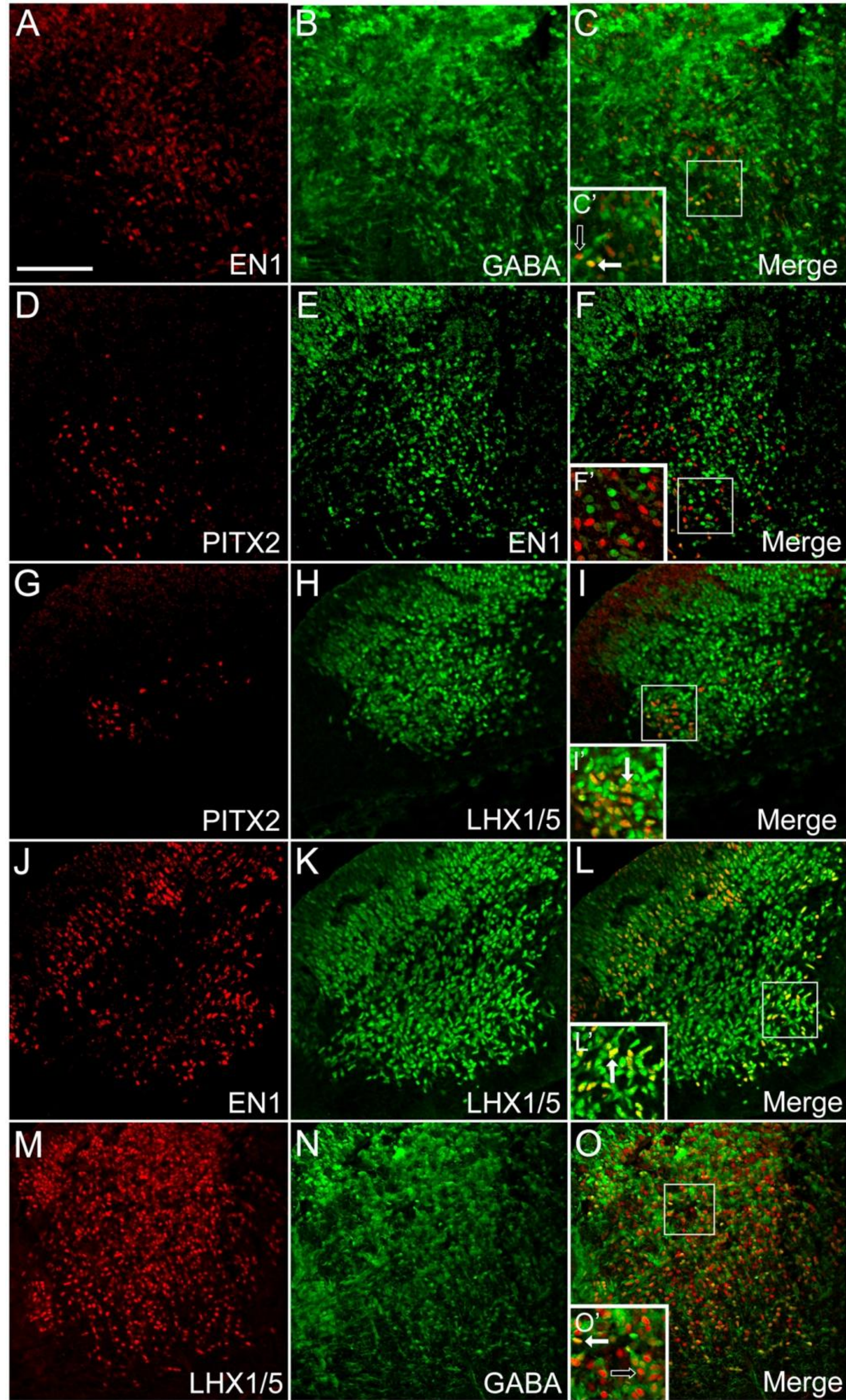


Figure 2.3. GABAergic r1 neurons express PITX2, EN1, and LHX1/5. Double immunolabeling of E12.5 mouse brain tissues sectioned transversely at the level of r1 (orientation as in Figure 2.2A) reveals distinct patterns of overlap among GABA and several transcription factors. Insets in C-O are enlarged in 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.

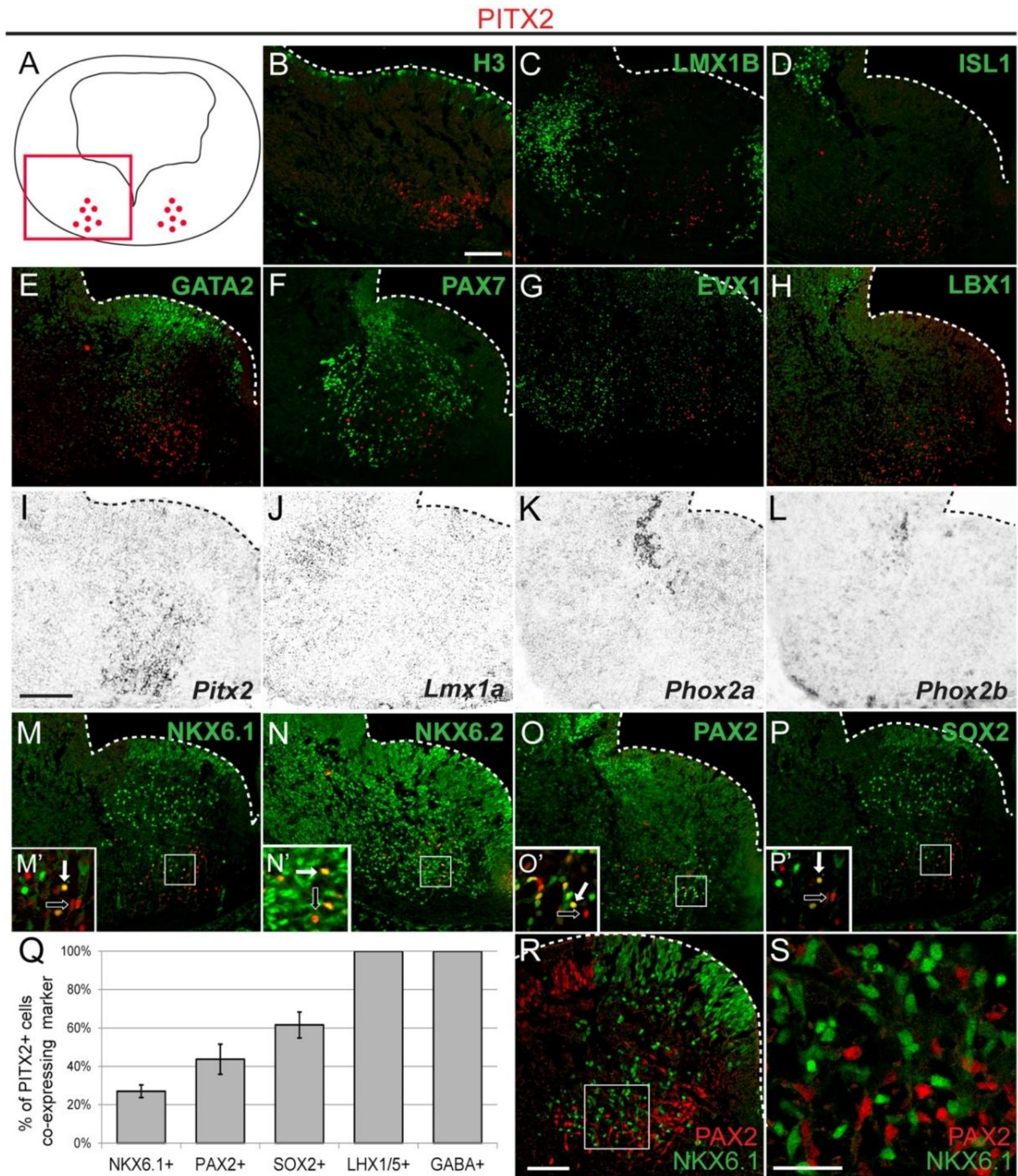


Figure 2.4. PITX2-positive GABAergic neurons occupy distinct regions of the ventral hindbrain. Double immunolabeling (A-H, M-P, R, S) or *in situ* hybridization (I-L) of E12.5 mouse brain tissues sectioned transversely at the level of r1 (orientation as in panel A) reveals distinct patterns of overlap 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.

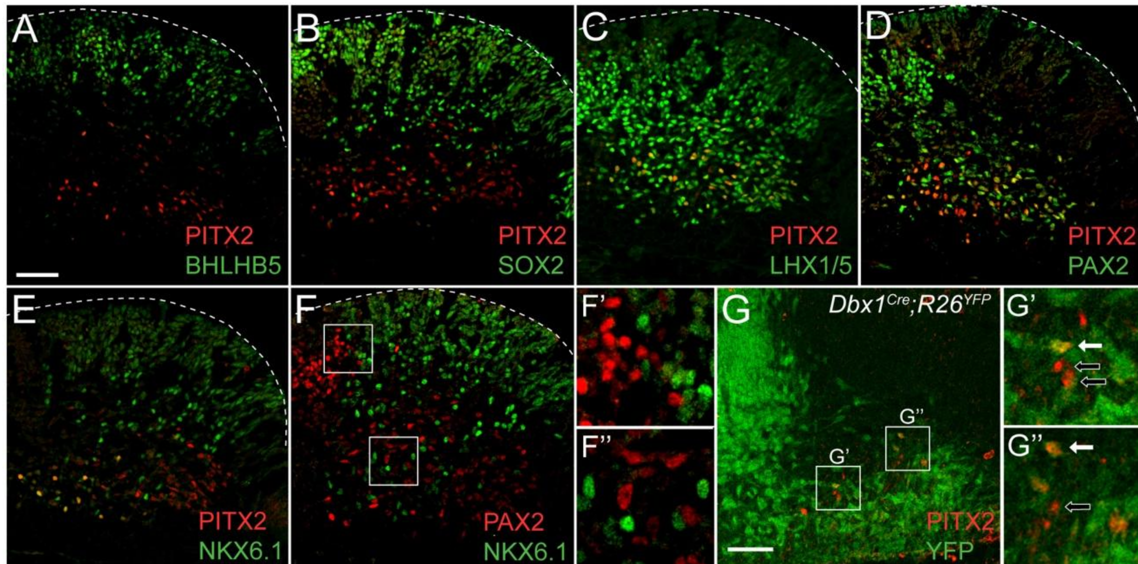


Figure 2.5. Early expression of PITX2 is similar to E12.5 patterning and includes *Dbx1*-lineage cells. Immunolabeling of E11.5 (A-G'') *Pitx2*$^{+/+}$ or *Dbx1*^{Cre};*R26*^{YFP} embryos sectioned transversely at the level of r1 (orientated as in Figure 2.4A). (A-E) E11.5 PITX2-positive cells in r1 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.

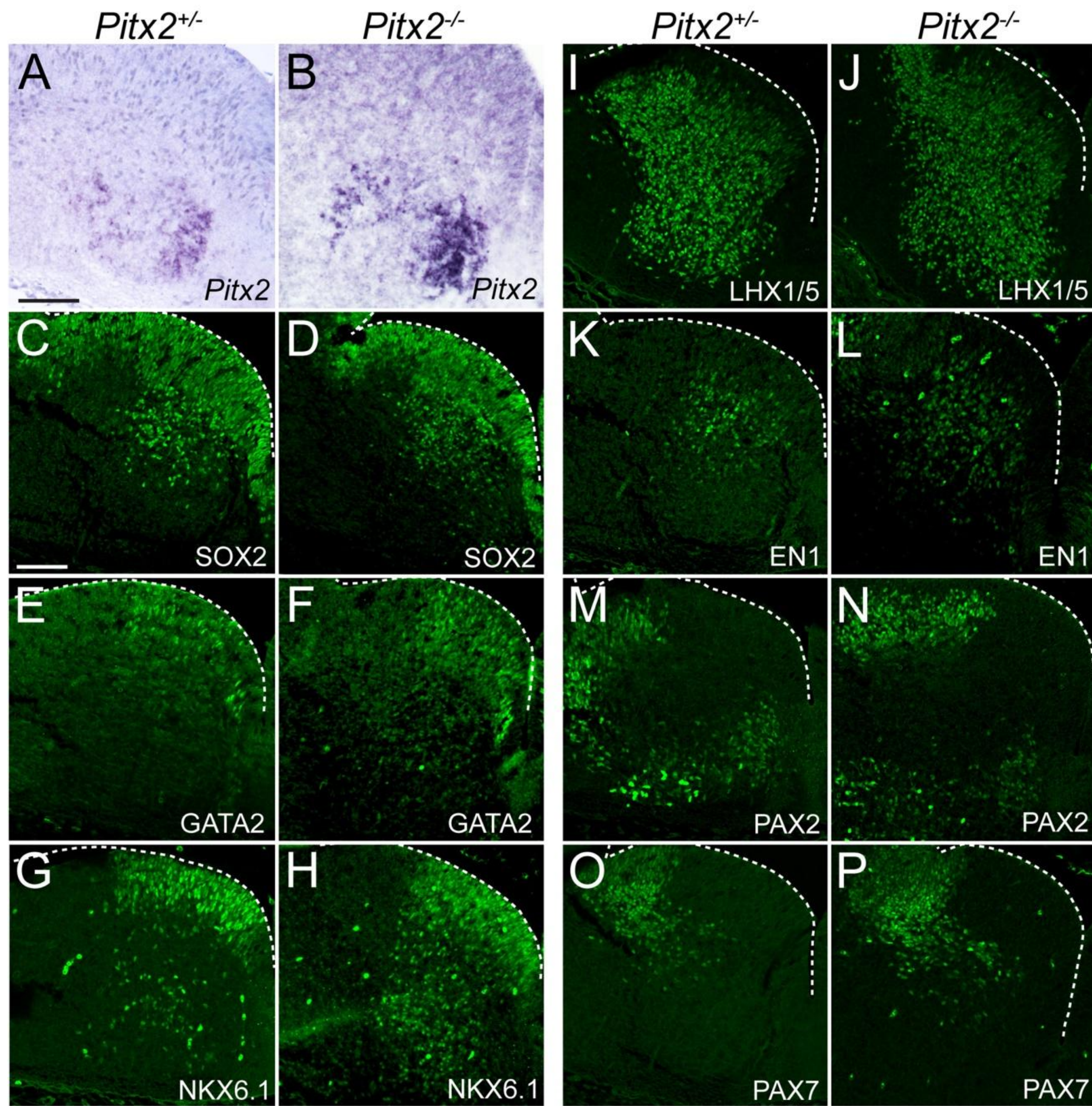


Figure 2.6. PITX2 is dispensable for early neuronal migration and ventral r1 patterning. *In situ* hybridization (A-B) or immunolabeling (C-P) of E12.5 *Pitx2*$^{+/-}$ and *Pitx2*$^{-/-}$ mouse brain tissues sectioned transversely at the level of r1 (orientation as in Figure 2.4A) reveals normal transcription 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.

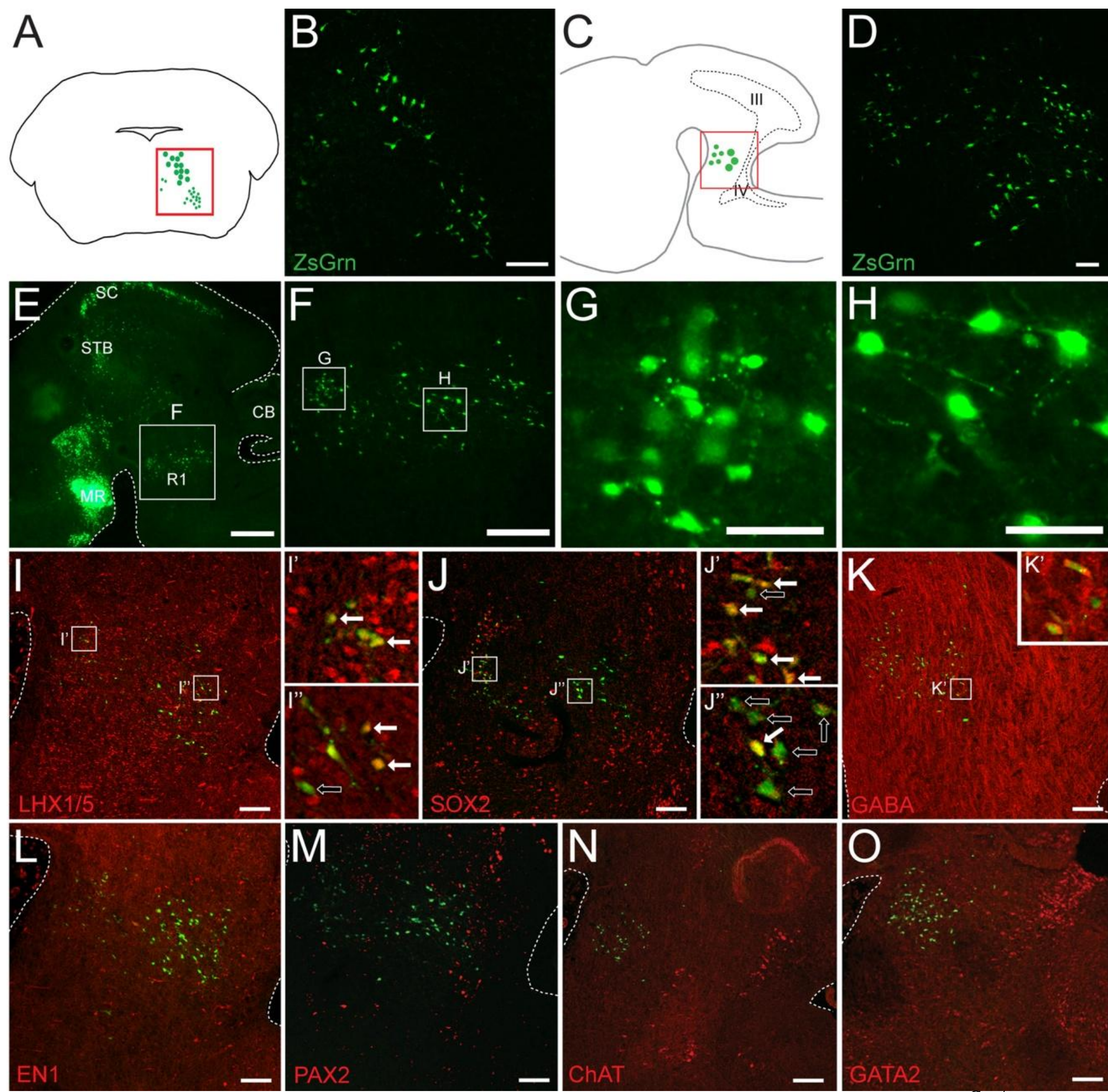


Figure 2.7. Two PITX2-positive populations span ventral r1. E18.5 *Pitx2*$^{Cre/+}$*;ZsGrn* transverse (B) and sagittal (D-O) sections processed for immunofluorescence with antibodies against transcription factors, ChAT, and GABA in the hindbrain. (A) Schematic of a transverse section identifying the 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.

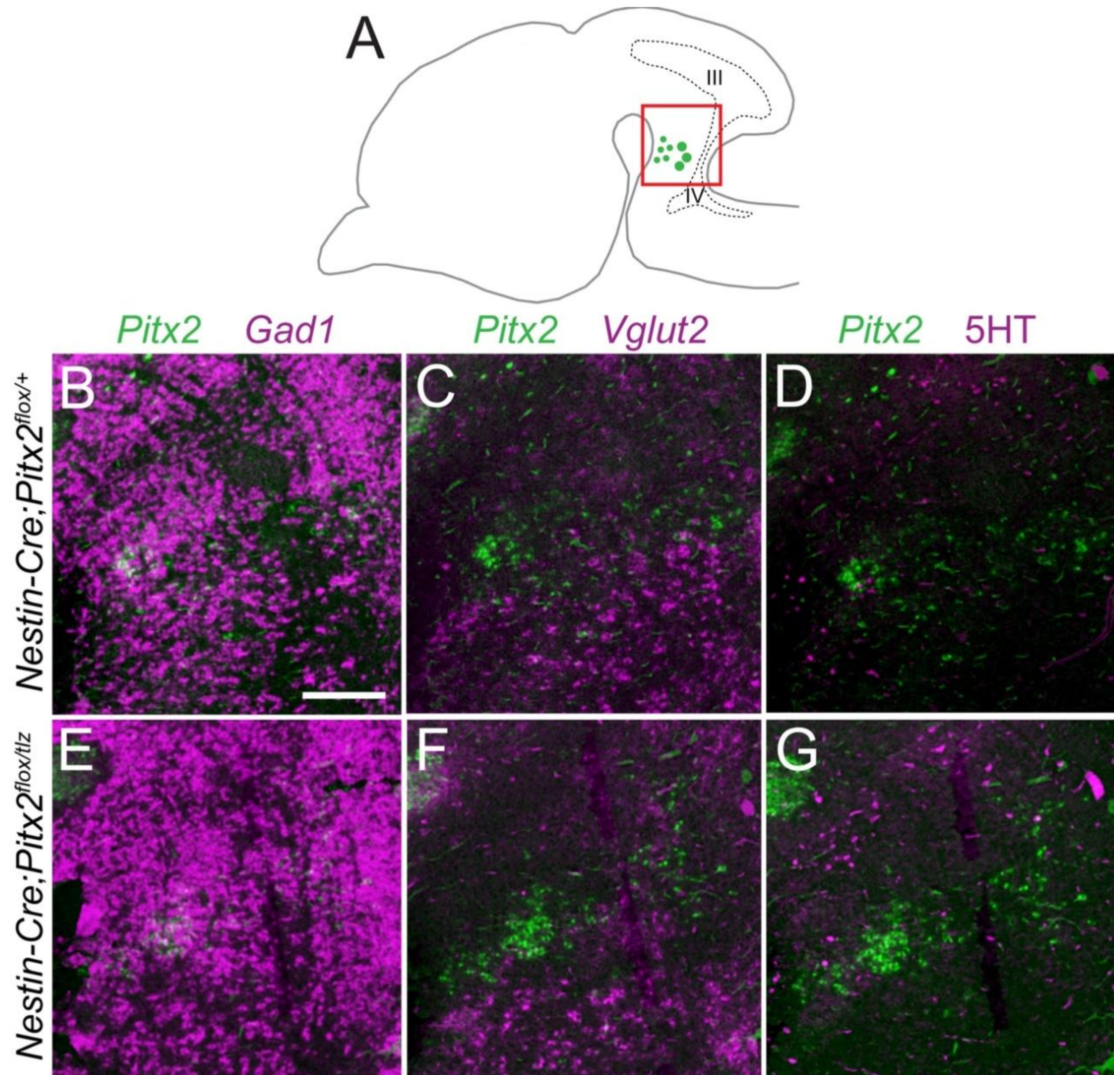
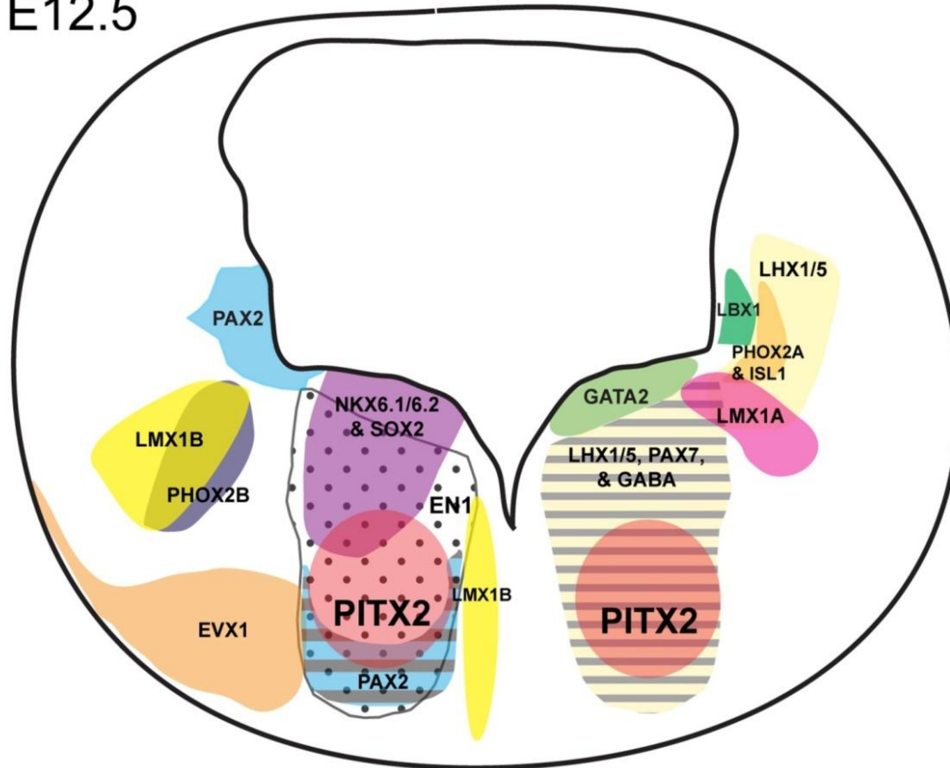


Figure 2.8. Ventral r1 GABAergic identity is PITX2-independent. Sagittal E18.5 *Nestin-Cre;Pitx2*$^{flox/+}$ and *Nestin-Cre;Pitx2*$^{flox/tlz}$ littermate brains processed for single *in situ* hybridization for *Pitx2, Gad1*, or *Vglut2* or immunohistochemistry with antibodies against 5-HT. (A) Cartoon 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.



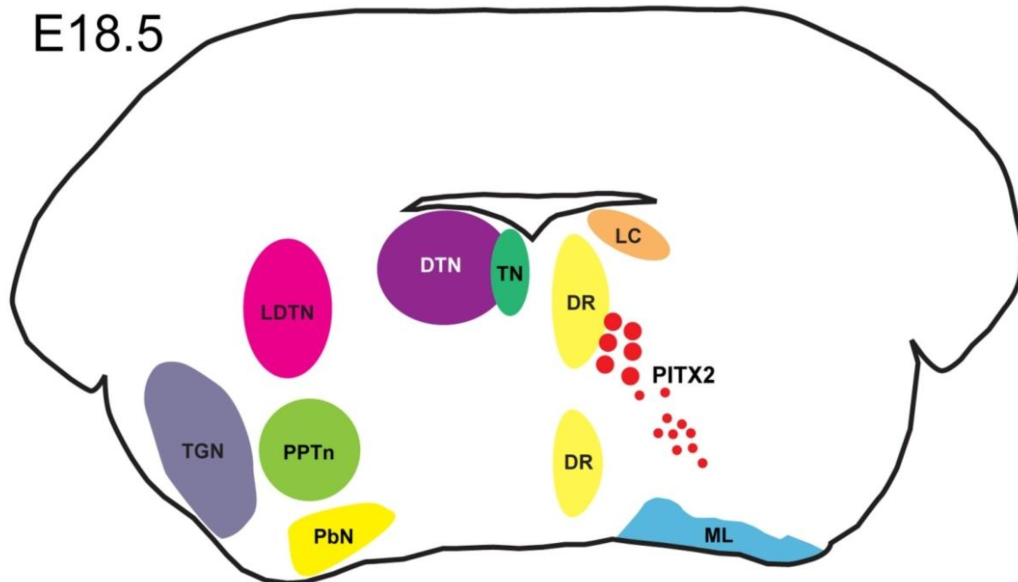


Figure 2.9. Schematic of transverse sections through the developing mouse brain at the level of rhombomere 1. (A) E12.5 transverse section through r1 showing transcription factor and GABA patterning in relation to PITX2. (B) E18.5 transverse section through r1 showing nuclei patterning based on "Atlas 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.

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Chapter III

GABAergic and glutamatergic identities of developing midbrain *Pitx2* neurons[1]

Abstract

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 which promote GABAergic and glutamatergic differentiation.

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 (Meredith and Stein, 1986; Wickelgren, 1971). 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 (Kala et al., 2009; Ono et al., 2007). 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 (Garey et al., 1968; Mehler et al., 1960; Valverde, 1973). These inputs are important for movement of the head and limbs in response to stimuli, attention, and mediating saccades (Kustov and Robinson, 1996; Lunenburger et al., 2001; Sparks and Mays, 1990). 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 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 (Feenstra et al., 1992; Le Moal and Simon, 1991; 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, 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 (Andersson et al., 2008; Bayly et al., 2007), 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 (Kala et al., 2009; Ono et al., 2007), 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.

Materials and Methods

<u>Mice</u>

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 the Jackson Laboratory 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 vaginal plug identification designated as E0.5. Pregnant dams were euthanized using cervical dislocation. Embryos were fixed in 4% paraformaldehyde for 30 minutes-2 hours 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, USA), 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 minutes to 3 hours. P8 pups were anesthetized as described above and perfusion fixed. Brains were removed and fixed at 4°C for 3 hours 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, USA) for cryosectioning. Frozen sections were post-fixed with 0.5% glutaraldehyde fixative, washed in X-Gal Wash Buffer, and stained with X-Gal Staining Solution overnight at 37° as previously described (Sclafani et al., 2006). Slides were washed in PBS and X-Gal Wash Buffer, eosin counterstained and mounted with Permount (Fisher, Waltham, MA).

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; Martin et al., 2004) with rabbit anti-PITX2 at 1:8000 (Zagoraiou et al., 2009), rabbit anti-PITX2 at 1:4000 (Capra Science, Ängelholm, Sweden), guinea pig anti-BRN3A at 1:400 (Fedtsova and Turner, 1995), rabbit-anti VGLUT2 at 1:1000 (Millipore), rabbit anti-GABA at 1:1000 (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) 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; Martin et al., 2004) using a cRNA probe for *Pitx2*.

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 and markers of specific neurotransmitters. In the E14.5 superior colliculus, most PITX2-positive cells were positive for GABA (Fig. 3.1C-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. 3.1F-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. 3.1I-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. 3.1L-N’). PITX2-positive cells were located superficial to LHX1/5 positive cells (Fig. 3.1O-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. 3.1R) and *Pitx2*-expressing cells were identified within the intermediate AChE positive layer (Fig. 3.1S,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. 3.2A-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. 3.2D-F’). This is consistent with known glutamatergic afferents projecting to the SGI including cortico-collicular, retino-collicular, and colliculo-collicular pathways (Mize and Butler, 1996; Olivier et al., 2000; Woo et al., 1985). 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; Ono et al., 2007). BRN3A-positive cells were distributed throughout the E14.5 intermediate and medial superior colliculus (Fig. 3.2G-I’). At P8, BRN3A-positive nuclei were tightly associated with VGLUT2-positive label throughout the colliculus (Fig. 3.2J-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. 3.2M-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. 3.2P-R). Thus, collicular layers can be identified by unique transcription factor patterns.

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. 3.3A). At E14.5, GATA2 was restricted to cells located superficial to the ventricular zone and did not co-localize with PITX2 (Fig. 3.3B). At E12.5, LHX1/5-positive cells were localized to the mantle zone, more superficially than GATA2, and found just deep to PITX2-positive neurons (Fig. 3.3C). At E14.5, LHX1/5-positive cells extended from the ventricular zone to the sub-pial surface (Fig. 3.3D). At both E12.5 and E14.5, only a few cells were positive for both PITX2 and LHX1/5 (Fig. 3.3C',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 deep to PITX2-positive cells, whereas at E14.5 BRN3A was expressed throughout the ventral and intermediate colliculus (Fig. 3.3E,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 (Thompson et al., 2004; Thompson et al., 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. 3.3G-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. 3.4A,A'). In *Pitx2*$^{Cre/-}$*;NL* midbrains, βGAL-positive cells were medially mislocalized in a GABA-poor layer and were GABA-negative (Fig. 3.4B,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. 3.4C-F, Fig. 3.11). Additionally, the GABAergic precursor markers GATA2 and LHX1/5 and the glutamatergic precursor marker BRN3A were correctly localized in *Pitx2*$^{Cre/-}$*;NL* midbrains (Fig. 3.4G-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. 3.5A-I). In the m1-m5 domains, LHX1/5-positive neurons become GABAergic, whereas in the m6 domain, LHX1/5-positive cells become glutamatergic (Ono 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. 3.5J-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. 3.5M-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 and m4 post-mitotic cells (Ono 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. 3.6A). *Nkx2.2* and *Gata2* are expressed in m5 GABAergic progenitors and precursors, respectively (Joksimovic et al., 2009; Kala et al., 2009; Ono et al., 2007). Neither NKX2.2 nor GATA2 co-localized with PITX2 (Fig. 3.6B,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. 3.6D).

To determine whether PITX2 is necessary for early transcription factor patterning of the ventral midbrain, we compared gene expression in E12.5 $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ littermate midbrains (Fig. 3.7). Transcription factor patterning in the midbrain was similar in $Pitx2^{Cre/+}$ and wildtype embryos (Fig. 3.5). BRN3A (green) was dispersed throughout the ventromedial zone and red nucleus in both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ midbrains (Fig. 3.7A-J). FOXA2 was present in the ventricular zone, ventromedial population, and the red nucleus and was unchanged in $Pitx2^{Cre/-}$ embryos (Fig. 3.7A,B). LHX1/5-positive cells were distributed throughout the ventromedial population and the red nucleus in both $Pitx2^{Cre/+}$ and $Pitx2^{Cre/-}$ midbrains (Fig. 3.7C,D). Loss of PITX2 did not affect the localization of NKX6.1 cells, which are BRN3A-negative (Fig. 3.7E,F). The oculomotor nucleus marker, ISL1, also appeared normal in $Pitx2^{Cre/-}$ midbrains (Fig. 3.7G,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. 3.7I,I'); this pattern of expression was unchanged in $Pitx2^{Cre/-}$ embryos (Fig. 3.7J,J'). Transcription factor patterns also appeared unchanged in $Pitx2^{Cre/-}$ mutants in the rostral-caudal plane (Fig. 3.11). 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. 3.8A), in contrast with the E12.5 PITX2-positive red nucleus (Fig. 3.5). At E14.5,

the few ventral PITX2-positive cells were also BRN3A-positive, suggesting that these neurons become glutamatergic (Fig. 3.8B-C'). In contrast, most GABA immunofluorescence was localized to the m5 domain and was not present in PITX2-positive cells (Fig. 3.8D-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 staining in midbrain red nucleus neurons during development (Fig. 3.8F).

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. 3.8G-I'). We also identified co-localization between βGAL and BRN3A in the red nucleus of *Pitx2*$^{Cre/+}$*;NL* embryos (Fig. 3.8J-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 (Kele et al., 2006; Prakash and Wurst, 2006; Sanders et al., 2002). Our studies on E12.5 and E14.5 ventral midbrains suggested that PITX2 may be downregulated in the red nucleus during development (Fig. 3.5M-U, 3.8A-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. 3.9A,E,I). *Pitx2* mRNA expression was maintained in the superior colliculus, red nucleus, and ventromedial populations through E18.5 (Fig. 3.9C,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. 3.9D,H,L).

Previous studies showed that *Pitx2* 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 cells in the ventromedial population and red nucleus (Fig. 3.9M,N), even though neighboring sections showed significant *Pitx2* mRNA expression in both populations (Fig. 3.9O,P). At E12.5, many PITX2-positive cells in the red nucleus can be identified by immunofluorescence (Fig. 3.5), 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 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 (Kala et al., 2009; Ono et al., 2007). 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. 3.10). We identified PITX2-positive cells in all seven midbrain domains, with strongest expression in the m1-m4 and m6 domains (Fig. 3.10A,B). In m1-m2 domains, *Pitx2* is co-expressed with *Lhx1/5* and GABA (Fig. 3.10B) 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. 3.10C,D). In the m6 domain, *Pitx2* is co-expressed with *Lhx1/5, Brn3a, Foxa2, Nkx6.2,* and *Vglut2* (Fig. 3.10B,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 which 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 (Kala et al., 2009; Miyoshi et al., 2004). 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 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.

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Chapter III Notes

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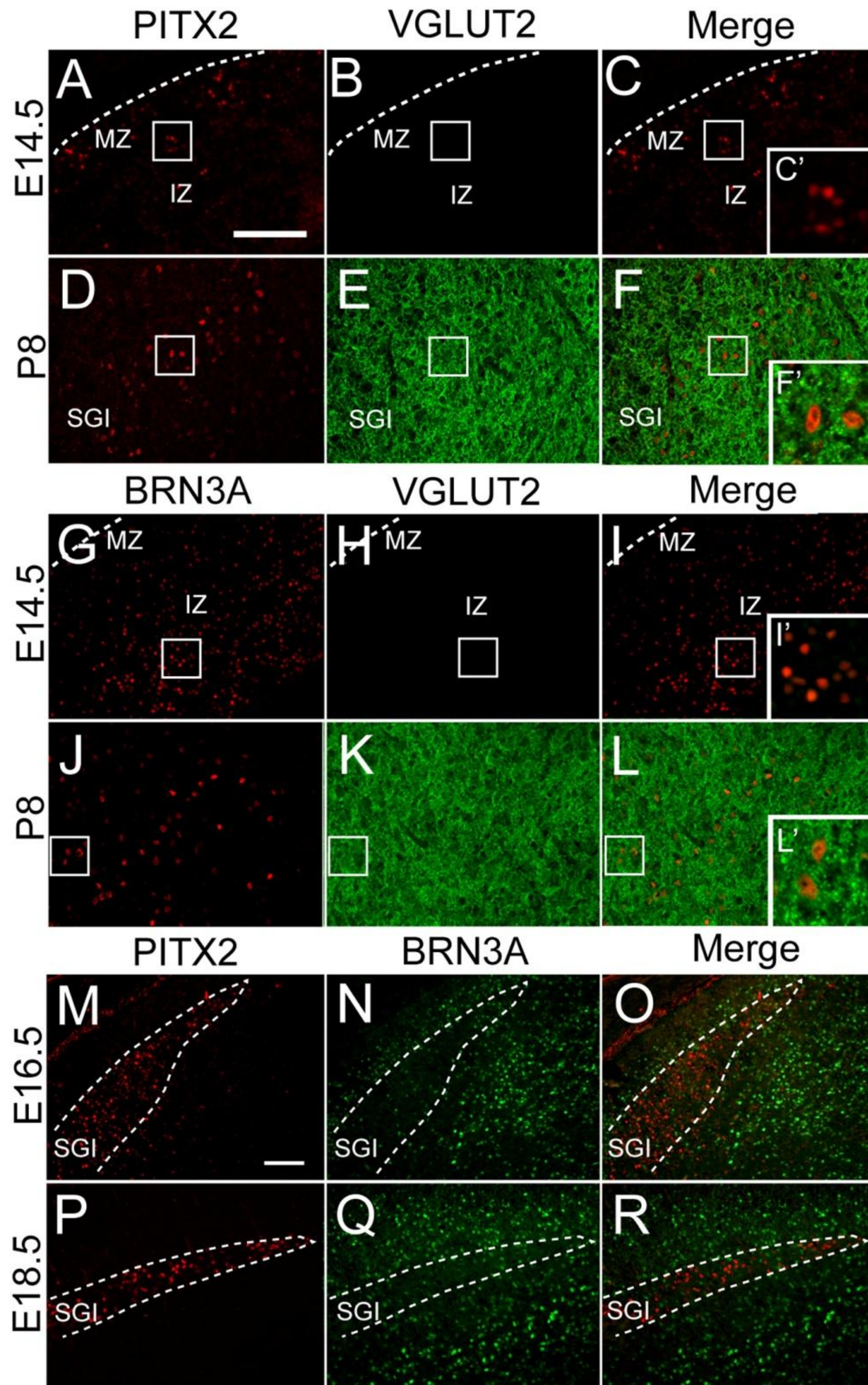
Figure 3.1. PITX2 identifies GABAergic interneurons in an intermediate layer of the dorsal midbrain. (A) Cartoon showing sagittal view of an embryonic mouse brain with midbrain highlighted in pink and a dotted line indicating the location of coronal sections shown in C-T. (B) Cartoon of a coronal midbrain 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). Boxes in E and H are enlarged 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. Boxes in K and N are enlarged 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 is 100 µm and applies to panels C-E and I-K. Scale bar in F is 100 µm and applies to panels F-H and L-Q. Scale bar in R is 250 µm and applies to panels R-T. Figures in L-N' were imaged using confocal microscopy. Abbreviations: mantle zone (MZ); intermediate zone (IZ); stratum griseum superficiale (SGS); stratum opticum (SO); stratum griseum intermedium (SGI); stratum album intermedium (SAI).

Figure 3.2. 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 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 is 100 µm and applies to panels A-C and G-1. Scale bar in D is 100 µm and applies to panels D-F and J-L. Scale bar in M is 100 µm and applies to panels M-R. Panels D-F' and J-L' were imaged using confocal microscopy. Abbreviations: mantle zone (MZ); intermediate zone (IZ); stratum griseum intermedium (SGI).


PITX2
VGLUT2
Merge
E14.5
A
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SGI
E
SGI
F
SGI
F'
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VGLUT2
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E14.5
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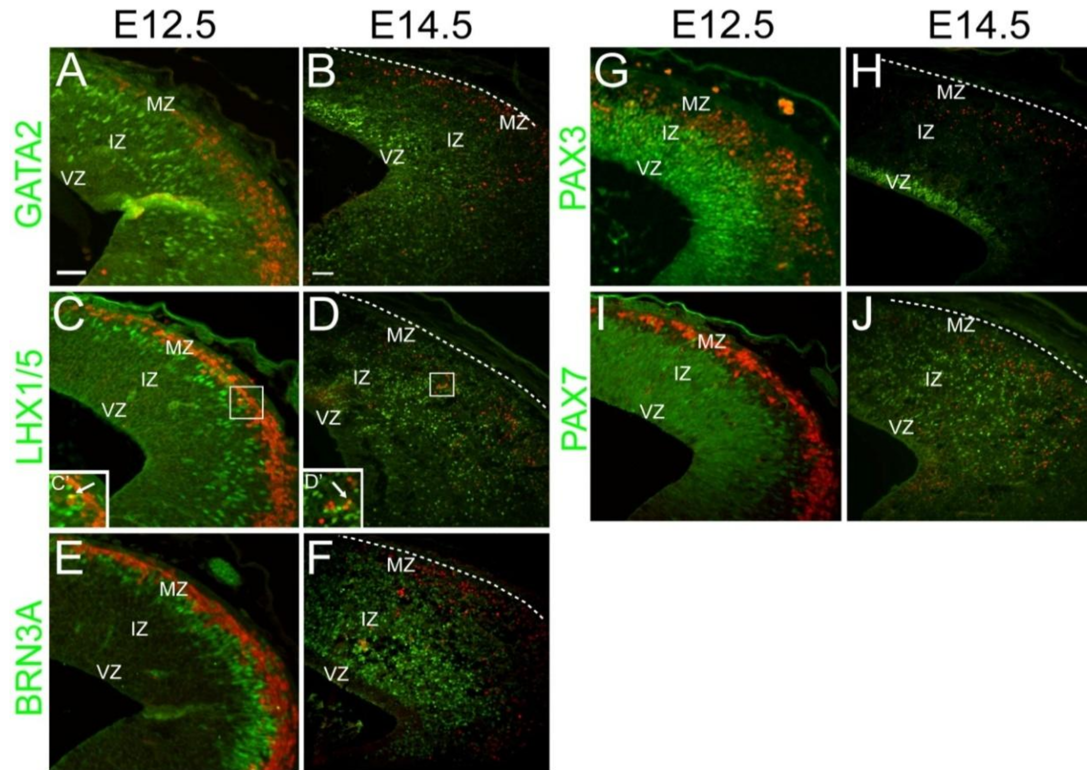


Figure 3.3. PITX2-positive cells represent a unique population of GABAergic dorsal midbrain precursors. Coronal sections of E12.5 and E14.5 midbrains were processed for double immunofluorescence with antibodies against markers of neuronal precursors. At E12.5 and E14.5, PITX2-positive cells (red) reside 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 (inserts 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 are 100 μm and apply to panels A, C, E, G, I and B, D, F, H, J, respectively. Abbreviations: mantle zone (MZ); intermediate zone (IZ); ventricular zone (VZ).

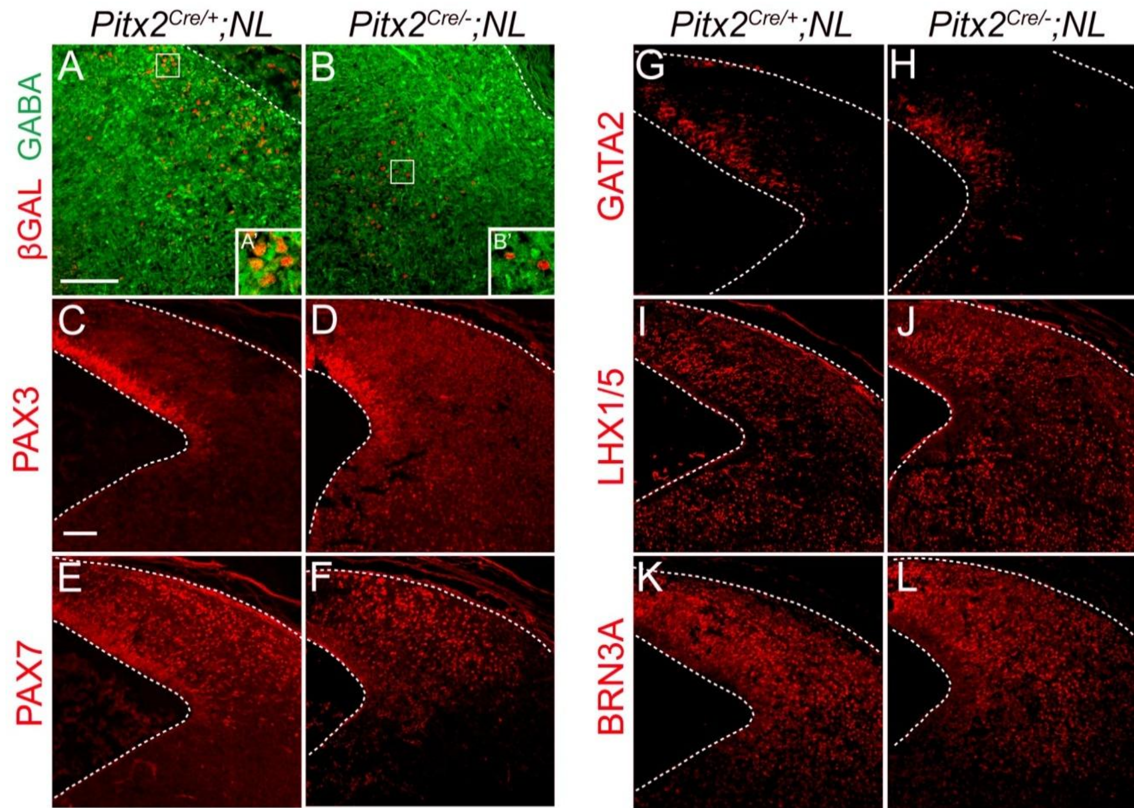


Figure 3.4. PITX2 is required for GABAergic differentiation. Coronal sections of E14.5 *Pitx2$^{Cre/+}$;NL* and *Pitx2$^{Cre/-}$;NL* midbrains were processed for double immunofluorescence with antibodies against βGAL and GABA (A,B). Boxes in A and B are enlarged in A' and B'. (A,A') PITX2-lineage cells 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. Panels A-L were imaged using confocal microscopy. Scale bars in A and C are 100 μm and apply to A-B and C-L, respectively.

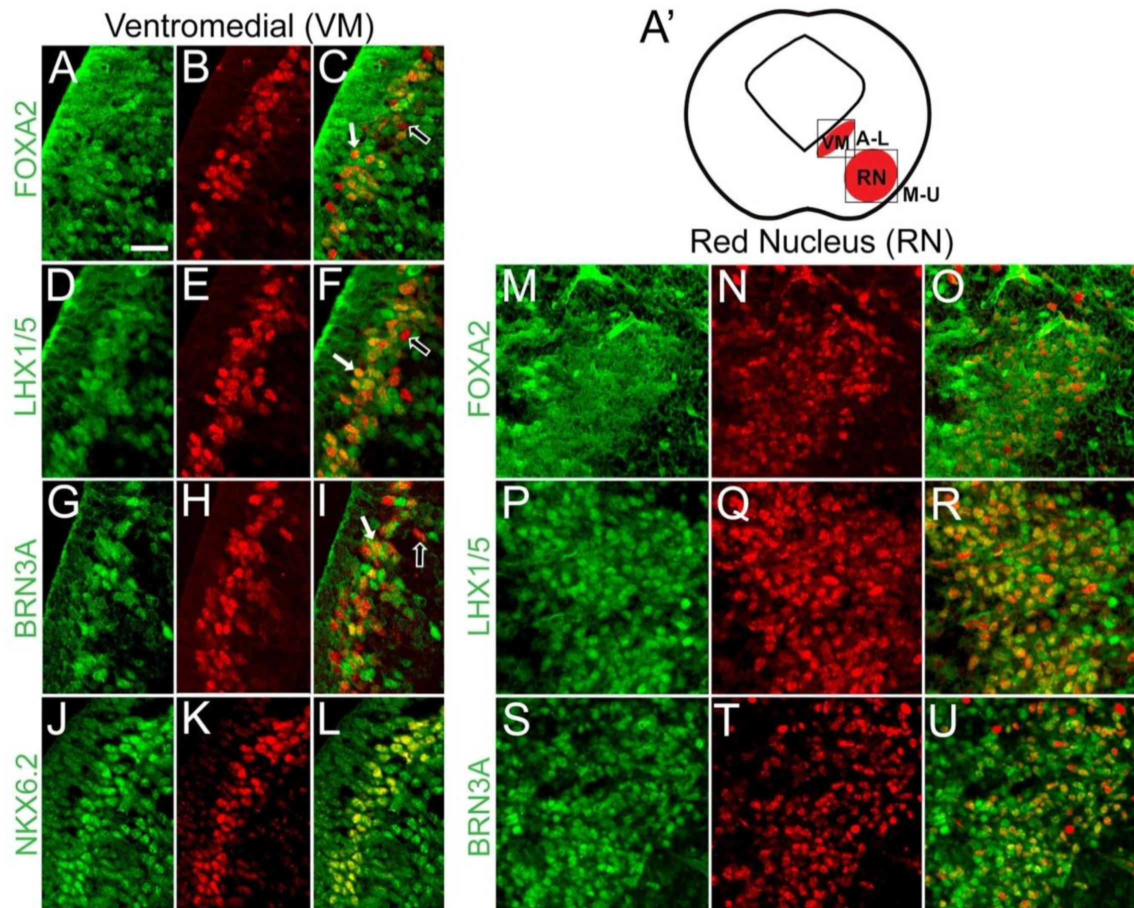


Figure 3.5. PITX2 identifies restricted populations of ventromedial midbrain precursors. Immunofluorescence of E12.5 midbrain coronal sections processed with antibodies against PITX2 (red) and other ventral midbrain markers (green) and imaged with confocal microscopy**.** (A') Cartoon showing coronal 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 panels 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. Panels 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 panels A-U and is 25 μm.

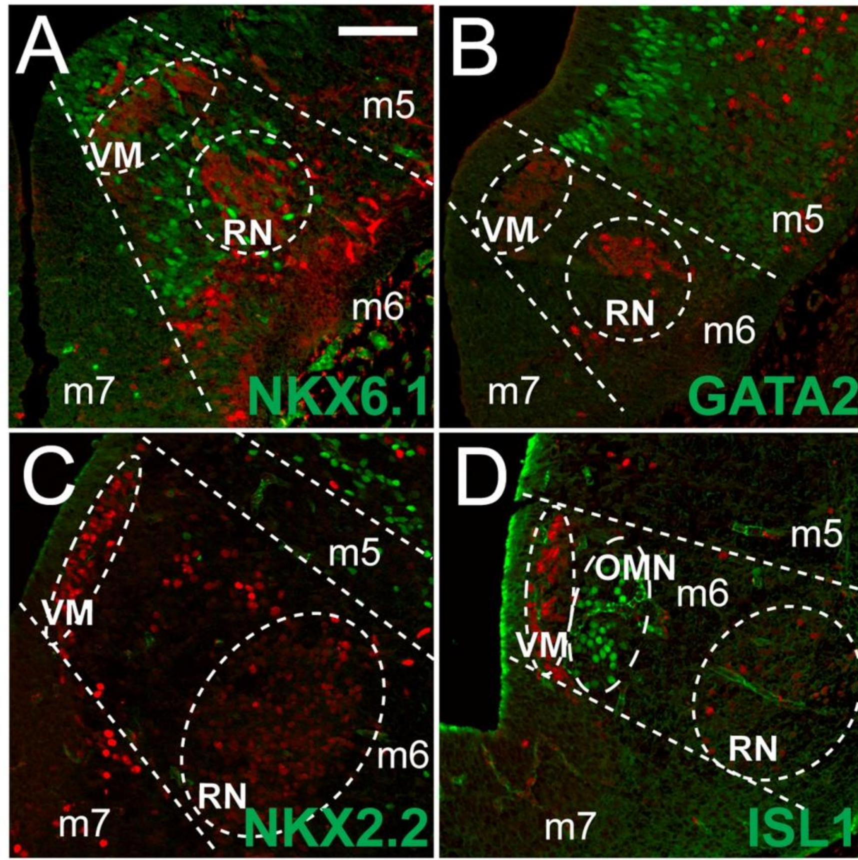


Figure 3.6. Ventral midbrain domains are delineated by transcription factor patterning. E12.5 coronal sections were processed for immunofluorescence with antibodies against PITX2 (red) and other ventral midbrain markers (green) and imaged with confocal microscopy. (A) NKX6.1 is restricted to m6 progenitors 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 superficial to the deep PITX2 population in m6. Scale bar in A is 50 μm and applies to panels A-D. Abbreviations: ventromedial population (VM), red nucleus (RN), and oculomotor nucleus (OMN).

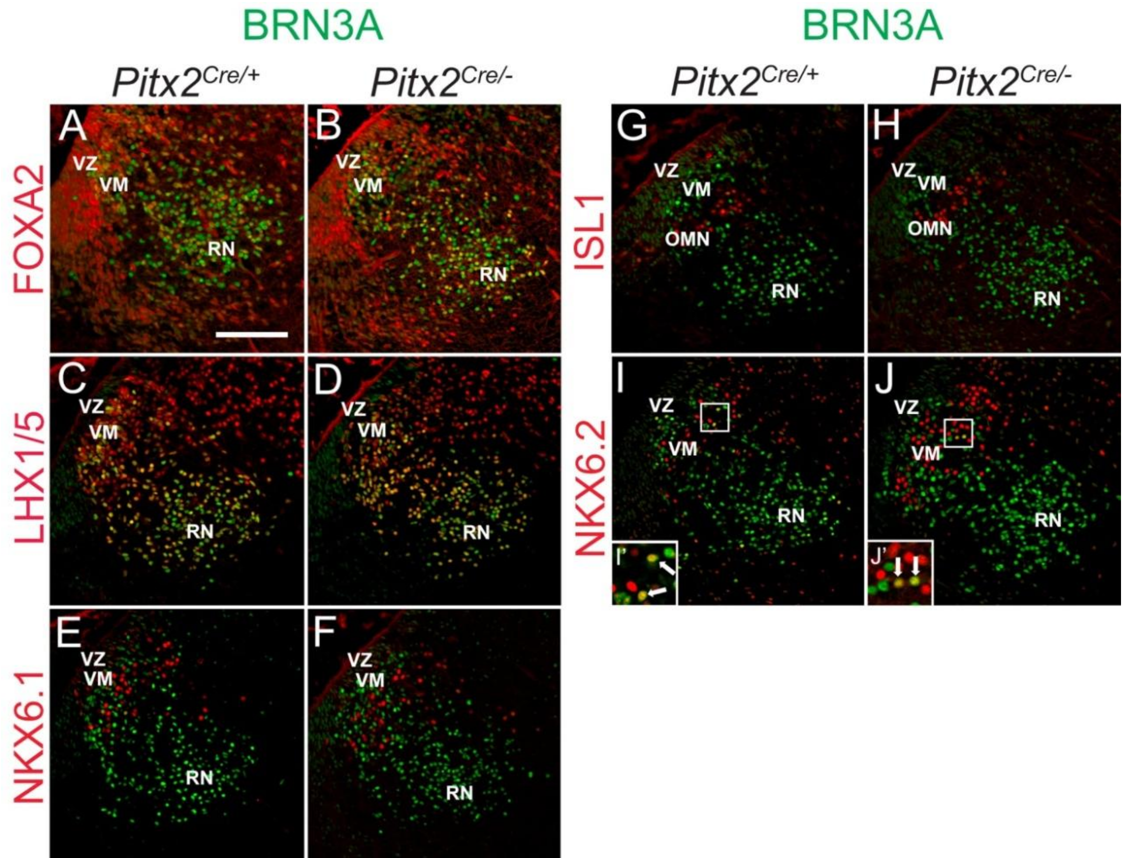


Figure 3.7. Early ventral midbrain patterning is PITX2-independent. E12.5 coronal sections were processed for immunofluorescence with antibodies against BRN3A (green) and FOXA2, LHX1/5, NKX6.1, ISL1, or NKX6.2 (red) and imaged with confocal microscopy. (A-B) FOXA2-positive cells in m6 are localized in 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 is 100 µm and applies to panels A-J. Abbreviations: ventricular zone (VZ) ventromedial population (VM), and red nucleus (RN).

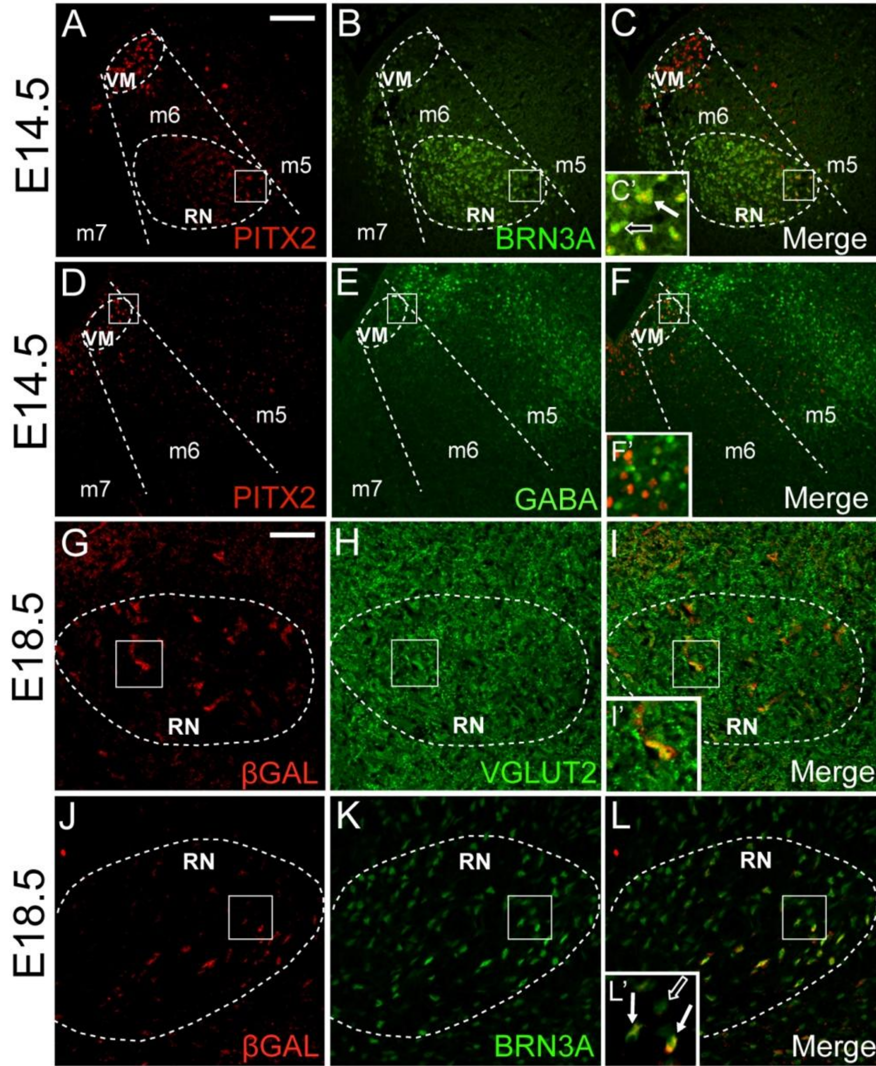


Figure 3.8. *Pitx2*-lineage neurons are glutamatergic and sparse in the red nucleus. (A-F) Coronal sections of E14.5 midbrains processed for PITX2 and BRN3A (A-C) or GABA (D-F) immunofluorescence. Dotted areas demarcate cells in the PITX2-positive deep ventromedial population and the red nucleus. Boxes 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 are 100 μm and 50 μm and apply to panels A-F and G-L, respectively. Abbreviations: ventromedial population (VM) and red nucleus (RN).

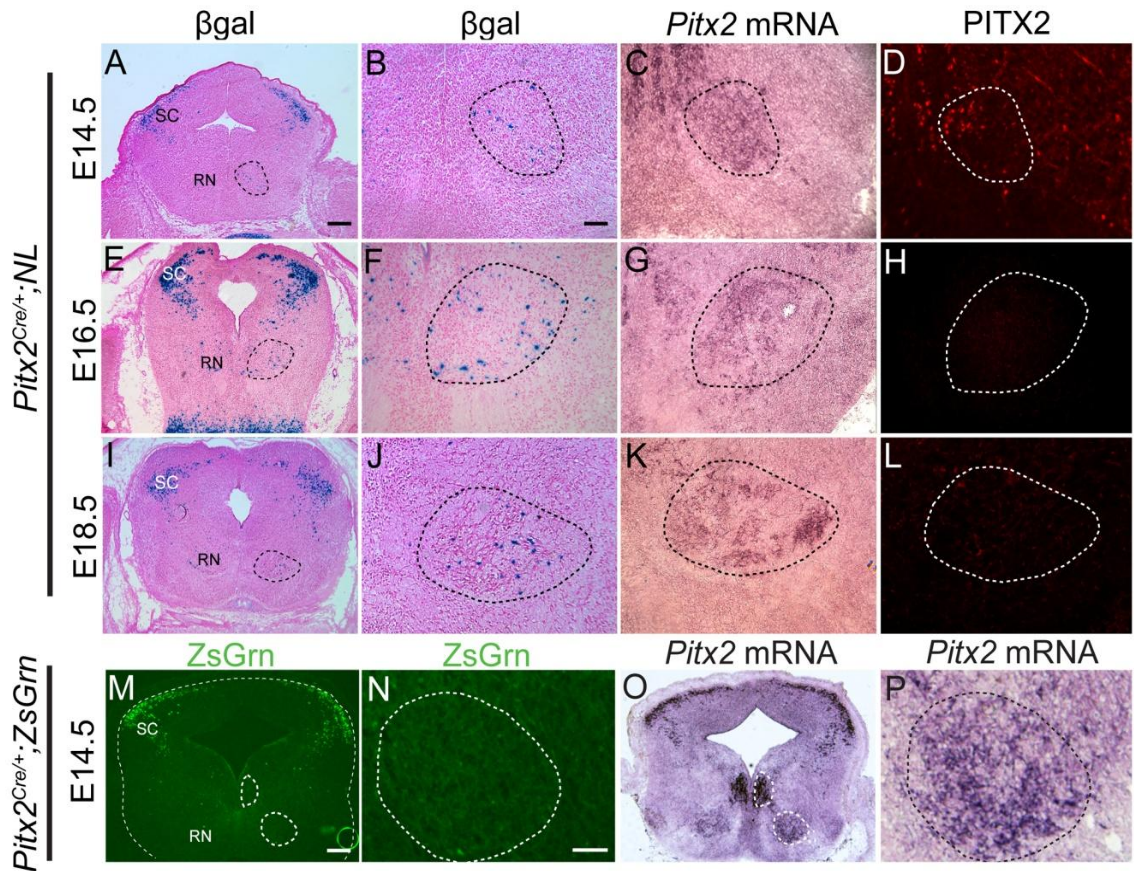


Figure 3.9. ***Pitx2* expression is transient in the ventral midbrain.** (A-L) Adjacent coronal sections of *Pitx2*$^{Cre/+}$*;NL* midbrains processed for βGAL histochemistry (A, B, E, F, I, J), *Pitx2* mRNA (C, G, K) or PITX2 immunofluorescence (D, H, L). The dotted line demarcates the red nucleus. (A) At E14.5, *Pitx2*-lineage neurons are located in the superior colliculus (SC) and red nucleus (RN). (B) High magnification of panel 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. Few cells are fluorescent in the ventral midbrain as seen in a high magnification image of the red nucleus (N). (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 is 125 μm and applies to panels A, E, and I. Scale bar in B is 32 μm and applies to panels B-D, F-H, and J-L. Scale bars in M and N are 100 μm and 50 μm and apply to panels M,O and N,P, respectively.

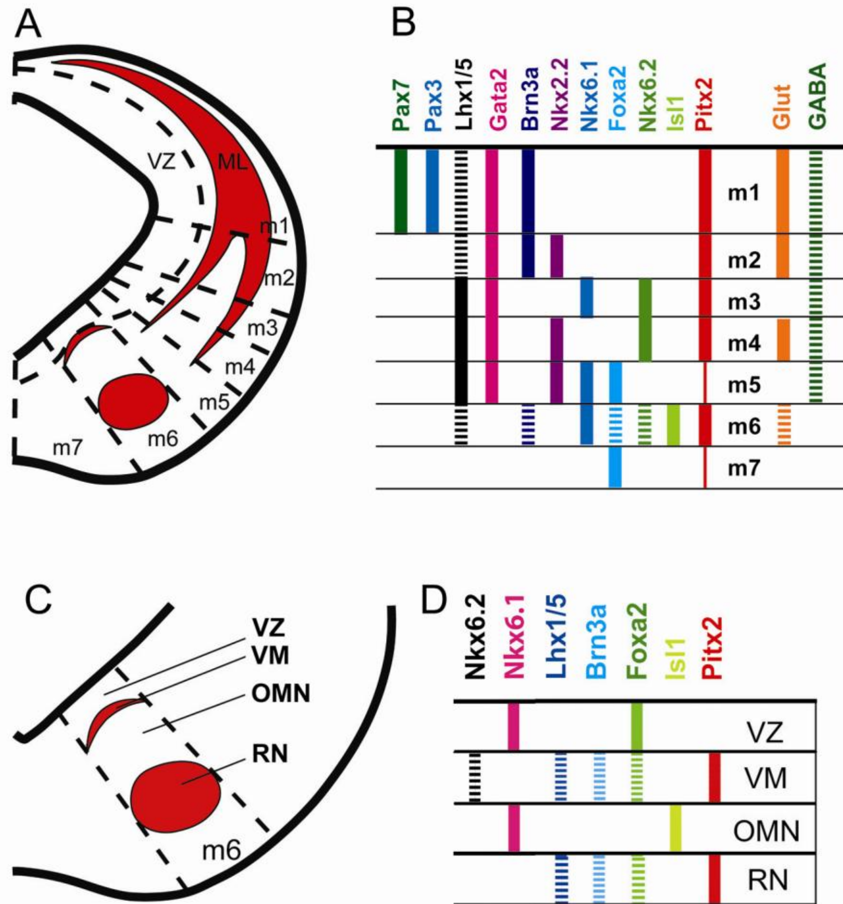
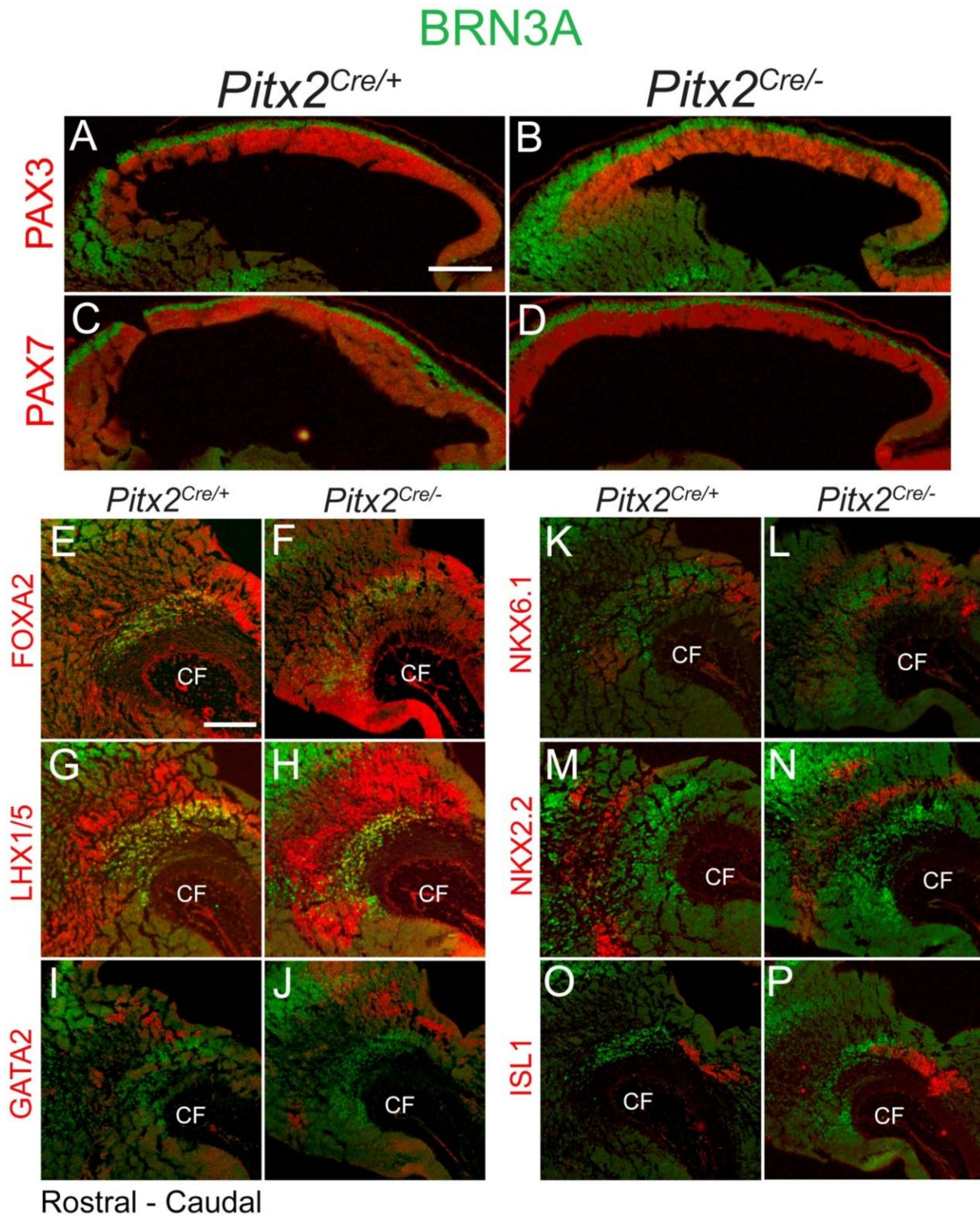


Figure 3.10. Summary of *Pitx2* expression in the developing dorsal and ventral midbrain. Schematic is based on previously published models (Nakatani et al., 2007; Kala et al., 2009), wherein early developmental transcription factors were mapped by domain. (A,C) Cartoons of typical E12.5 coronal sections 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. Abbreviations: dopaminergic neurons (DA); glutamatergic neurons (GLUT); GABAergic neurons (GABA); ventricular zone (VZ); ventromedial population (VM); oculomotor nucleus (OMN); red nucleus (RN); mantle layer (ML).

3.11 Supplementary Figure S1. Early rostral-caudal patterning of the midbrain is PITX2-independent. E12.5 *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ littermate midbrain sagittal sections were processed for immunofluorescence with antibodies against BRN3A (green) and other midbrain markers (red). (A-D) PAX3 and PAX7-positive cells are found throughout the ventricular and medial zones and extend across the entire rostral-caudal dorsal midbrain. (E-F) FOXA2-positive cells are located throughout the ventral midbrain in both the ventricular and intermediate zones. (G-H) LHX1/5-positive cells are found throughout the ventral midbrain and many cells surrounding the cephalic flexure (CF) demonstrate co-localization of LHX1/5 and BRN3A (yellow cells). (I-J) GATA2-positive cells occupy a dorso-ventral stripe just caudal to the cephalic flexure. (K-L) NKX6.1-positive cells form an arc bordering the cephalic flexure. (M-N) NKX2.2 is patterned in two stripes located dorsal to the cephalic flexure. (O-P) The ISL1-positive oculomotor nucleus sits on the rostral aspect of the cephalic flexure. Rostral is to the left in all panels. Scale bars in A and E are 250 μm and apply to panels A-D and E-P, respectively.


BRN3A
$Pitx2^{Cre/+}$
$Pitx2^{Cre/-}$
A
B
PAX3
C
D
PAX7
$Pitx2^{Cre/+}$
$Pitx2^{Cre/-}$
$Pitx2^{Cre/+}$
$Pitx2^{Cre/-}$
E
F
FOXA2
CF
G
H
LHX1/5
I
J
GATA2
K
L
NKX6.1
M
N
NKX2.2
O
P
ISL1
Rostral - Caudal

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Chapter IV

Pleiotropic and isoform-specific functions for *Pitx2* in superior colliculus and hypothalamic neuronal development

Abstract

Transcriptional regulation of gene expression during development is critical for proper neuronal differentiation and migration. Alternative splicing and differential isoform expression have been demonstrated for most mammalian genes, but their specific contributions to gene function are not well understood. In the mouse, the transcription factor gene *Pitx2* is expressed as three different isoforms (*Pitx2a, Pitx2b,* and *Pitx2c*) which have unique amino termini and common DNA binding homeodomains and carboxyl termini. The specific roles of these isoforms in neural development are not known. Here we report the onset of *Pitx2ab* and *Pitx2c* isoform-specific promoter activity by E9.5 in the developing mouse brain. Using isoform-specific *Pitx2* deletion mouse strains, we show that collicular neuron migration requires PITX2AB and that collicular GABAergic differentiation and targeting of hypothalamic projections require unique *Pitx2* isoform dosage. These results provide insights into *Pitx2* dosage and isoform-specific requirements underlying midbrain and hypothalamic development, and suggest that these isoforms may have unique genetic targets which direct region-specific neuronal differentiation.

Introduction

Gene expression is a tightly controlled process known to direct critical aspects of neuronal migration and differentiation (Briscoe and Novitch, 2008; Dessaud et al., 2008; Wilson and Maden, 2005). Alternative splicing adds an additional layer of regulation, wherein a single gene gives rise to multiple protein isoforms with distinct functions, greatly increasing functional capacity. Alternative splicing occurs in up to 98% of human genes with multiple exons (Dessaud et al., 2008; Pan et al., 2008; Wang et al., 2008). Recent data on mouse gene splicing is not available, but previous studies found that the mouse genome undergoes slightly less splicing than the human genome (Chacko and Ranganathan, 2009; Kim et al., 2007; Modrek and Lee, 2003). Organs with increased cellular and functional complexity, such as the central nervous system (CNS), tend to utilize gene splicing more frequently than other tissues such as muscle (Modrek et al., 2001; Yeo et al., 2004). Nonetheless, there are few detailed studies of isoform function in the developing brain. The morphogen *fibroblast growth factor 8* (*Fgf8*) gene is expressed as eight unique isoforms with variable receptor binding properties and roles in midbrain/hindbrain development (Guo et al., 2010). Several transcription factor genes expressed in the brain, including the forkhead-domain containing gene *FOXP2* and the basic helix-loop helix domain containing gene *TCF4* (mutated in human Pitt-Hopkins syndrome) exhibit alternative splicing, but the specific roles of individual isoforms for these two genes in neuronal development are also unclear (Santos et al., 2011; Sepp et al., 2011). A critical unanswered question is whether different transcription factor isoforms also exhibit unique functions during brain development.

PITX2 is a bicoid-like homeodomain transcription factor gene. Heterozygous *PITX2* mutations in humans result in Rieger Syndrome, characterized by developmental defects in the eyes, teeth, umbilicus, heart, and brain (Amendt et al., 2000; Childers and Wright, 1986; Cunningham et al., 1998; Idrees et al., 2006; Semina et al., 1997). Mouse models of *Pitx2* deficiency exhibit ocular, tooth, and brain phenotypes similar to humans with *PITX2* mutations, but the molecular mechanisms underlying these defects are only partially understood (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Liu et al., 2003; Martin et al., 2004; Skidmore et al., 2012; Waite et al., 2011). In the mouse CNS, *Pitx2* is expressed in discrete populations of neurons in the hypothalamus, midbrain,

rhombomere 1, and spinal cord. In the hypothalamus, *Pitx2* is necessary for formation of the mammillothalamic tract (MTT) and midbrain *Pitx2* is critical for neuronal migration and GABAergic differentiation (Skidmore et al., 2012; Waite et al., 2011). In the midbrain, *Pitx2* is expressed downstream of a GABAergic cell-fate signaling cascade involving *Helt* and *Gata2* (Cazorla et al., 2000; Miyoshi et al., 2004; Nakatani et al., 2007). *In vitro* studies have shown that *Pitx2* is capable of activating *Gad1* expression for GABA synthesis (Chen et al., 2011; Westmoreland et al., 2001), suggesting *Pitx2* may act indirectly or directly as a terminal GABAergic differentiation factor.

In chick, mouse, and rat, *Pitx2* gives rise to three unique isoforms (PITX2A*,* PITX2B*,* and PITX2C*)* that arise from alternative promoter usage and exon splicing. These isoforms have distinct N-termini which are necessary to modulate of gene expression and exhibit dosage and tissue-specific requirements (Kioussi et al., 2002; Simard et al., 2009). In mouse, PITX2C (but not PITX2A or PITX2B) is required for left-sided morphogenesis of the heart, lungs, and ovaries, as well as for looping of the gut (Guioli and Lovell-Badge, 2007; Liu et al., 2001; Liu et al., 2002). *In vitro,* PITX2C is necessary for retention of myoblasts in an undifferentiated state and for continued proliferation (Martinez-Fernandez et al., 2006), whereas in HeLa cells PITX2A regulates actin-myosin changes to promote cell spreading and migration (Wei and Adelstein, 2002). Interestingly, no unique *in vivo* requirements for PITX2A or PITX2B have been identified, although PITX2AB appears to be sufficient for tooth development (Liu et al., 2003).

All three *Pitx2* isoforms appear to be expressed at similar levels in the mature rodent brain (Smidt et al., 2000). Therefore, we hypothesized that *Pitx2* isoforms may have unique functions during brain development. To test this hypothesis, we characterized the onset of *Pitx2* isoform expression in the brain and the effects of global, conditional, or isoform-specific *Pitx2* deficiency on hypothalamic and midbrain neuronal development. Our results suggest the presence of brain-region, dosage, and isoform-specific roles for *Pitx2* in neuronal migration, differentiation, and axon tract formation.

Materials and Methods

Mice

C57BL/6J mice were obtained from the Jackson Laboratory (JAX #000664). Mouse alleles used in this study are shown in Figure 1. *Pitx2*$^{\Delta ab/+}$ and *Pitx2*$^{\Delta c/+}$ mice were obtained from James Martin (Liu et al., 2001; Liu et al., 2002). *Pitx2c-lacZ* mice were created by Hiroshi Hamada and contain *lacZ* knocked into the *Pitx2c* promoter (manuscript in preparation). To generate *Pitx2*$^{+/-}$*;ZsGrn* mice, *ZsGrn/ZsGrn* reporter mice obtained from Jackson Laboratories (JAX #007006) (Madisen et al., 2010) were crossed with *Pitx2*$^{+/-}$ mice (Gage et al., 1999). To generate *Pitx2*$^{Cre/-}$*;ZsGrn* embryos, *Pitx2*$^{Cre/+}$ mice (Liu et al., 2002; Skidmore et al., 2008; Waite et al., 2011) were crossed to *Pitx2*$^{+/-}$*;ZsGrn* mice. *Pitx2*$^{tlz/+}$ mice were as previously described (Skidmore et al., 2012). *Nestin-Cre* (*NCre)* transgenic mice (Tronche et al., 1999) were crossed with *Pitx2*$^{tlz/+}$ (Skidmore et al., 2012) to produce *NCre;Pitx2*$^{tlz/+}$ mice. *NCre;Pitx2*$^{tlz/+}$ mice were then crossed with *Pitx2*$^{flox/flox}$ mice (Gage et al., 1999) to generate *NCre;Pitx2*$^{tlz/flox}$ and *Pitx2*$^{tlz/flox}$ littermates.

Tissue Preparation

The morning of vaginal plug identification was designated as E0.5. Embryos were then fixed in 2-4% paraformaldehyde for 15 minutes to 4 hours, depending on the age and genotype. For frozen sections, embryos were cryoprotected overnight in 30% sucrose-PBS, flash frozen in O.C.T. embedding compound (Tissue Tek, Torrance, CA), and stored at -80°C until being sectioned at 12-30 µm. For paraffin sections, embryos were dehydrated in an ethanol gradient, embedded in paraffin, and sectioned at 7-9 µm. From each embryo, amniotic sac or tails were used for genotyping. All procedures were approved by the University Committee on Use and Care for Animals at the University of Michigan.

ES cell isolation and chimera generation

On Day 1, *Pitx2*$^{tlz/+}$ females, aged 28 days, were treated with 5 IU of pregnant mare's serum gonadotropin. On Day 3, pregnant females were treated with 5 IU human chorionic gonadotropin and subsequently crossed to *Pitx2*$^{+/-}$ males overnight. On Day 7,

pregnant females were sacrificed and blastocysts were collected. ES cell lines were prepared from blastocysts, genotyped, and cryopreserved. 3 clones each of *Pitx2*$^{tlz/+}$ and *Pitx2*$^{tlz/-}$ ES cells were expanded, checked for chromosomal euploidy, and one clone of each genotype was injected into wild type blastocysts to generate chimeric mice with assistance from The Transgenic Animal Model Core at the University of Michigan. At E14.5, chimeric embryos were dissected from the females, cryoprepared as described below, and sectioned at 30 μm for X-gal staining. Midbrain X-gal staining was scored as normal or medially mislocalized and performed blind to the genotype.

Immunofluorescence, immunohistochemistry, and *in situ* hybridization

Immunofluorescence on paraffin embedded tissues was done as previously described (Martin et al., 2002; Martin et al., 2004). In preparation for frozen-section immunofluorescence, sections were fixed for 5 minutes in 4% PFA, rinsed in PBS, and washed in 0.1% PBS-Tween. Immunofluorescence was then performed as for paraffin sections. Antibodies used were rabbit 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-BRN3A at 1:800 (provided by Dr. Eric Turner, University of California-San Diego), and rabbit anti-GABA (Sigma). DAB immunohistochemistry was performed using a mouse anti-Neurofilament at 1:100 (2H3, Developmental Studies Hybridoma Bank) (Skidmore et al., 2008) and processed for immunohistochemistry using the Vectastain ABC reagent (Vector labs) and DAB (3,3′-Diaminobenzidine, Sigma). *In situ* hybridization on frozen sections was done as previously described (Martin et al., 2002; Martin et al., 2004) using cRNA probes created from PCR-amplified cDNA for *Pitx2* (Suh et al., 2002).

β-galactosidase and cresyl violet histochemistry

To generate embryonic tissues for X-Gal staining, *Pitx2*$^{\Delta ab/+}$, *Pitx2*$^{\Delta c/+}$, *or Pitx2*$^{+/-}$ female mice were crossed with *Pitx2*$^{\Delta ab/+}$, *Pitx2*$^{+/-}$, or *Pitx2*$^{tlz/+}$ males. E9.25-E14.5 whole embryos and E18.5 brains were isolated and fixed in 2-4% paraformaldehyde for 10 minutes to 4 hours, depending on age. Samples for cryosectioning were washed with PBS, cryoprotected in 30% sucrose-PBS with 2 mM MgCl overnight, and frozen in

O.C.T. embedding medium (Tissue Tek, Torrance, CA). Frozen sections were post-fixed in 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). Stained slides were washed in PBS, followed by eosin counterstaining, and then mounted using Permount (Fisher). For vibratome sections, whole embryos were washed in X-Gal wash buffer, incubated at 37º C for 3-7 days in X-Gal staining solution, then fixed in 4% PFA for up to 7 days. Stained embryos were embedded in 4% low-melt agarose and vibratome sectioned at 150 μm. To visualize tract formation, paraffin sections were stained with cresyl violet.

Microscopy

Confocal fluorescent images were taken using a Leica TCS SP5 X Supercontinuum Confocal System with Upright Fluorescent Microscope. For neighboring merged images, non-fluorescent sections were photographed in brightfield and converted into pseudo-fluorescent color, then overlaid in Photoshop. Brightfield and some fluorescent sections were imaged on a Leica DM500B upright microscope. For vibratome sections, sections were photographed in brightfield on a Leica MZ10F dissecting microscope. Digital images were processed with Adobe Photoshop CS3 software.

RNA isolation and real-time PCR

E14.5 wild type embryos were isolated and midbrains and hypothalamic tissue microdissected into ice-cold TRIzol (Invitrogen) and mechanically homogenized. Isolated RNA was treated with DNase I (Qiagen) prior to cDNA synthesis. cDNA was created using the Superscript First-Strand cDNA Synthesis system for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen) using random primers. Relative expression levels were assayed using POWER SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and primers specific for *Pitx2a* (Forward: GGCCTGCGTGCAATTAGAGAAAGAT, Reverse: GCTGCTGGCTAGTGAAATGAGTCCT), *Pitx2b* (Forward: TGGACCAACCTTACGGAAGC, Reverse: CATGTCATCGTAGGGCTGCAT), *Pitx2c*

(Forward: GAGGTGCATACAATCTCCGATAC, Reverse: AGCGGTTTCTCTGGAAAGTG), and *GAPDH* (Forward: TGCCCCCATGTTTGTGATG, Reverse: TGTGGTCATGAGCCCTTCC). Reactions were run in triplicate on an Applied Biosystems StepOne-Plus Real-Time PCR System. The level of *GAPDH* was used as an internal control. In order to compare unique primer sets, all target genes were given the same threshold, appropriately positioned within the exponential phase of amplification. CT was measured for each gene and ΔCT calculated as the difference between the gene of interest (GOI) and *Gapdh* (Livak and Schmittgen, 2001). ΔΔCT was measured as the difference in ΔCT between GOIs. The fold difference between GOIs was defined as $2^{-\Delta\Delta CT}$.

Calculation of PCR Efficiency

The ΔΔCT method requires primer efficiencies at 100% +/-10% (Livak and Schmittgen, 2001). The amplification efficiencies of real-time PCR primers for *Pitx2* isoforms and *Gapdh* were analyzed as previously published (Mygind et al., 2002). Briefly, a 5-fold dilution series of cDNA was used as a template in real-time PCR to generate a log plot of copy numbers at different dilutions versus the corresponding cycle threshold (CT). The slope for each primer set is determined by linear regression and applied to the equation slope=-(1/log E) where E=efficiency. All primers used tested near 100% efficiency (+/-10%).

Results

Pitx2 isoforms and alleles

The mouse *Pitx2* gene is composed of two promoters and six exons (Fig. 4.1A). Alternative splicing and promoter usage generates three different *Pitx2* isoforms, PITX2A, PITX2B, and PITX2C (Fig. 4.1A,B). All three isoforms have unique N-termini, but share the same C-terminus composed of exons 5 and 6. Exon 5 contains the homeodomain which is required for proper DNA binding, specificity, and transactivation potential of *Pitx2* (Amendt et al., 1998; Saadi et al., 2001). PITX2C is the largest isoform at 324 amino acids due to the large size of exon 4, whereas PITX2A is the smallest with 271 amino acids.

To determine the functions and expression patterns of *Pitx2* isoforms in the developing mouse brain, we used various combinations of mouse *Pitx2* alleles (Fig. 4.1C). *Pitx2*$^{\Delta ab}$ is a *Pitx2ab*-specific knockout allele, wherein part of exon 2 and all of exon 3 are replaced by the *lacZ* gene, rendering PITX2AB non-functional and leaving PITX2C intact (Liu et al., 2001). Conversely, the *Pitx2*$^{\Delta c}$ allele lacks exon 4, rendering PITX2C non-functional and leaving PITX2AB intact (Liu et al., 2002). The *Pitx2*^{flox} allele contains two *loxP* sites flanking exon 5, where *Cre* recombination acts to excise exon 5 and create a null allele (Gage et al., 1999). The *Pitx2*^{null} (or *Pitx2*$^{-}$) allele is missing exon 5 and is functionally null (Gage et al., 1999). The *Pitx2*^{Cre} allele contains a *Cre* sequence in place of exon 5, rendering *Pitx2* non-functional with *Cre* expression under the control of both *Pitx2* promoters (Liu et al., 2002; Liu et al., 2003; Skidmore et al., 2008). The *Pitx2*^{tlz} allele contains an IRES-*TauLacZ* (*tlz*) sequence in place of exon 5, resulting in disrupted *Pitx2* function and expression of β-galactosidase under control of *Tau* bovine neurofilament in neuronal axons (Skidmore et al., 2012).

Pitx2ab and _Pitx2c_ are expressed in the mouse midbrain by E9.25

Pitx2 expression in the developing mouse embryo begins at E8.0 in the lateral plate mesoderm and is later expressed in multiple tissues, including brain, craniofacial structures, eyes, heart, and thoracic/abdominal viscera (Campione et al., 1999; Liu et al., 2001; Mucchielli et al., 1997; Porter et al., 2001). *Pitx2* expression continues through adulthood in the brain, eyes, and heart (Kirchhof et al., 2011; Porter et al., 2001; Smidt et al., 2000). To determine which *Pitx2* isoforms are expressed in the developing mouse brain, we performed quantitative RT-PCR (QPCR) on RNA obtained from E14.5 midbrain tissue samples (Fig. 4.2A). All three *Pitx2* isoforms were expressed in the midbrain at E14.5 (*N*=3 embryos) (Fig. 4.2A). *Pitx2a* cDNA was most abundant, at levels 23.66-fold (+/- 0.839) higher than *Pitx2c,* and *Pitx2b* cDNA was present at levels 4.46-fold (+/- 0.849) higher than *Pitx2c*. *In situ* hybridization using a cRNA probe that detects all three isoforms (Fig. 4.1A) showed that *Pitx2* mRNA is present at E8.5 in the branchial arches, but not in the neuroepithelium (Fig. 4.2B). Consistent with this, X-gal staining of E8.5 *Pitx2*$^{\Delta ab/+}$ tissues (in which β-gal is expressed under the control of the *Pitx2ab* promoter) revealed no *Pitx2ab*-positive cells in the neuroepithelium (data not

shown). However, by E9.5, PITX2 immunofluorescence was detected in post-mitotic ventral midbrain neurons, as determined by co-staining with anti-PITX2 and anti-phosphohistone H3 (Fig. 4.2C). Therefore, *Pitx2* is expressed very early in the neuroepithelium, and by E14.5 all isoforms are expressed, albeit at different levels.

To determine the onset and isoform specificity of *Pitx2* expression in the brain, we performed X-gal staining on E9.25-E10.5 *Pitx2*$^{\Delta ab/+}$ and *Pitx2c-lacZ* embryos. At E9.25, a single βgal positive *Pitx2ab*-positive cell was present in the ventral midbrain (Fig. 4.2D-D''), and by E9.5, numerous βgal positive cells were detected (Fig. 4.2E-E''). At E10.5, βgal positive cells in *Pitx2*$^{\Delta ab/+}$ embryos were visible throughout the midbrain (Fig. 4.2F-F''). In *Pitx2c-lacZ* embryos, βgal positive cells were absent from the ventral midbrain at E9.25 (Fig. 4.2G') but present at E9.5-E10.5 (Fig. 4.2H-I''), indicating an onset of *Pitx2c* expression in the brain that is similar to *Pitx2ab*. Interestingly, *Pitx2ab*- and *Pitx2c*-positive cells in E9.25-E10.5 embryos were always localized to the ventrolateral midbrain neuroepithelium, and did not intermingle with cells closer to the ventricle, suggesting that *Pitx2* isoforms are expressed early in brain development in post-mitotic neurons.

***Pitx2ab* and *Pitx2c* are expressed in E14.5 collicular neurons**

To confirm these *Pitx2* isoforms are present at the protein level in the superior colliculus, we analyzed PITX2 immunofluorescence in the midbrains of various *Pitx2* isoform-knockout mice. At E14.5, PITX2 protein in wild type mice localized to the collicular surface and to a ventromedial (VM) neuron population (Fig. 4.3B) (Martin et al., 2002; Waite et al., 2011). *Pitx2*$^{\Delta c/\Delta c}$ embryos, which lack the PITX2C isoform but produce two alleles of PITX2AB protein, exhibited a pattern of PITX2 immunofluorescence similar to wild type (Fig. 4.3C). In *Pitx2*$^{\Delta ab/-}$ and *Pitx2*$^{\Delta ab/\Delta ab}$ embryos, which produce only PITX2C protein, PITX2 immunofluorescence was shifted medially and deep into the neuroepithelium (Fig. 4.3E,F), consistent with prior reports of delayed collicular neuron migration in *Pitx2* null embryos (Martin et al., 2004; Waite et al., 2011). Interestingly, PITX2 is normally present in the VM population (Fig. 4.3B); however, ventromedial PITX2 expression was absent in embryos lacking PITX2AB (*Pitx2*$^{\Delta ab/-}$ and *Pitx2*$^{\Delta ab/\Delta ab}$) (Fig. 4.3E,F). This suggests that either *Pitx2c* is not expressed

in the VM population or that *Pitx2ab* is required for the formation of the VM population. Additionally, medial mislocalization of *Pitx2*-expressing neurons in $Pitx2^{\Delta ab/-}$ and $Pitx2^{\Delta ab/\Delta ab}$ embryos (Fig. 4.3E,F and Fig. 4.4), suggests roles for *Pitx2ab* in neuronal migration.

Distinct dosage requirements for *Pitx2* isoforms in collicular neuronal migration

Previous studies showed that *Pitx2* is necessary for superior collicular neuronal migration (Martin et al., 2004; Waite et al., 2011); however, the *Pitx2* isoforms required for this function were not known. Embryos heterozygous for *Pitx2 tlz* ($Pitx2^{tlz/+}$) and *Pitx2ab* null ($Pitx2^{\Delta ab/+}$) alleles displayed normal localization of *Pitx2*-expressing cells at the dorsal-most aspect of the superior colliculus (N=7 embryos) (Fig. 4.4A,D), whereas loss of all *Pitx2* isoforms (in $Pitx2^{tlz/-}$ embryos) resulted in medial or deep mislocalization of *Pitx2*-expressing cells (Fig. 4.4B). $Pitx2^{tlz/\Delta c}$ embryos, which have no functional *Pitx2c* and a single allele of *Pitx2ab*, exhibited normal collicular neuron localization (Fig. 4.4C). Thus, a single allele of *Pitx2ab* appears sufficient for superior collicular neuronal migration, whereas *Pitx2c* is dispensable. Interestingly, many collicular cells in $Pitx2^{\Delta ab/-}$ midbrains were also mislocalized (N=7 embryos) compared to controls ($Pitx2^{\Delta ab/+}$), although the phenotype was not as severe as in *Pitx2* null embryos (compare Fig. 4.3E and 4.4E to Fig. 4.3D, 4.4A, and 4.4B). $Pitx2^{\Delta ab/\Delta ab}$ midbrains also exhibited intermediate phenotypes (N=6 embryos), where some neurons were medially mislocalized although less severely than in $Pitx2^{\Delta ab/-}$ embryos (Fig. 4.4F and Fig. 4.3F). These data suggest that *Pitx2* isoforms and their dosage are both important in collicular neuron migration.

To determine whether *Pitx2ab* is required for the timing of collicular neuron migration, we analyzed conditional- and isoform-specific *Pitx2* knockout embryos at a later gestational age (E18.5), when collicular layering is nearing completion (Edwards et al., 1986). We assessed collicular lamination using anti-BRN3A and *Pitx2* expression, since *Brn3a* and *Pitx2* mark neighboring laminae (Waite et al., 2011). At E18.5, βGAL-positive neurons in $Pitx2^{\Delta ab/+}$, $Pitx2^{\Delta ab/\Delta ab}$, and $Pitx2^{\Delta ab/-}$ colliculi were properly localized between BRN3A-positive layers (Fig. 4.5A-I), although several βgal-positive neurons were present in deeper layers in $Pitx2^{\Delta ab/\Delta ab}$ and $Pitx2^{\Delta ab/-}$ embryos (see * in Fig. 4.5D-I).

To determine the migrational phenotype of E18.5 midbrains in the absence of all *Pitx2* isoforms, *NCre;Pitx2^{tlz/flox}* embryos were analyzed for *Pitx2 in situ* hybridization. In *NCre;Pitx2^{tlz/flox}* E18.5 conditional mutants, most *Pitx2*-expressing neurons were mislocalized to the deep BRN3A-positive layer previously identified as the stratum album intermedium (see * in Fig. 4.5J-L) (Waite et al., 2011), although a few neurons were properly localized between BRN3A-positive layers. Thus, complete loss of *Pitx2* leads to severely disrupted collicular neuron localization, whereas isoform-specific deletion results in milder phenotypes. Unique combinations of *Pitx2* isoforms may therefore regulate the timing or efficiency of collicular neuronal migration.

Evidence against extrinsic influences on migration of *Pitx2*-deficient collicular neurons

Pitx2 exhibits both cell autonomous and non-cell autonomous requirements during tissue development. For example, *Pitx2* is required non-cell autonomously in the thalamus for formation of the mammillothalamic tract and in the eye for optic stalk development (Evans and Gage, 2005; Skidmore et al., 2012), but cell autonomously for survival of extraocular muscle (Zacharias et al., 2011). The cell autonomous nature of *Pitx2* functions in migration and differentiation of collicular neurons has not been studied. To address this, we generated chimeric embryos by injecting wild type blastocysts with either *Pitx2^{tlz/+}* or *Pitx2^{tlz/-}* embryonic stem cells. Chimeric embryos were harvested at E14.5 and brain sections analyzed by X-gal staining to visualize locations of *Pitx2*-expressing cells. In *wild type;Pitx2^{tlz/+}* midbrains (*N*=4 embryos), βgal-positive cells were properly localized to the dorsal-most aspect of the colliculus (Fig. 4.6A), whereas βgal-positive cells in *wild type;Pitx2^{tlz/-}* embryos (*N*=4 embryos) were shifted deeper in the neuroepithelium, consistent with migratory delay or arrest (Fig. 4.6B). The lack of βgal-positive cells at more superficial locations in the *wild type;Pitx2^{tlz/-}* colliculus argues against non-cell autonomous functions for *Pitx2*.

Collicular GABAergic differentiation is *Pitx2* dosage-dependent but isoform-independent

We previously showed that *Pitx2* is necessary for GABAergic differentiation of a subpopulation of midbrain neurons (Waite et al., 2011), wherein loss of *Pitx2* results in lack of GABAergic differentiation (Waite et al., 2011) (Fig. 4.7B,B'). Here, we found that embryos null for *Pitx2c* ($Pitx2^{\Delta c/\Delta c}$) and those with only a single allele of *Pitx2ab* ($Pitx2^{\Delta c/tlz}$), display normal PITX2 co-localization with GABA (Fig. 4.7C-D'), suggesting a single allele of *Pitx2ab* is sufficient for GABAergic differentiation. Similarly, embryos null for *Pitx2ab* ($Pitx2^{\Delta ab/\Delta ab}$) and those with a single allele of *Pitx2c* ($Pitx2^{\Delta ab/-}$) also display normal GABA expression, suggesting a single allele of *Pitx2c* is sufficient for GABAergic differentiation of PITX2-positive collicular neurons (Fig. 4.7E-F'). Because $Pitx2^{\Delta ab/-}$ and $Pitx2^{\Delta c/tlz}$ collicular PITX2-positive neurons are GABAergic, a single allele of either *Pitx2ab* or *Pitx2c* appears sufficient for GABAergic differentiation of collicular *Pitx2*-positive neurons. Additionally, because $Pitx2^{\Delta ab/\Delta ab}$ and $Pitx2^{\Delta c/\Delta c}$ collicular PITX2-positive neurons are GABAergic, neither isoform is individually necessary.

PITX2AB is necessary for tract formation in the developing brain

These studies suggest that unique *Pitx2* isoforms are required for development of the midbrain through regulation of neuronal migration and differentiation, but we were also interested in the isoform-specific requirements for axon outgrowth. Previous studies showed that *Pitx2* is expressed in hypothalamic neurons and is required non-cell autonomously for development of the mammillothalamic tract (MTT), which projects from the mammillary body to the anterior nucleus of the thalamus (Skidmore et al., 2012); however the isoforms responsible were not identified. To determine which *Pitx2* isoforms are expressed in the developing mouse forebrain, we performed quantitative RT-PCR (QPCR) on RNA obtained from E14.5 hypothalamic tissues (*N*=3 embryos) (Fig. 4.8B). In contrast to the midbrain, *Pitx2a* and *Pitx2b* cDNA was equally abundant in the hypothalamus at levels 16.85-fold (+/- 0.409) and 15.88-fold (+/- 0.623) higher than *Pitx2c*. At E18.5, embryos heterozygous or null for *Pitx2c* ($Pitx2^{\Delta c/+}$, $Pitx2^{\Delta c/\Delta c}$, or $Pitx2^{\Delta c/-}$) also displayed a normal MTT (Fig. 4.8C-H). Similarly, embryos null for *Pitx2ab* ($Pitx2^{\Delta ab/\Delta ab}$) displayed a normal MTT (Fig. 4.8I-J). However, embryos with only a single allele of *Pitx2c* ($Pitx2^{\Delta ab/-}$) failed to form the MTT (Fig. 4.8K-L), similar to embryos with *NCre*-mediated conditional *Pitx2* deletion (Skidmore et al., 2012). Thus, a

single allele of *Pitx2c* is not sufficient for MTT formation which requires either two alleles of *Pitx2c* or one of *Pitx2ab*.

Discussion

Conclusion

Ours is the first study to identify unique transcription factor isoform requirements in the developing brain. This is also the first report of a requirement for PITX2AB in tissue development. We show that all three *Pitx2* isoforms are expressed in the developing midbrain and hypothalamus, and that *Pitx2a* and *Pitx2b* isoforms are expressed at higher levels than *Pitx2c* in a region-specific manner. We also demonstrate that a subpopulation of collicular neurons requires *Pitx2ab* for proper migration, yet requires a single allele of *Pitx2ab* or *Pitx2c* for GABAergic differentiation. Finally, we show that formation of the mammillothalamic tract requires any combination of two isoform-specific *Pitx2* alleles.

Pitx2 isoforms exhibit unique dosage effects during brain development

Pitx2a and *Pitx2b* are expressed at higher levels than *Pitx2c* in the developing midbrain and hypothalamus. This suggests that these isoforms have unique functions and dosage requirements during brain development. For example, GABAergic differentiation of collicular neurons requires only a single allele of *Pitx2ab* or *Pitx2c,* suggesting low dosage of *Pitx2* may be sufficient (Table 4.1). In contrast, MTT formation requires either a single allele of *Pitx2ab* or two alleles of *Pitx2c,* suggesting it requires higher *Pitx2* dosage than midbrain GABAergic differentiation. The highest dosage is required by collicular neurons undergoing migration which require one allele of *Pitx2ab,* although two *Pitx2c* alleles are partially sufficient. *Pitx2* isoforms may be partially functionally redundant or there may be isoform-specific gene regulation. In the developing branchial arches, *Pitx2* isoforms are interchangeable and contribute distinct dosages which translate into unique developmental functions (Liu et al., 2003).

Pitx2a and Pit2b are the major Pitx2 isoforms during brain development

The embryonic brain may be unique from other organs in its requirement for *Pitx2a* and *Pitx2b*. *Pitx2a* and *Pitx2b* are the most highly expressed *Pitx2* isoforms in the embryonic midbrain and hypothalamus, so it is not surprising that their loss results in more severe phenotypes. To date, no studies have identified a requirement for *Pitx2ab* in development of any non-CNS tissue, consistent with prior reports that loss of *Pitx2ab* is not lethal in mice (Liu et al., 2001). *Pitx2ab* is co-expressed with *Pitx2c* in the developing eyes, craniofacial tissues, pituitary, liver hematopoietic stem cells, body wall, and weakly in the lungs (Gage and Camper, 1997; Kieusseian et al., 2006; Kitamura et al., 1999; Liu et al., 2001; Liu et al., 2003). Minor roles for *Pitx2ab* have been identified in lung development (Liu et al., 2001), and *Pitx2*ab is sufficient but dispensable for tooth development (Liu et al., 2003). However, $Pitx2^{\Delta ab/\Delta ab}$ embryos often have medially displaced eyes (unpublished observations), suggesting an unexplored requirement for *Pitx2ab* in eye development. Consistent with this, overexpression of *Pitx2a* in the eye causes severe eye defects similar to those seen in Rieger Syndrome such as corneal hypertrophy and opacification, irido-corneal adhesions, and glaucoma-like retinal degeneration (Pillai et al., 2007). Therefore, the developing CNS and non-CNS appear to have different requirements for *Pitx2* isoforms.

In vitro studies on PITX2A and PITX2B functions have provided additional functional information. In cell lines, PITX2A regulates cellular migration and cell spreading through activation of *RhoA* and *Rac1* (Liu et al., 2001; Wei and Adelstein, 2002). Additionally, PITX2A regulates cell cycle genes such as *P21* and *CyclinD1* in epithelial cells (Zhao et al., 1999). Both PITX2A and PITX2B are capable of transactivating the same genes as *Pitx2c,* but with different efficiencies which are dependent upon cell type and the presence of other proteins (Cox et al., 2002; Ganga et al., 2003; Smidt et al., 2000). Of the three PITX2 isoforms, PITX2B often has the lowest transactivation efficiency (Cox et al., 2002; Ganga et al., 2003; Smidt et al., 2000), but can heterodimerize with PITX2A and PITX2C for increased gene activation (Cox et al., 2002). Isoform heterodimerization is likely facilitated by the homeodomain or C-terminal tail, both of which have also been implicated in PITX2 homodimerization (Amendt et al., 1999; Green et al., 2001). However, the mechanism by which *Pitx2* isoform heterodimerization influences gene expression is unknown.

PITX2C is redundant during brain development

In the hypothalamus and midbrain, *Pitx2c* is expressed at lower levels than *Pitx2a* and *Pitx2b,* suggesting it may be less important for brain development, although expression level does not always correlate with functional importance. This contrasts strongly with developmental studies in other tissues where *Pitx2c* is essential for development of internal organs such as the heart, lungs, and gut (Liu et al., 2001; Liu et al., 2002). *Pitx2c* is first expressed at E8.5 in the left lateral plate mesoderm (L-LPM) downstream of *Shh* and *Nodal,* and LPM induction of *Pitx2c* is necessary for later *Pitx2c* expression in left-sided organs (Brennan et al., 2002; Campione et al., 1999; Kahr et al., 2011; Pagan-Westphal and Tabin, 1998; Shiratori and Hamada, 2006). *Pitx2c* is required for left-sided morphogenesis of the heart and lungs and for looping of the gut (Liu et al., 2001; Liu et al., 2002). Later, *Pitx2c* is necessary for heart development where it induces expression of *atrial natriuretic factor (ANF)* and *Plod1* and cardiac transcription factors including *Isl1, Mef2c* and *Gata4* (Lozano-Velasco et al., 2011). *In vitro* studies suggest that heart development requires synergism specifically between PITX2C and NKX2.5 to regulate cardiac development genes, and that other *Pitx2* isoforms are inadequate (Ganga et al., 2003; Simard et al., 2009; Warren et al., 2011). Consistent with this, PITX2C/NKX2.5 synergism requires the unique PITX2C N-terminus (Simard et al., 2009). Interestingly, continued *Pitx2c* expression through adulthood appears to be required for cardiac fitness, as loss of *Pitx2c* in the cardiac atrium results in susceptibility to atrial fibrillation (Chinchilla et al., 2011; Kirchhof et al., 2011).

In the developing CNS, two alleles of *Pitx2c* are sufficient for MTT formation and only partially sufficient for collicular neuron migration, whereas a single allele of *Pitx2ab* is sufficient for both. One possible explanation for this discrepancy is that *Pitx2ab* is simply more abundant than *Pitx2c*¸ and therefore compensating for the loss of *Pitx2ab* requires the expression of multiple *Pitx2c* alleles. Alternatively, PITX2C may be less efficient at regulating genes which promote MTT and collicular development than is PITX2AB. Other studies have shown that *Pitx2a* induces the expression of *Pitx2c* (Guioli and Lovell-Badge, 2007; Kala et al., 2009), and *Pitx2c* is expressed in all organs known to express *Pitx2ab* (Gage and Camper, 1997; Kieusseian et al., 2006; Liu et al.,

2001; Liu et al., 2003), suggesting that *Pitx2c* in the developing midbrain may be induced by the presence of PITX2AB. Regardless, PITX2C appears redundant with PITX2AB function in the MTT and midbrain and independent functions of PITX2C are yet unknown.

Transcriptional regulation of collicular neuron migration and differentiation

In the superior colliculus, PITX2 is downstream of *Helt* and *Gata2* and is necessary for GABAergic differentiation (Kala et al., 2009; Miyoshi et al., 2004; Waite et al., 2011). *In vitro*, *Pitx2* is capable of inducing expression of *Gad1,* the gene encoding glutamate decarboxylase, which is an enzyme that catalyzes GABA synthesis and is necessary for GABAergic identity (Chen et al., 2011; Westmoreland et al., 2001). Therefore, *Pitx2* may be the first terminal differentiation factor identified in a subpopulation of GABAergic neurons in the superior colliculus. Other transcription factors such as *Pax3/7, Gata2, Lhx1/5,* and *Brn3a* are expressed during superior colliculus development and are required at various developmental stages; however, the spatiotemporal distribution of their expression and prior functional studies suggest they act earlier than terminal differentiation. *Pax3* and *Pax7* are expressed in progenitors, whereas *Gata2, Lhx1/5,* and *Brn3a* are expressed during or after neurogenesis. The paired-box transcription factors, *Pax3* and *Pax7,* are expressed throughout the dorsal neural tube, and are important for dorsal brain identity and polarity (Jostes et al., 1990; Kawakami et al., 1997; Matsunaga et al., 2001; Thomas et al., 2004). Midbrain progenitors continue to express *Pax3* but down-regulate *Pax7* later in development (Thompson et al., 2008). *Pax7* then becomes restricted to precursors and mature neurons, where it is thought to somehow establish regional identity neuronal maintenance (Jostes et al., 1990; Stoykova and Gruss, 1994; Thomas et al., 2004). While *Pax7* is expressed during terminal differentiation, it is unknown whether *Pax7* is involved in the terminal differentiation process.

Pax3/7 midbrain neural progenitors express *Gata2* as they undergo neurogenesis and continue *Gata2* expression as collicular precursors (Kala et al., 2009; Willett and Greene, 2011). *Gata2* is necessary for GABAergic neuronal identity determination and migration of neural precursors, but its expression turns off prior to terminal

differentiation (Kala et al., 2009; Willett and Greene, 2011). *Lhx1* and *Lhx5* (*Lhx1/5*) are LIM-homeodomain transcription factors that are expressed in *Gata2*-lineage collicular neurons (Kala et al., 2009). In the colliculus, *Lhx1/5* are expressed downstream of *Gata2* in progenitors undergoing neurogenesis and continue to be expressed in neuronal precursors and mature GABAergic neurons (Kala et al., 2009; Waite et al., 2011). *Lhx1/5* are required for neurogenesis, precursor differentiation, and maintenance of neuronal identity, but their roles in terminal differentiation are unclear (Pillai et al., 2007; Taira et al., 1994; Zhao et al., 1999). Unlike *Lhx1/5* and *Gata2,* the POU domain transcription factor *Brn3a* is expressed in post-mitotic glutamatergic precursors and mature glutamatergic neurons (Fedtsova and Turner, 1995; Lanier et al., 2009; Nakatani et al., 2007; Waite et al., 2011). No studies have identified the function of *Brn3a* in these collicular precursors. In trigeminal ganglion neurons, *Brn3a* is required for the expression of early fate markers and repression of alternate differentiation programs (Lanier et al., 2009), suggesting that it may also act earlier than terminal differentiation in the colliculus. Thus, unlike *Pitx2*, *Gata2, Lhx1/5* and *Brn3a* have not been associated with GABAergic terminal differentiation.

Independent regulation of collicular neuron migration and differentiation by Pitx2

It is unknown whether *Pitx2* regulation of midbrain neuronal migration and GABAergic differentiation are independent or linked processes. For example, the location of collicular neurons within the neuroepithelium may influence local inputs that direct terminal differentiation. If true, then *Pitx2* requirements for collicular neuron migration could be linked to its requirements for GABAergic differentiation. Alternatively, *Pitx2* could regulate a cell-autonomous differentiation program independent of its migrational functions. Interestingly, the tumor suppressor $p27^{Kip1}$ is capable of independently regulating both migration and differentiation by inhibiting *RhoA/ROCK* to promote neuronal migration and Ngn2 to promote differentiation (Nguyen et al., 2006). Different termini of the $\mathrm{p27^{Kip1}}$ protein regulate neuronal migration (N-terminal) and differentiation (C-terminal) (Nguyen et al., 2006). PITX2A regulates *RhoA* signaling to facilitate migration in HeLa cells and activates *Gad1* in developing neurons (Kirchhof et al., 2011; Morselli et al., 1999; Wei and Adelstein,

2002), suggesting that *Pitx2* could regulate midbrain neuronal migration and differentiation as independent processes. *Pitx2*$^{\Delta ab/-}$ midbrains exhibit medially mislocalized, yet GABA-positive neurons at E14.5, indicating that midbrain neurons can be medially mislocalized but still undergo GABAergic differentiation. Therefore, *Pitx2* is capable of independently regulating different developmental processes in the midbrain.

As genetic sequencing techniques have improved, the ability to identify causative variant mutations and link these mutations to developmental brain phenotypes has also advanced. Accurate assignment of functionality for sequence variants, however, requires an understanding of the developmental consequences produced by sequence variation. Our results highlight the unique developmental requirements for *Pitx2* isoforms, which could be critical for functional annotation of future sequence analyses in humans. Ultimately, this could improve our ability to diagnose and treat a variety of neurodevelopmental disorders.

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Chapter IV Notes

[1]A revised version of Chapter IV has been submitted for publication as Waite, M.R., Skidmore, J.M., Shiratori, H., Hamada, H., Martin, J.F., and Martin, D.M. (2012). Pleiotropic and isoform-specific functions for *Pitx2* in superior colliculus and hypothalamic neuronal development.

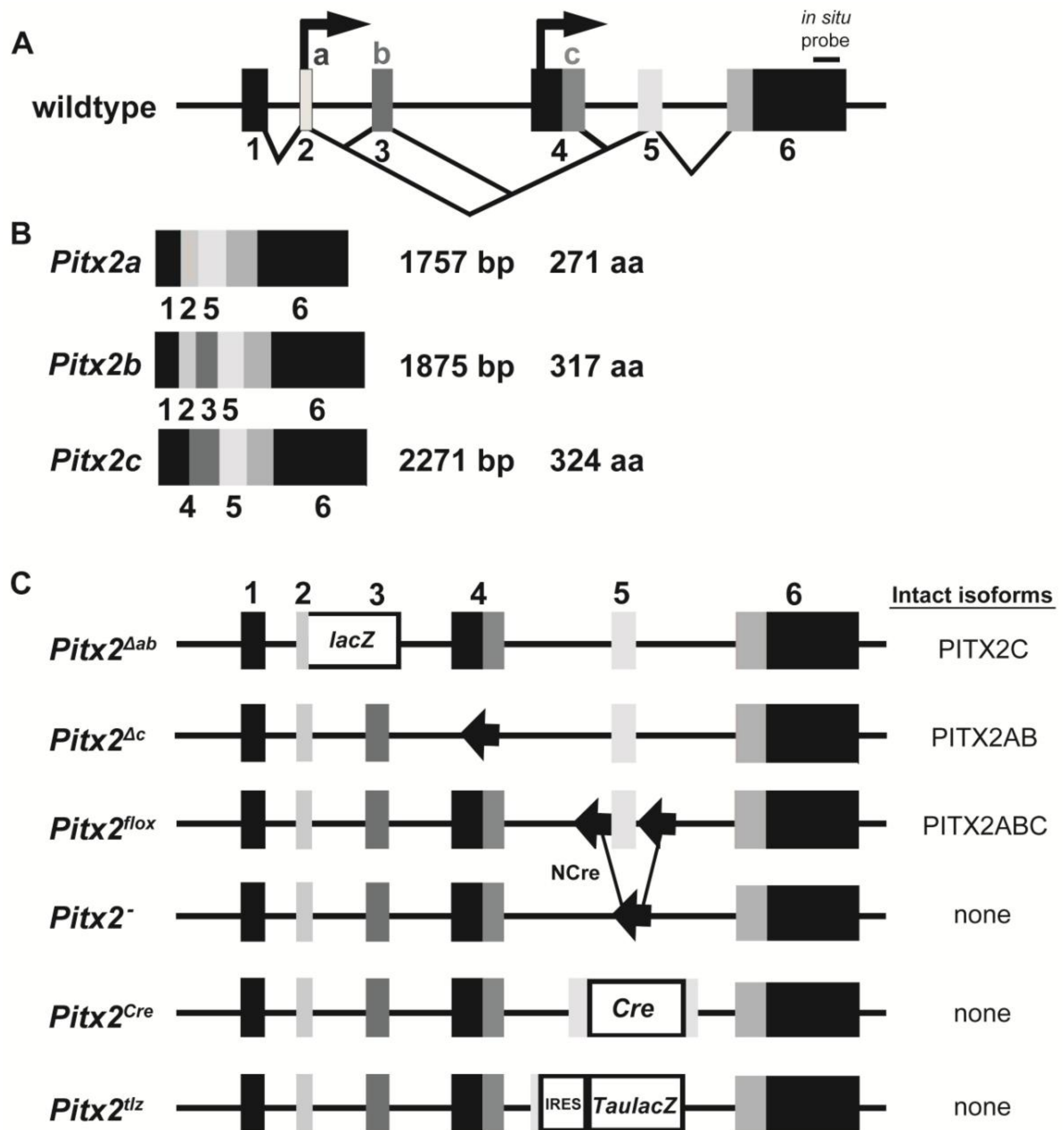
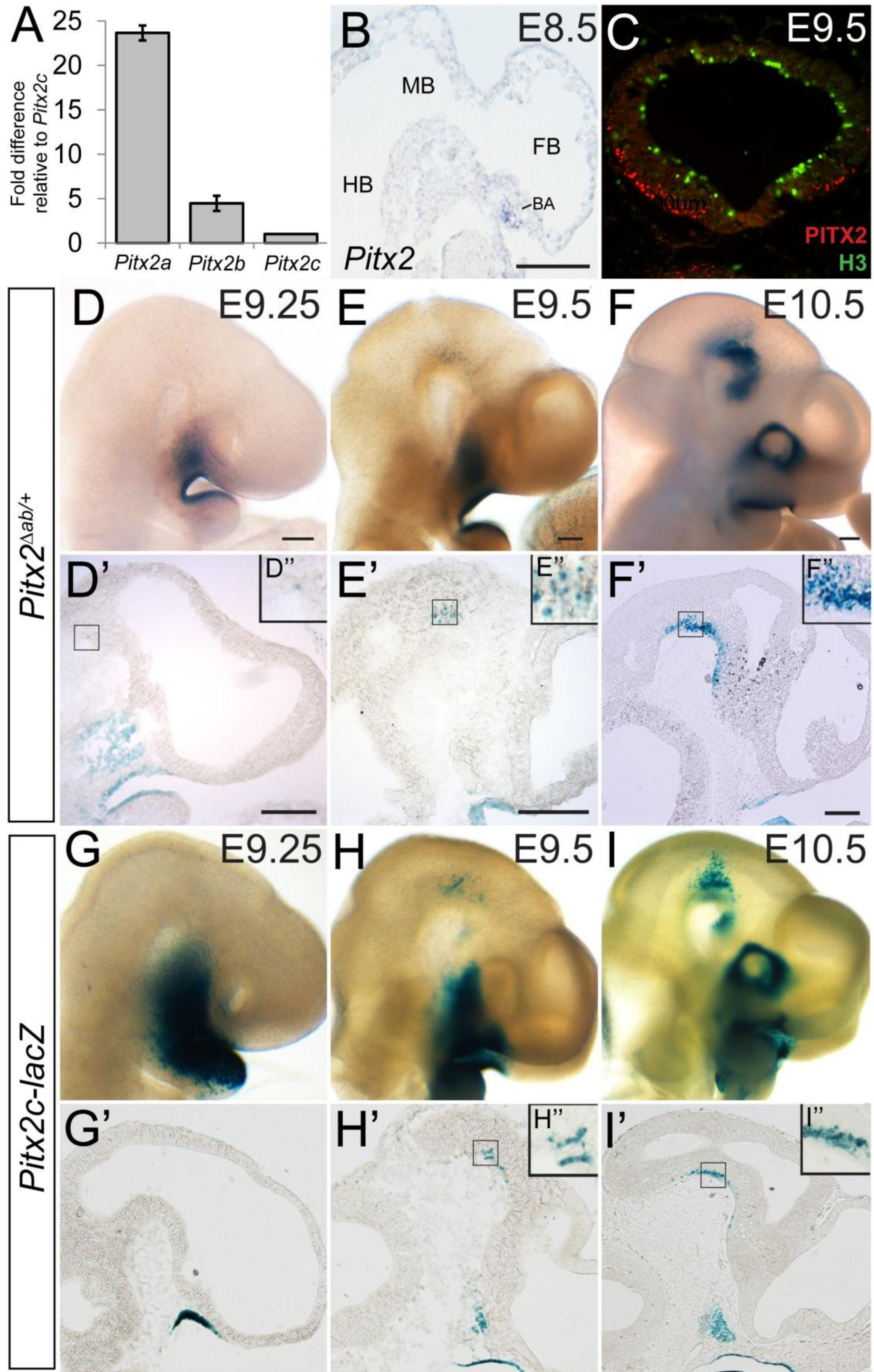


Figure 4.1. ***Pitx2* isoforms and alleles.** (A) Map of the *Pitx2* gene showing exons, introns, and isoforms. Arrows indicate alternate transcription start sites. (B) Summary of exon usage and size of *Pitx2* isoforms. (C) List of mouse *Pitx2* alleles used to generate unique *Pitx2* deficient embryos.

Figure 4.2. *Pitx2* is expressed in early post-mitotic midbrain neurons. (A) QPCR for *Pitx2a, Pitx2b,* and *Pitx2c* from E14.5 midbrain RNA shows that *Pitx2a* and *Pitx2b* are 23.66-fold and 4.46-fold more abundant than *Pitx2c,* respectively. (B) Sagittal section of an E8.5 wild type embryo processed for *in situ* hybridization shows *Pitx2* mRNA in the branchial arch (BA). (C) Coronal section of an E9.5 wild type midbrain immunostained for PITX2 (red) and H3 (green). $Pitx2^{\Delta ab/+}$ (D-F) and *Pitx2c-lacZ* (G-I) embryos (E9.25-E10.5) processed for wholemount X-gal staining. (D'-I') Sagittal sections from embryos shown in D-I. Boxes in D'-I' are enlarged in D''-I''. *Pitx2ab* expression is visible in the ventral midbrain in rare cells at E9.25, and is easily detected at E9.5 and E10.5. *Pitx2c* expression is first visible in the ventral midbrain at E9.5, and is more abundant at E10.5. Abbreviations: BA, branchial arch; FB, forebrain; HB, hindbrain; MB, midbrain. Scale bar in B is 100 µm. Scale bars in D, E, and F are 200 µm and apply to panels D-I. Scale bars in D', E', and F' are 250 µm and apply to panels D':G', E':H', and F':I'.


A
25
20
15
10
5
0
Fold difference relative to Pitx2c
Pitx2a
Pitx2b
Pitx2c
B
E8.5
MB
FB
HB
BA
Pitx2
C
E9.5
PITX2
H3
D
E9.25
E
E9.5
F
E10.5
Pitx2^Δab/+
D'
D''
E'
E''
F'
F''
G
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H
E9.5
I
E10.5
Pitx2c-lacZ
G'
H'
H''
I'
I''

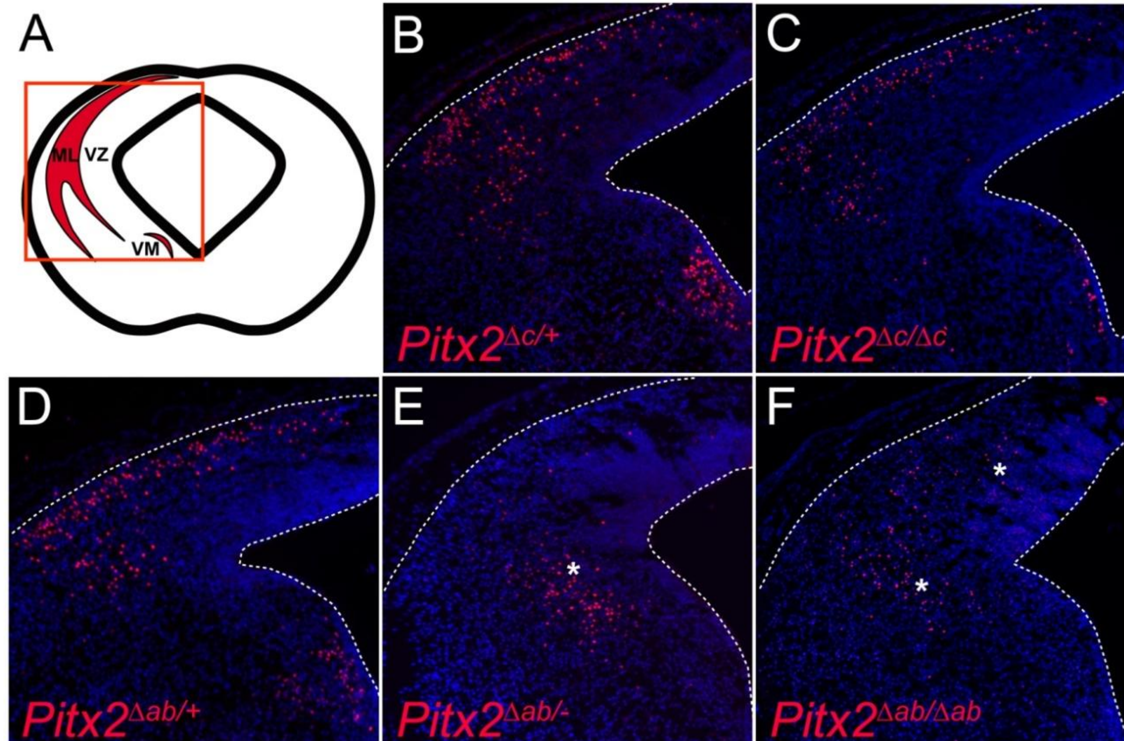
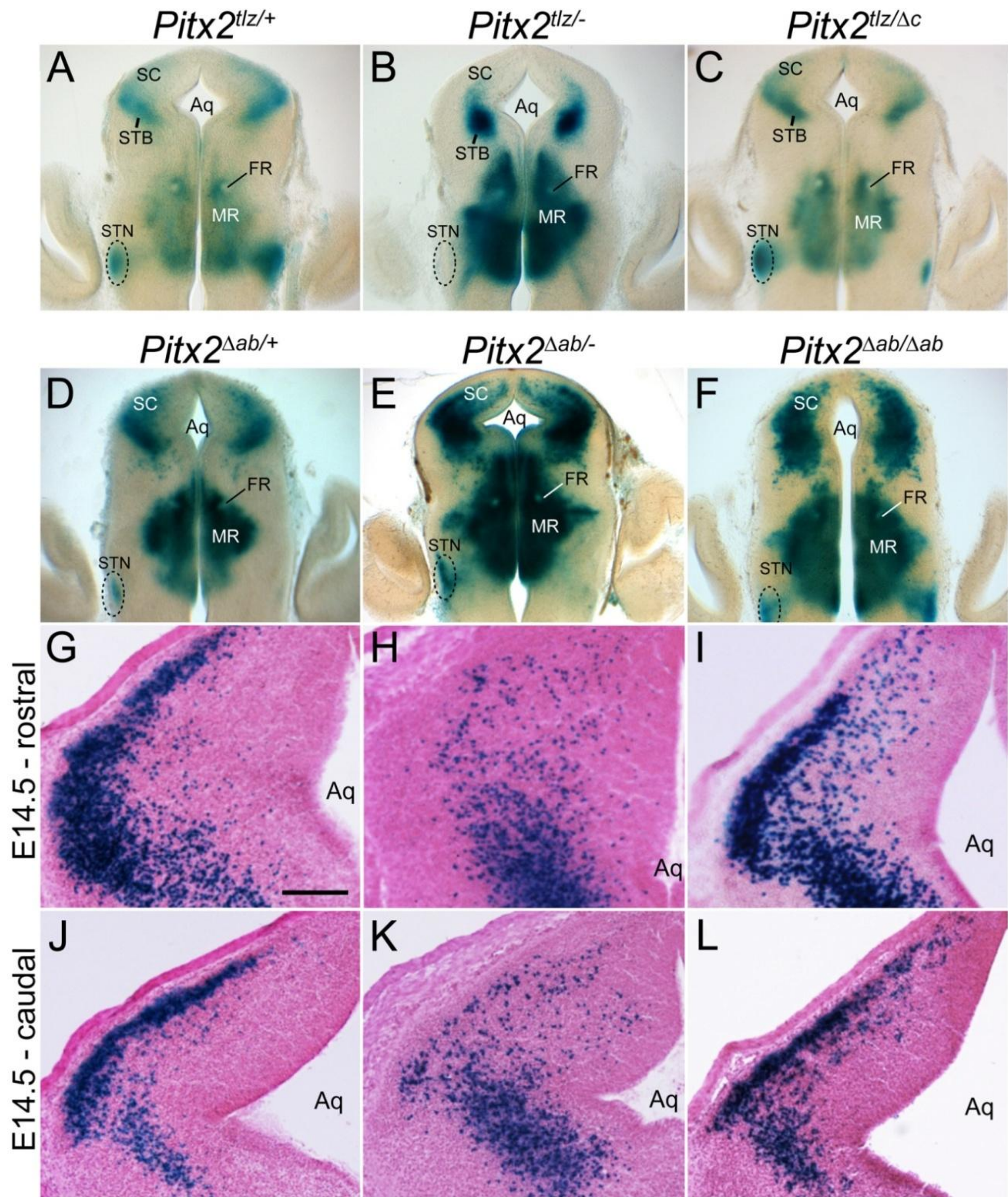


Figure 4.3. ***Pitx2ab* and *Pitx2c* are expressed in midbrain neurons.** (A) Schematic of coronal midbrain section highlighting *Pitx2* expression as shown in panels B-F. (B-F) E14.5 coronal midbrain sections processed for PITX2 immunofluorescence. (B) $Pitx2^{\Delta c/+}$, (C) $Pitx2^{\Delta c/\Delta c}$, and (D) $Pitx2^{\Delta ab/+}$ midbrains exhibit PITX2-positive cells at the collicular pial surface and in the ventromedial (VM) population. (E-F) $Pitx2^{\Delta ab/-}$ midbrains exhibit medially mislocalized PITX2-positive cells (*), whereas $Pitx2^{\Delta ab/\Delta ab}$ collicular PITX2-positive cells exhibit an intermediate location (*).

Figure 4.4. ***Pitx2*** **isoforms exhibit differential contributions to midbrain neuron migration.** (A-F) Coronal midbrain sections of E14.5 X-gal stained, vibratome-sectioned (150 μm) embryos. (A,D) Embryos heterozygous for *Pitx2* ($Pitx2^{tlz/+}$) and *Pitx2ab* ($Pitx2^{\Delta ab/+}$) exhibit X-gal staining in the superior colliculus (SC), mammillary region (MR), and subthalamic nucleus (STN). (B) $Pitx2^{tlz/-}$ mutants exhibit medial mislocalization of collicular βgal-positive neurons and absence of label in the subthalamic nucleus. (C) $Pitx2^{tlz/\Delta c}$ embryos display normal βgal patterning in both midbrain and hypothalamus. (E) $Pitx2^{\Delta ab/-}$ embryos exhibit medially denser label. (F) $Pitx2^{\Delta ab/\Delta ab}$ embryos exhibit an intermediate phenotype, with some collicular neurons reaching the pial surface and others occupying deeper locations. X-gal stained coronal cryosections of E14.5 (G,J) $Pitx2^{\Delta ab/+}$, (H,K) $Pitx2^{\Delta ab/-}$, or (I,L) $Pitx2^{\Delta ab/\Delta ab}$ colliculi. Panels are arranged rostral (G-I) to caudal (J-L) with $Pitx2^{\Delta ab/-}$ (H,K) and $Pitx2^{\Delta ab/\Delta ab}$ (I,L) rostral sections showing more severe mislocalization phenotypes than caudal sections. Scale bar in G is 150 μm and applies to panels G-L. Other abbreviations: Aq, aqueduct; FR, fasciculus retroflexus; MR, mammillary region; SC, superior colliculus; STB, subtectal band; STN, subthalamic nucleus.



Pitx2tlz/+
Pitx2tlz/-
Pitx2tlz/Δc
A
B
C
SC
Aq
STB
FR
MR
STN
Pitx2Δab/+
Pitx2Δab/-
Pitx2Δab/Δab
D
E
F
G
H
I
J
K
L
E14.5 - rostral
E14.5 - caudal

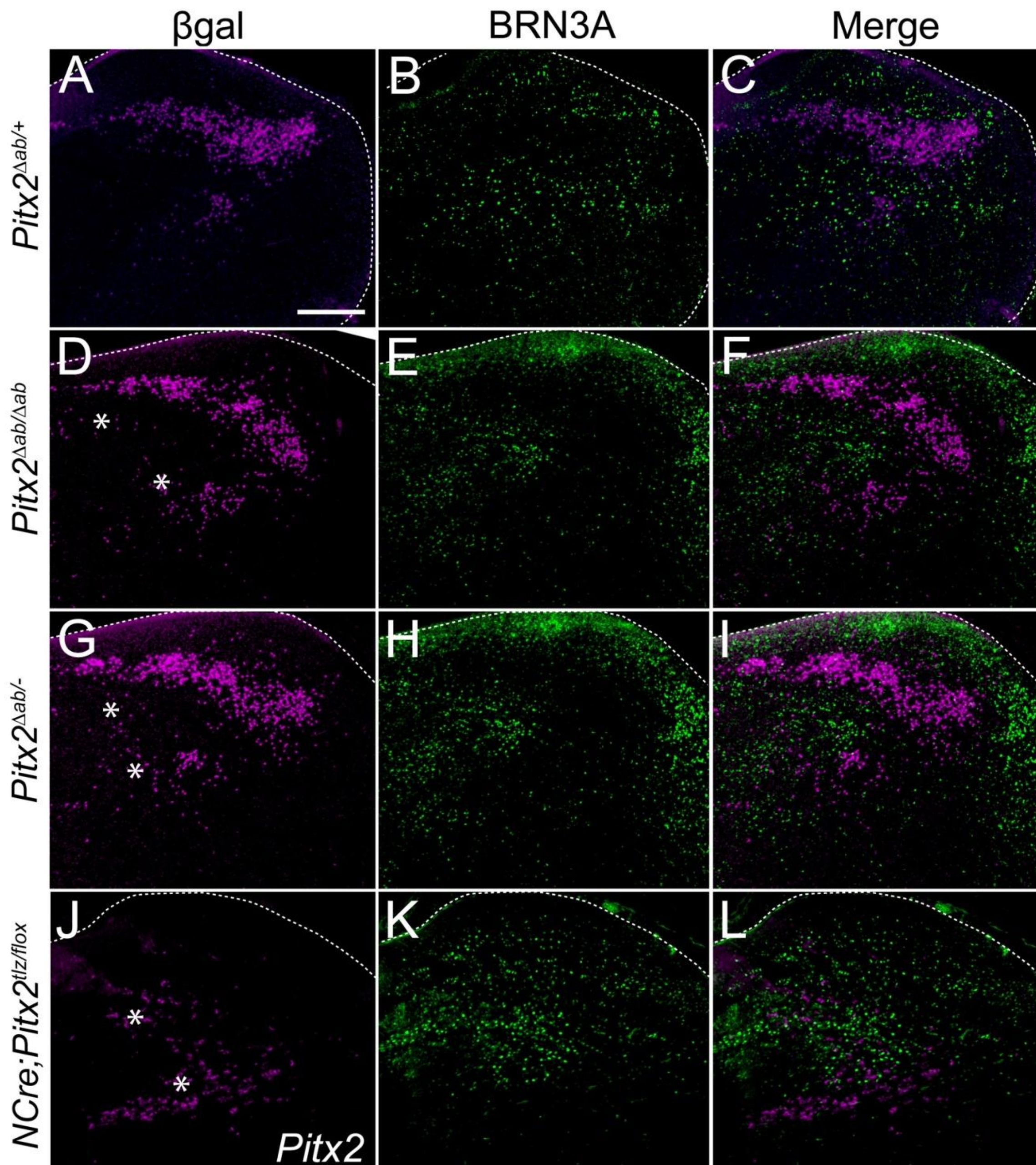


Figure 4.5. *Pitx2ab* regulates the timing of midbrain neuronal migration. (A-L) Pseudocolored and merged images of neighboring coronal midbrain cryosections of E18.5 *Pitx2*$^{\Delta ab/+}$, *Pitx2*$^{\Delta ab/-}$, and *Pitx2*$^{\Delta ab/\Delta ab}$*,* and *NCre;Pitx2*$^{tlz/flox}$ alleles processed for X-gal (A, D, G) or *Pitx2 in situ* (J) and adjacent sections processed for BRN3A immunofluorescence (B, E, H, K). Merged images show some relatively normal β-gal positive neuron localization (C, F, I) with some genotypes exhibiting a number of medially mislocalized neurons (*) (F, I, L). Scale bar in A is 250 μm and applies to panels A-L.

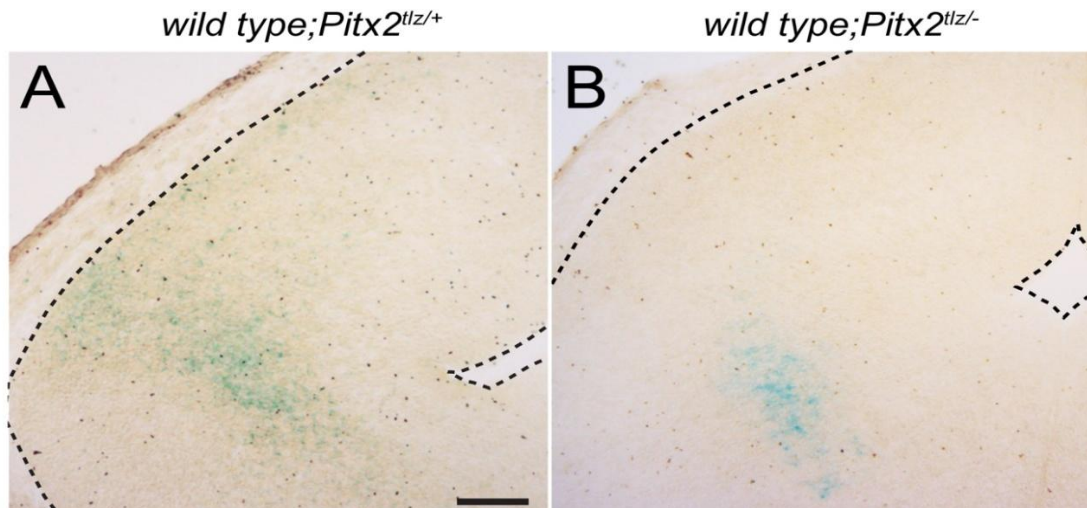


Figure 4.6. Evidence for cell-autonomous effects of *Pitx2* deficiency on collicular neuronal migration. Coronal midbrain cryosections from E14.5 *wild type;Pitx2*$^{tlz/+}$ (A) or *wild type*;*Pitx2*$^{tlz/-}$ (B) chimeras produced from mouse embryonic stem (ES) cells and processed for X-gal histochemistry. (A) *wild type;Pitx2*$^{tlz/+}$ sections display proper patterns of collicular X-gal stained neurons, whereas neurons in the *wild type;Pitx2*$^{tlz/-}$ colliculus are mislocalized deeper in the neuroepithelium (B). Scale bar in A is 200 µm and applies to panels A and B.

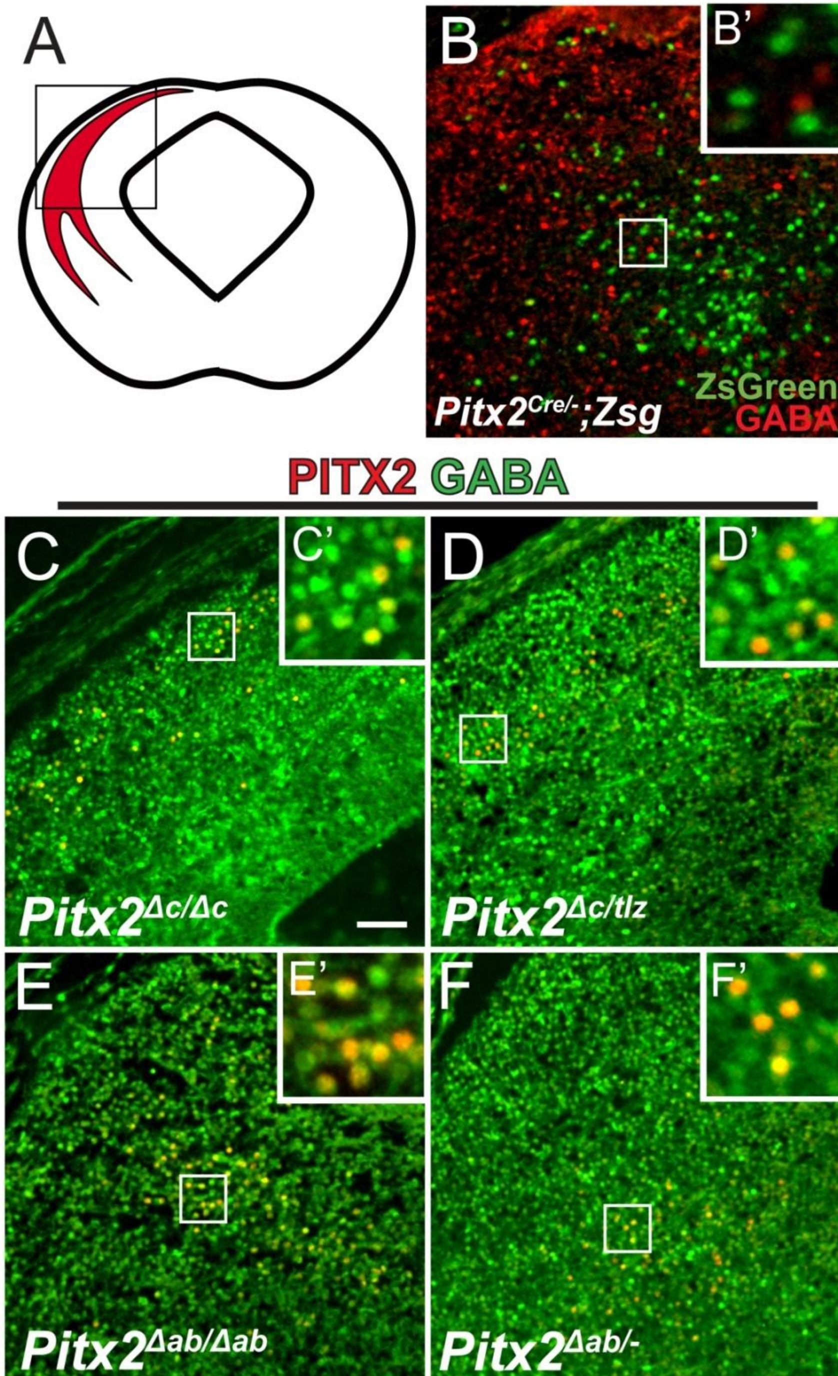
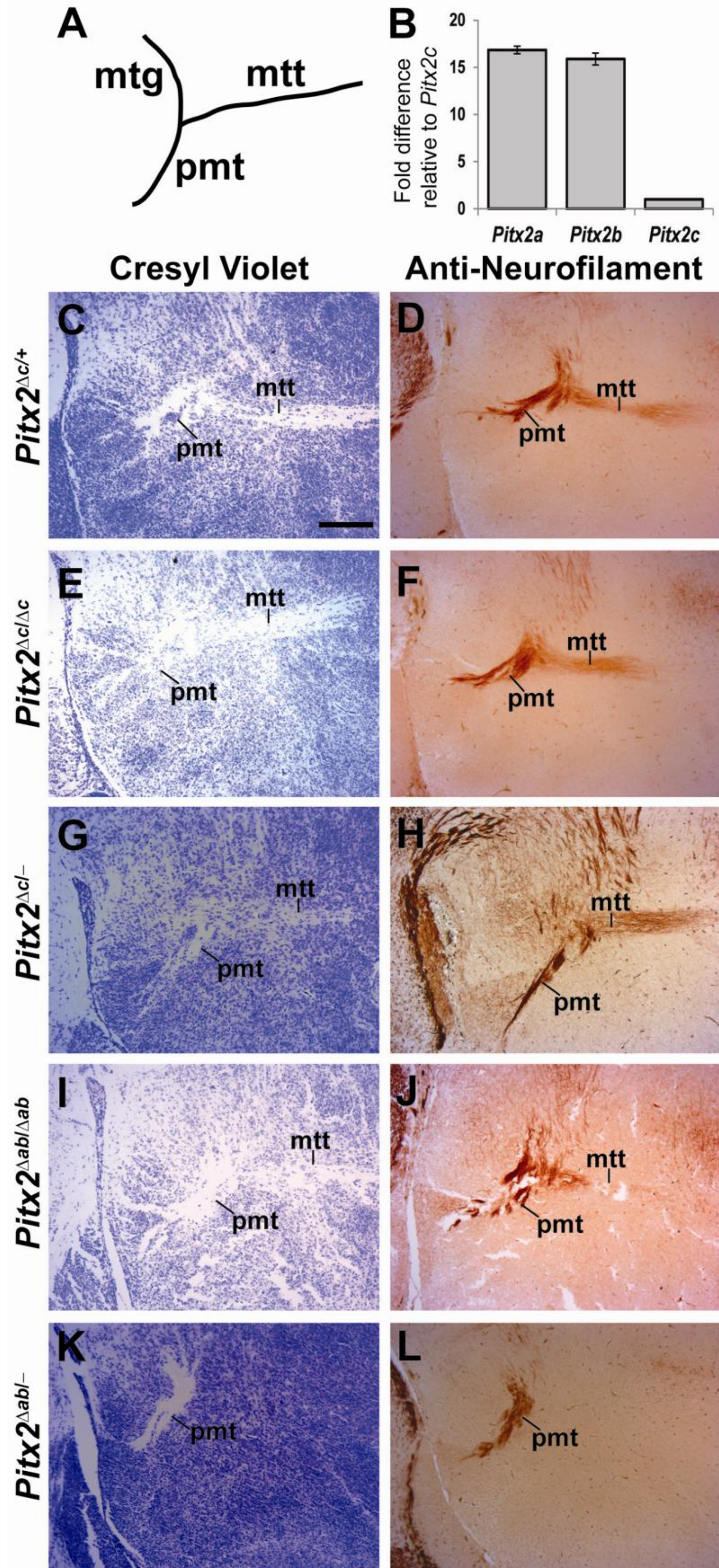


Figure 4.7. Collicular GABAergic differentiation requires a single allele dose of either *Pitx2ab* or *Pitx2c*. (A) Cartoon showing coronal view of an embryonic mouse midbrain identifying the dorsal *Pitx2*-positive population. Box indicates location of *Pitx2*-positive neurons magnified in panels B-F. (B) E14.5 $Pitx2^{Cre/-}$*;Zsg* (green) coronal midbrain section processed for immunofluorescence against GABA (red). (C-F) E14.5 coronal midbrain sections processed for double-immunofluorescence against PITX2 (red) and GABA (green). (C) $Pitx2^{\Delta c/\Delta c}$, (D) $Pitx2^{\Delta c/tlz}$, (E) $Pitx2^{\Delta ab/\Delta ab}$, and (F) $Pitx2^{\Delta ab/-}$ colliculi exhibit similar co-localization of PITX2 and GABA. Scale bar in B is 50 µm and applies to panels B-F.

Figure 4.8. PITX2AB is necessary for formation of the mammillothalamic tract (MTT). (A) Cartoon of a sagittal section identifying tracks in the forebrain. (B) QPCR for *Pitx2a, Pitx2b,* and *Pitx2c* from E14.5 midbrain RNA shows that *Pitx2a* and *Pitx2b* are 13.83-fold and 16.93-fold more abundant than *Pitx2c,* respectively. E18.5 sagittal brain sections processed were for cresyl violet staining (C, E, G, I, K) or immunohistochemistry for Neurofilament (D, F, H, J, L). (C-D) $Pitx2^{\Delta c/+}$, (E-F) $Pitx2^{\Delta c/\Delta c}$, (G-H) $Pitx2^{\Delta c/-}$, and (I-J) $Pitx2^{\Delta ab/\Delta ab}$ embryos exhibit normal MTT. (K-L) $Pitx2^{ab/-}$ embryos lack the MTT stemming from principal mammillary tract (PMT). Scale bar in B is 200 µm and applies to panels B-I. Abbreviations: mtt, mammillothalamic tract; pmt, principle mammillary tract.


A
mtg
mtt
pmt
B
Fold difference relative to Pitx2c
20
15
10
5
0
Pitx2a
Pitx2b
Pitx2c
Cresyl Violet
Anti-Neurofilament
C
D
E
F
G
H
I
J
K
L
Pitx2Δc/+
Pitx2Δc/Δc
Pitx2Δc/−
Pitx2Δab/Δab
Pitx2Δab/−
mtt
pmt

	Pitx2 dosage: High ——— Low					
Function	$Pitx2^{+/-}$	$Pitx2^{\Delta c/+}$	$Pitx2^{\Delta c/\Delta c}$	$Pitx2^{\Delta ab/\Delta ab}$	$Pitx2^{\Delta ab/-}$	$Pitx2^{-/-}$
GABAergic differentiation	nl	nl	nl	nl	nl	negative
MTT formation	nl	nl	nl	nl	absent	absent
Neuronal migration	nl	nl	nl	interm	disrupted	disrupted

Table 4.1. Different functions during collicular development require unique *Pitx2* dosage. Left side of table lists developmental functions. Mouse genotypes at top of table are in order from highest *Pitx2* dose (left) to lowest (right). Green boxes indicate a normal phenotype, yellow indicates an intermediate (int) phenotype, and red boxes indicate abnormal phenotypes. The box referencing MTT formation in $Pitx2^{-/-}$ embryos refers to results from E18.5 conditional *Pitx2* knockout embryos ($NCre;Pitx2^{tlz/flox}$) (Skidmore et al., 2012).

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Chapter V

Conclusion

My thesis work has focused on the pleiotropic requirements for transcription factors during brain development. Specifically, I have identified requirements for the homeodomain transcription factor *Pitx2* during development of unique neuronal subpopulations.

Identities of CNS *Pitx2*-positive neurons

The findings in this dissertation have contributed to our understanding of the identities of *Pitx2*-positive neuronal populations in the developing brain. In the dorsal midbrain (superior colliculus), *Pitx2*-positive neurons are GABAergic, but do not express early markers of GABAergic differentiation. At later ages, collicular *Pitx2*-positive neurons are localized to the intermediate layer (stratum griseum intermedium [SGI]) and are surrounded by BRN3A-positive layers. In contrast, *Pitx2*-expressing neurons in the ventral midbrain are glutamatergic and localized to a ventromedial population and the red nucleus. The ventromedial population co-expressed *Brn3a, Lhx1/5, Foxa2,* and *Nkx6.2,* whereas the red nucleus population co-expressed *Brn3a, Lhx1/5,* and *Foxa2.*

In r1, *Pitx2*-positive neurons are GABAergic, but can be subdivided into at least two unique, intermingled subpopulations. The two *Pitx2*-positive populations express *Pax2* and *Nkx6.1* in a mutually exclusive manner. Additionally, some *Pitx2*-positive r1 neurons arise from a *Dbx1*-lineage progenitor pool, suggesting that these are similar to spinal cord V0 interneurons. *Pitx2*-positive neurons which are *Dbx1*-lineage negative likely constitute a population similar to spinal cord V2-V3 neural populations. These data suggest that *Pitx2*-positive populations throughout the CNS may be subdivided into neuronal subpopulations based on localization and transcription factor identity.

Significance and Future Directions

The results presented here describe the unique lineage mapping, transcription factor expression patterns, and neurotransmitter identity of *Pitx2*-positive subpopulations throughout the developing brain. The data from the midbrain and r1 were incorporated to create maps detailing transcription factor expression patterns and unique neuronal nuclei in the context of *Pitx2* expression. These maps improve our ability to identify unique neuronal populations, collicular layers, and brain nuclei and will also improve the clarity of our dialogue when discussing specific neuronal populations. As such, these brain expression maps are important for applying local context to future studies on brain development and can be used as a basis for further transcription factor mapping studies. Additionally, transcription factor maps provide basic expression data for developing neuronal populations, and as such may be useful for extrapolating the differentiation requirements for each population. Thus, these maps may be an important starting point for studies identifying the unique transcription factor combinations required to promote development of *in vitro* stem cells/neural progenitors.

Although we created expression maps of the developing brain, further refinements identifying the *Pitx2*-positive populations are necessary. We were able to identify the transcription factors co-expressed by *Pitx2* ventromedial neurons, but could not identify neurotransmitter identity or mature nucleus structure. To determine the neurotransmitter identity of these ventromedial neurons, *in situ* hybridization could be performed for *Pitx2, Gad67, Vglut2,* and *VAChT* or immunofluorescence for PITX2, GABA, VGLUT2 or ChAT on E18.5 midbrains. Localization of the *Pitx2*-positive neurons suggests they may belong to the Edinger-Westphal nucleus, supraoculomotor nucleus cap, or the nucleus of Darkschewitsch (Cornwall et al., 1990; Joksimovic et al., 2009; Moreno-Bravo et al., 2010). To identify the nucleus containing these neurons, one could look at population-specific markers such as *Nenf* or *Ghsr* for the Edinger-Westphal nucleus, *NADPH-diaphorase* for the supraoculomotor cap, and *urocortin* for the nucleus of Darkschewitsch (Burnell et al., 2009; Carrive and Paxinos, 1994; Weninger et al., 1999).

In r1, we determined that some *Pitx2*-positive neurons are from *Dbx1*-lineage progenitors. However, we did not identify the lineage of the remaining *Pitx2*-positive neurons. Based on PITX2 co-localization with NKX6.1, we hypothesized that these

neurons might be V2 or V3 interneurons as in the spinal cord. However, to properly identify this population, lineage tracing for *Olig2* and *Nkx2.2* is necessary. According to spinal cord progenitor expression maps, *Olig2* expression is specific to V2 progenitors, whereas *Nkx2.2* is expressed only in V3 progenitors (Caspary and Anderson, 2003; Liu et al., 2007; Ulloa and Briscoe, 2007). On a broader scale, it would be interesting to see whether ventral r1 recapitulates the expression patterns of the spinal cord and whether analogous groups of neurons in the two structures have similar functions.

We have either identified or at least narrowed down the identities of several *Pitx2*-positive populations throughout the CNS, but we have not determined their axonal targets. This is unfortunate, because knowing the synaptic pathways of *Pitx2*-positive neurons could aid in determining their mature identity and functions. To determine the projections of *Pitx2*-expressing neurons in the brain, *Pitx2*$^{Cre/+}$*;Zsg* adult brains could be analyzed for ZsGreen fluorescence, which localizes to *Pitx2*-lineage cell bodies and axons. Cryosections would provide single-axon focus, whereas vibratome sections would highlight entire axon tracts. It would be relatively simple, and interesting, to create a map of *Pitx2*-positive tracts throughout the CNS which could be useful for future tract and developmental studies.

Although the identities of the *Pitx2*-positive neuronal populations could use further refinement, our current conclusions allow for speculation as to the function of mature *Pitx2*-positive neural populations. In the ventral midbrain, we identified one *Pitx2*-expressing population in the red nucleus, which is made up of glutamatergic neurons projecting to both the cervical and lumber spinal cord (Liang et al., 2011). The red nucleus has been implicated in regulating rhythmic jaw movements, limb posture coordination, and extremity motor control during skilled tasks (Hermer-Vazquez et al., 2004; Jarratt and Hyland, 1999; Satoh et al., 2006; Zelenin et al., 2010). These data suggest that *Pitx2*-positive neurons in the red nucleus may also be performing any or all of these tasks.

The superior colliculus is a structure required for producing saccadic eye movement in response to sensory inputs (King, 2004; Schiller and Stryker, 1972). Neuronal activity in the superficial collicular layers is responsible for encoding a map of the visual field, whereas the SGI encodes a map focused on a single position targeting the

next saccadic movement (Goldberg and Wurtz, 1972a, b; Robinson, 1972; Sparks, 1978; Sparks et al., 1976; Wurtz and Goldberg, 1972). GABAergic neurons in the SGI are believed to be responsible for suppressing superficial collicular activity during saccadic eye movement, thereby preventing an infinite feed-forward loop of saccades (Isa and Hall, 2009; Lee et al., 2007; Phongphanphanee et al., 2011). It is unknown whether PITX2-positive neurons are required for proper saccadic activity through superficial neuron inhibition or even whether *Pitx2*-positive SGI neurons project to the superficial layers. To assess whether loss of *Pitx2* in the developing colliculus results in changes to saccadic performance, brain-specific conditional *Pitx2* knockout mice (such as *En1-Cre;Pitx2*$^{flox/-}$*)* could be analyzed for saccadic behavior. Previous studies have generally focused on ablation of the entire colliculus or only the superficial layers; therefore, it is not known exactly which types of behavioral phenotypes would result from an abnormal intermediate layer. However, knowing that the SGI is responsible for focusing saccadic movements to a single external target, we may expect that loss of proper SGI function would result in delayed or deficient ability to change focus to a visual target, such as a blinking light, or even reduced ocular motility during saccades (Goodale and Murison, 1975; Zhou et al., 2009).

Additionally, we have pieced together possible *Pitx2* expression pathways from other studies, but future studies on *Pitx2* in the brain could benefit from a deeper knowledge of the region-specific pathways regulating *Pitx2* expression. Specifically, it would be beneficial to know which proteins are regulating *Pitx2* expression in the different developing CNS areas. To do this, we could use a Yeast-One-Hybrid (Y1H) strategy with the *Pitx2* promoter as bait and cDNA libraries from different regions within the embryonic and adult mouse brain as prey. We could then validate the results by evaluating candidate activation of the *Pitx2* promoter in luciferase assays or by assessing *Pitx2* expression in the brains of available candidate gene knockout mice. Another option would be to perform *in vivo* electroporation to either overexpress or knock down genes of interest and evaluate the effects on *Pitx2* expression.

Axial-level and isoform-specific requirements for *Pitx2* during brain development

Here, we have mapped the expression of *Pitx2* isoforms in the early developing brain using reporter mouse strains. Additionally, we have identified unique requirements for *Pitx2* during brain development. We showed that *Pitx2* is not involved in early transcription factor patterning in the midbrain or hindbrain, but that *Pitx2* is required for superior colliculus neuronal migration and GABAergic differentiation, as well as MTT formation. Each of these functions has unique *Pitx2* isoform-specific requirements: neural migration requires at least one allele of *Pitx2ab,* GABAergic differentiation requires one copy of any allele, and MTT formation requires either one allele of *Pitx2ab* or two alleles of *Pitx2c.*

Significance and Future Directions

Our results identifying a requirement for *Pitx2* in MTT development are the first to implicate *Pitx2* in axon tract formation and are unusual in that *Pitx2* appears to be required in a non-cell autonomous manner. These data suggest that *Pitx2* may also have non-cell autonomous effects on other axon tracts throughout the CNS. To determine whether this is true, we could analyze Neurofilament immunofluorescence to identify axon tract morphology throughout the CNS in embryonic littermates heterozygous or null for *Pitx2*. Because some axon tracts, such as the MTT, do not form until later stages of development, Neurofilament could also be analyzed in the CNS on postnatal mice with conditional *Pitx2* knockout in the CNS using a *Cre* line such as *NCre* (brain), *En1-Cre* (brain), or *Bcre32* (spinal cord) crossed with *Pitx2*$^{flox/-}$ (Kala et al., 2009; Sclafani et al., 2006; Wine-Lee et al., 2004; Yu et al., 2011; Zervas et al., 2004). Additionally, the peripheral nervous system could be analyzed for axon development by crossing the *Peripherin-EGFP* mouse line, which expresses EGFP throughout the peripheral nervous system, with *Pitx2* null and conditional knockout strains (McLenachan et al., 2008). Wholemount embryos and vibratome sections could be easily compared for EGFP patterning throughout the body.

Our studies are also the first to identify unique dosage requirements for transcription factor isoforms during brain development, and suggest that different isoform dosages may produce unique developmental functions. Specifically, *Pitx2ab* appears to

be required for proper brain development, whereas *Pitx2c* is dispensable. In contrast, previous studies in non-CNS tissues have focused on *Pitx2c* and identified requirements for *Pitx2c* in the heart, lungs and gut, but did not identify any *Pitx2ab* requirements. This requirement for specific isoforms adds a layer of complexity to brain development, and suggests that therapies targeting a specific protein or pathway should take into account specific isoforms or isoform functions. In this way, potential therapies could be more efficient and produce fewer side effects.

Although we have identified unique requirements for *Pitx2* isoforms during the development of some brain regions, the mechanisms behind these developmental functions are unknown and the function of *Pitx2* is still unknown in some regions (such as r1 and the spinal cord). In order to identify downstream targets of PITX2 in a region-specific manner (and therefore possibly identify functions for *Pitx2* in r1 and spinal cord), a microarray could be performed using microdissected CNS tissue (hypothalamus, midbrain, r1, spinal cord) from E12.5-E14.5 $Pitx2^{Cre/+}$*;Zsg* and $Pitx2^{Cre/-}$*;Zsg* littermates. A flow-sorting step could be added to restrict the assessment to *Pitx2*-expressing cells, thereby reducing signal dilution from extraneous tissue. However, we have shown here that *Pitx2* can function in a non-cell autonomous manner, and therefore flow-sorting could inappropriately eliminate downstream candidates. In addition to the microarray, a Western blot could also be performed to look for levels of GTP-bound RhoA and RAC1, as PITX2 may regulate neuronal migration by regulating activation of these proteins (GTP-bound) and not their expression levels (Wei and Adelstein, 2002).

Candidate gene results from the microarray could be validated using *in situ* hybridization on brain sections from *Pitx2* heterozygous versus null (or conditional knockout) littermates. While *in situ* hybridization is not quantitative, it is useful for determining qualitative gene expression in areas of interest. A more quantitative approach would be to analyze target gene expression in microdissected tissue using Real-Time PCR (QPCR). In order to determine whether *Pitx2* is capable of directly regulating validated candidate gene expression, luciferase assays could be performed using the gene of interest promoter as bait. One major caveat to the luciferase assay is that regulation of different developmental functions by *Pitx2* may require unique *Pitx2* dosages. Therefore,

the amount of transfected *Pitx2* may need to be taken into account when analyzing results.

PITX2 co-regulator binding proteins are also unknown. Identifying CNS region-specific binding partners of PITX2 may elucidate new functions of PITX2 and also provide a mechanistic understanding of how PITX2 performs unique functions in different CNS regions. PITX2 is already known to utilize unique binding partners to induce tissue-specific gene expression in other organs; in the pituitary, PITX2 binds the pituitary-specific protein PIT1 to induce *Prolactin* gene expression (Amendt et al., 1998; Quentien et al., 2002). In order to identify tissue-specific PITX2 binding partners, CNS tissue could be microdissected, protein purified and immunoprecipitated by targeting for PITX2, then analyzed by mass spectrometry. There are several technical caveats with this approach, but one of the most concerning is that some proteins which synergize with PITX2 do not physically bind the PITX2 protein. For example, NKX2.5 synergizes with PITX2C to enhance expression of heart-specific genes such as ANF and PLOD1, but PITX2 and NKX2.5 bind the promoter independently and do not physically interact with each other (Ganga et al., 2003).

Once the tissue-specific gene targets and binding proteins are identified *in vivo*, then *in vitro* experiments could be performed to identify which PITX2 amino acids are required for gene transactivation and protein binding. The results of this would help elucidate which *Pitx2* isoforms are responsible for individual functions. For example, if transactivation of a gene requires exon 4, then PITX2C must be the dominant isoform regulating expression of that gene. These data could improve our understanding of isoform-specific requirements during embryonic development.

Summary

The results reported in this dissertation improve our understanding of brain development at the molecular level. We have mapped *Pitx2* expression in the developing brain in relation to neurotransmitters and other transcription factors. Additionally, our data indicate that *Pitx2* is important for proper mouse brain development in an isoform- and region-specific manner, and suggest *Pitx2* dosage may dictate developmental function. A deeper understanding of *Pitx2* action during brain development will require

elucidation of the downstream mechanisms by which *Pitx2* and its individual isoforms function.

Our data suggest that transcription factors are critical regulators of regional neuronal development and that splicing of transcription factor genes provides an important layer of complexity. These data may help us better understand the cause and effect of improper neural development and could therefore be useful in diagnosing individuals with neurological disorders. An understanding of neural requirements during development will also be useful in developing new therapies targeted to neurological conditions. For example, embryonic and neural stem cell therapies often require manipulation of cell identity or function, which necessitates an understanding of the molecular requirements for different aspects of neural development (proliferation, migration, differentiation, survival, axon outgrowth). Therefore, greater focus on the mechanisms of CNS development will improve our understanding of neural disorders and is necessary for diagnosing, preventing, and repairing both the improperly developed and the injured CNS.

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Appendix

Methods

Mice

Nestin-Cre (*NCre)* transgenic mice (Tronche et al., 1999) were crossed with *Pitx2*$^{tlz/+}$ (Skidmore et al., 2012) to produce *NCre;Pitx2*$^{tlz/+}$ mice. *NCre;Pitx2*$^{tlz/+}$ mice were then crossed with *Pitx2*$^{flox/flox}$ mice (Gage et al., 1999) to generate *NCre;Pitx2*$^{tlz/flox}$ and *Pitx2*$^{tlz/flox}$ littermates.

Tissue Preparation

The morning of vaginal plug identification was designated as E0.5. Pregnant females underwent cervical dislocation and hysterectomy and embryos were dissected into PBS. For embryonic BrdU studies, pregnant dams were intraperitoneally injected with 300ul of 10mg/ml 5-bromo-2'-deoxyuridine (BrdU) (Roche). Embryos were fixed in 4% paraformaldehyde for 1-2 hours, depending on age. For frozen sections, embryos were cryoprotected overnight in 30% sucrose-PBS, flash frozen in O.C.T. embedding compound (Tissue Tek, Torrance, CA), and stored at -80°C until being sectioned at 12 µm. For paraffin sections, embryos were dehydrated in an ethanol gradient, embedded in paraffin, and sectioned at 7 µm. From each embryo, amniotic sac or tails were used for genotyping. All procedures were approved by the University Committee on Use and Care for Animals at the University of Michigan.

Immunofluorescence and *in situ* hybridization

Immunofluorescence on paraffin embedded tissues was done as previously described (Martin et al., 2002; Martin et al., 2004). Antibodies used were rabbit anti-phosphohistone H3 at 1:200 (Upstate Biotechnology, Inc., Lake Placid, NY) and rabbit anti-GABA (Sigma), and rat anti-BrdU (AbD Serotec). *In situ* hybridization on frozen

sections was done as previously described (Martin et al., 2002; Martin et al., 2004) using cRNA probes created from PCR-amplified cDNA for *Pitx2* (Suh et al., 2002), *Versican (Vcan)* (For: CAAACCTGAGACTTCCCAAGACTTC, Rev: TTCCGACAAGGGTTAGAGTGACATT), *RhoA* (For: AGACGTGGGAAGAAAAAGTCTG, Rev: CTCACCAGAGTTCTTGCAGTTG), *Rab2* (For: GCCACTTACTCTTCCCTTAGACAC, Rev: TCTCCTCTGACAGGACAGTGAAT), *Cullin3 (Cul3)* (For: CGCTGGTATTGTTTAAAGGTTTG, Rev: AAATGGTAGAATGGATGGATGTG), *Pkn1* (For: ATACATGCATTTTCAGCCTCTGT, Rev: AGATTCGGGCTCTCCATAAATAG), *Gdi1* (For: TGACCATGGACGAGGAATACGAC, Rev: AAAGCTGCCCTCCACCACCT) from (D'Adamo et al., 1998), *Tgfb1i4* (For: CTCATAAGCTTTTGGCTTGAAGA, Rev: TAAGCTTTTGGCTTGAAGAAATG), *Rac1,* and *Netrin1*. The *Rac1* probe was a gift from Ivan de Curtis (Corbetta et al., 2005) and the *Netrin1* probe was a gift from Lisa Goodrich (Abraira et al., 2008).

Microscopy

Brightfield and fluorescent sections were imaged on a Leica DM500B upright microscope. Digital images were processed with Adobe Photoshop CS3 software.

Statistical analyses

Cell counts were performed on at least three embryos of each genotype at each time point. A generalized linear model comparing differences between littermates was performed to determine the statistical significance. Standard error of the mean (s.e.m.) was calculated and plotted for each value.

Results

Expression patterns of possible downstream effectors of *Pitx2* in midbrain neuron migration

To identify downstream targets of PITX2, we performed a microarray on RNA isolated from whole brain, flow-sorted cells from E14.5 *Pitx2*$^{Cre/+}$*;mTmG* and *Pitx2*$^{Cre/-}$

;mTmG littermates, which express GFP under the control of *Cre* (Muzumdar et al., 2007). Several downregulated candidate target genes were significantly downregulated (*p*<0.05) in the *Pitx2*$^{Cre/-}$*;mTmG* samples and are expressed in the developing midbrain (Genepaint, Fig. A.1). To determine whether loss of *Pitx2* changed the expression patterns of our microarray candidates in the midbrain, we performed *in situ* hybridization on E12.5 and E14.5 *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ littermates. Candidate molecules such as *Versican (Vcan), Rac1, RhoA, Cullin3 (Cul3), Pkn1 (Prk1),* and *Necdin* (Alcantara et al., 2000; Ayala et al., 1990; Chen et al., 2009; Evanko et al., 1999; Kuwajima et al., 2010; Lachmann et al., 2011; Talamillo et al., 2003; Wei and Adelstein, 2002) have been shown to regulate cell migration. Other genes, such as *Rab2,* regulate neurite outgrowth, and the genes *Gdi1* and *Tgfb1i4* have unknown functions in neurons. We found that loss of *Pitx2* had no effect on the patterning of these genes in the dorsal midbrain at E12.5 or E14.5 (Fig. A.1). Some genes, such as *Netrin1* and *Pax6,* were only expressed in the ventral midbrain (data not shown).

Loss of *Pitx2* results in non-tissue autonomous defects

In mice, loss of *Pitx2* results in hypoplasia of the pituitary and right cardiac ventricle (Kitamura et al., 1999; Lin et al., 1999; Suh et al., 2002). To determine whether loss of *Pitx2* affects overall midbrain size, we measured midbrain dimensions in *Pitx2*$^{Cre/+}$ versus *Pitx2*$^{Cre/-}$ E14.5 embryos. In *Pitx2*$^{Cre/-}$ embryos (*N*=6), the ventricle height, lateral wall width, and total midbrain width were smaller (16%, 14%, and 5% smaller, respectively) than in their heterozygous *Pitx2*$^{Cre/+}$ littermates (*N*=6) (Fig. A.2A,A'), suggesting a small (5%) global reduction in midbrain size. This 5% reduction in midbrain size could be due to reduced cellular proliferation, increased cell death, altered cellular migration, or some combination of these. To determine whether *Pitx2* influences cellular proliferation during midbrain development, pregnant dams were injected with BrdU at E11.5, E12.5, E13.5, or E14.5 and embryos harvested for BrdU immunofluorescence at E14.5. Regardless of age at BrdU exposure, *Pitx2*$^{Cre/-}$ embryos had 13.8%-58.9% (E11.5-E14.5) fewer BrdU-positive cells in the dorsal midbrain (*N*=3 paired littermates) (Fig. A.2B,B'), suggesting that *Pitx2* is required for normal cellular proliferation between E11.5-E14.5. To determine the precise age at which *Pitx2* is

required for proliferation, pregnant dams were injected with BrdU at E11.5, E12.5, E13.5, or E14.5 and embryos harvested 30 minutes later. At E11.5, there was no significant difference in the number of BrdU-positive cells between *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ embryos; however, E12.5-E14.5 *Pitx2*$^{Cre/-}$ embryos exhibited 24.6%-58.9% (E12.5-E14.5) fewer BrdU-positive cells than *Pitx2*$^{Cre/+}$ littermates (*N*=3 paired littermates) (Fig. A.2C). Similarly, there were 35.3%-54.1% (E12.5-E14.5) fewer phosphohistone H3 (H3)-positive cells in *Pitx2*$^{Cre/-}$ embryos (*N*=3 paired littermates) (Fig. A.2D), suggesting that *Pitx2* is required for progenitor population development or maintenance.

The requirement for *Pitx2* in collicular progenitor maintenance and proliferation was surprising, given the lack of *Pitx2* expression in collicular progenitors (Fig. 4.2C). We therefore hypothesized that reduced progenitor proliferation in *Pitx2*$^{Cre/-}$ midbrains may reflect non-CNS specific effects of *Pitx2* deficiency such as hypoxia due to lung and cardiac malformations (Gage et al., 1999; Kieusseian et al., 2006; Kitamura et al., 1999; Yashiro et al., 2007). To analyze potential tissue-autonomous requirements for *Pitx2*, we used the *NCre* transgene to conditionally knock out *Pitx2* in the developing brain while leaving *Pitx2* expression in other organs intact (Sclafani et al., 2006; Skidmore et al., 2012; Waite et al., 2012). Unlike *Pitx2*$^{Cre/-}$, *NCre;Pitx2*$^{tlz/flox}$ and *Pitx2*$^{tlz/flox}$ E14.5 embryos did not exhibit reduced BrdU-positive or H3-positive collicular cells (*N*=3 paired littermates) (Fig. A.3A,B). Thus, *Pitx2* is required for collicular progenitor proliferation and maintenance, but in a non-tissue autonomous manner. In mice, embryonic death during the fetal period (E11.5-E15.5) can be attributed to defects in either cardiovascular circulation or liver haematopoiesis (Copp, 1995), both of which are defective in *Pitx2* null embryos (Gage et al., 1999; Kieusseian et al., 2006; Kitamura et al., 1999; Yashiro et al., 2007). Importantly, *NCre;Pitx2*$^{tlz/flox}$ embryos also exhibit neuronal migration defects in the midbrain, suggesting that CNS specific of *Pitx2* deficiency accounts for the migrational phenotype (Sclafani et al., 2006).

To determine whether *Pitx2* regulates cell death in the midbrain, E11.5-E13.5 *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ littermates were analyzed for TUNEL-positive cells (*N*=3 paired littermates) (Fig. A.3C). Regardless of age, only 1-3 midbrain cells were TUNEL-positive in both *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ embryos, and loss of *Pitx2* did not significantly

change the number of TUNEL-positive cells. Thus, *Pitx2* does not appear necessary for collicular cell survival. These data suggest that *Pitx2* promotes collicular progenitor proliferation in a non-tissue autonomous manner, but does not regulate cell death.

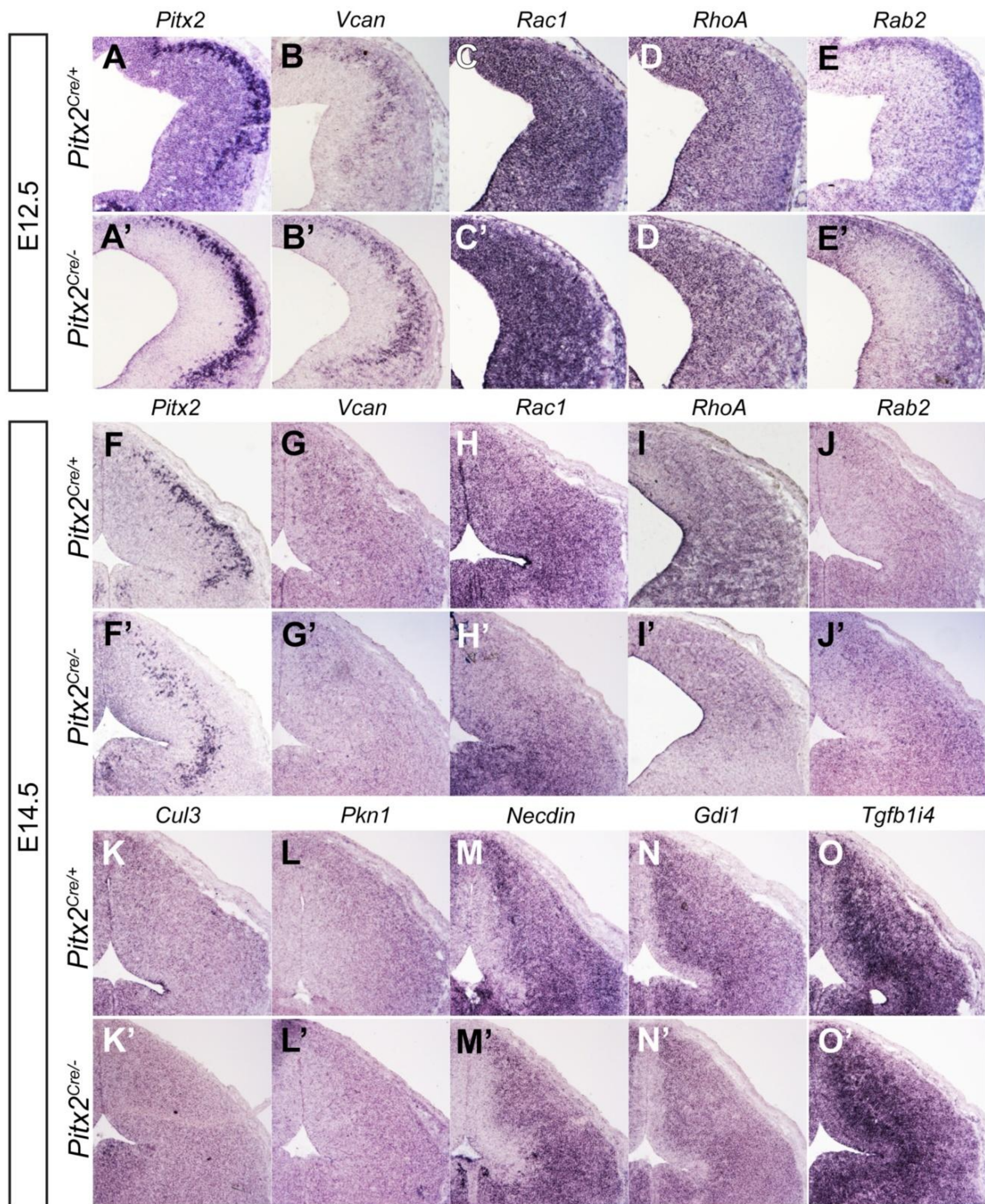

Figure A.1. PITX2 is dispensable for early patterning of migrational markers. *In situ* hybridization of (A-E') E12.5 or (F-O') E14.5 *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ mouse midbrain tissues sectioned coronally reveals normal patterning of genes associated with migration. Scale bars in A and F are 100 μm and apply to panels A-E' and F-O', respectively.

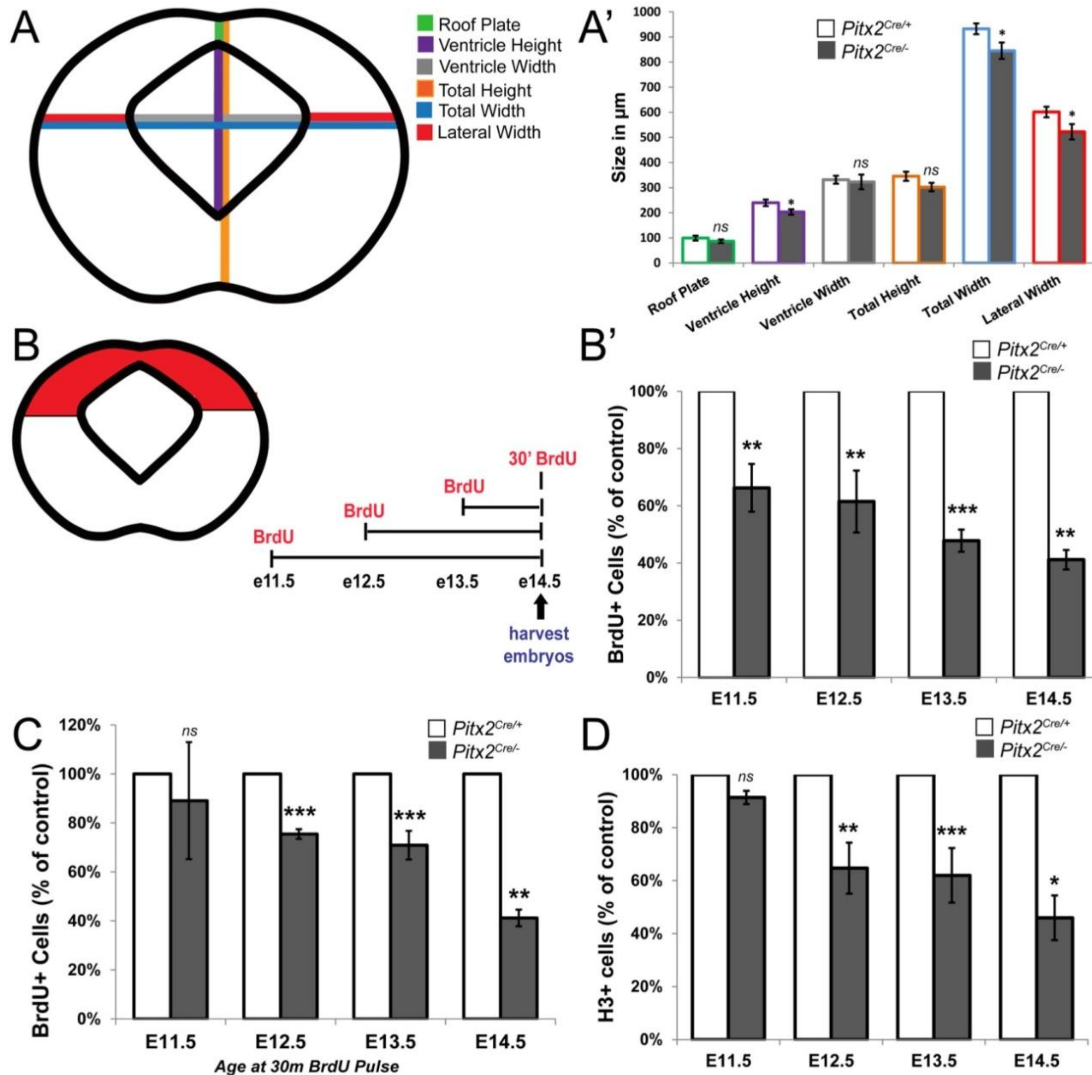

Figure A.2. ***Pitx2* is required for proliferation of midbrain progenitors.** (A) Schematic of a coronal section through the midbrain demonstrating measurements taken. (A') The length of each area highlighted in A in *Pitx2*$^{Cre/+}$ and *Pitx2*$^{Cre/-}$ E14.5 littermates. (B,B') BrdU-positive cells in embryos exposed to single BrdU injections between E11.5-E14.5 and harvested at E14.5. The number of BrdU (C) or H3 (D) positive cells in the dorsal midbrain of 30 minute BrdU-exposed *Pitx2*$^{Cre/-}$ embryos relative to their *Pitx2*$^{Cre/+}$ littermates. At E11.5, there was no significant difference in the number of BrdU or H3-positive cells between genotypes; however, at E12.5 and beyond, *Pitx2*$^{Cre/-}$ littermates had fewer BrdU and H3-positive cells. *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.001$, *ns*, not significant.

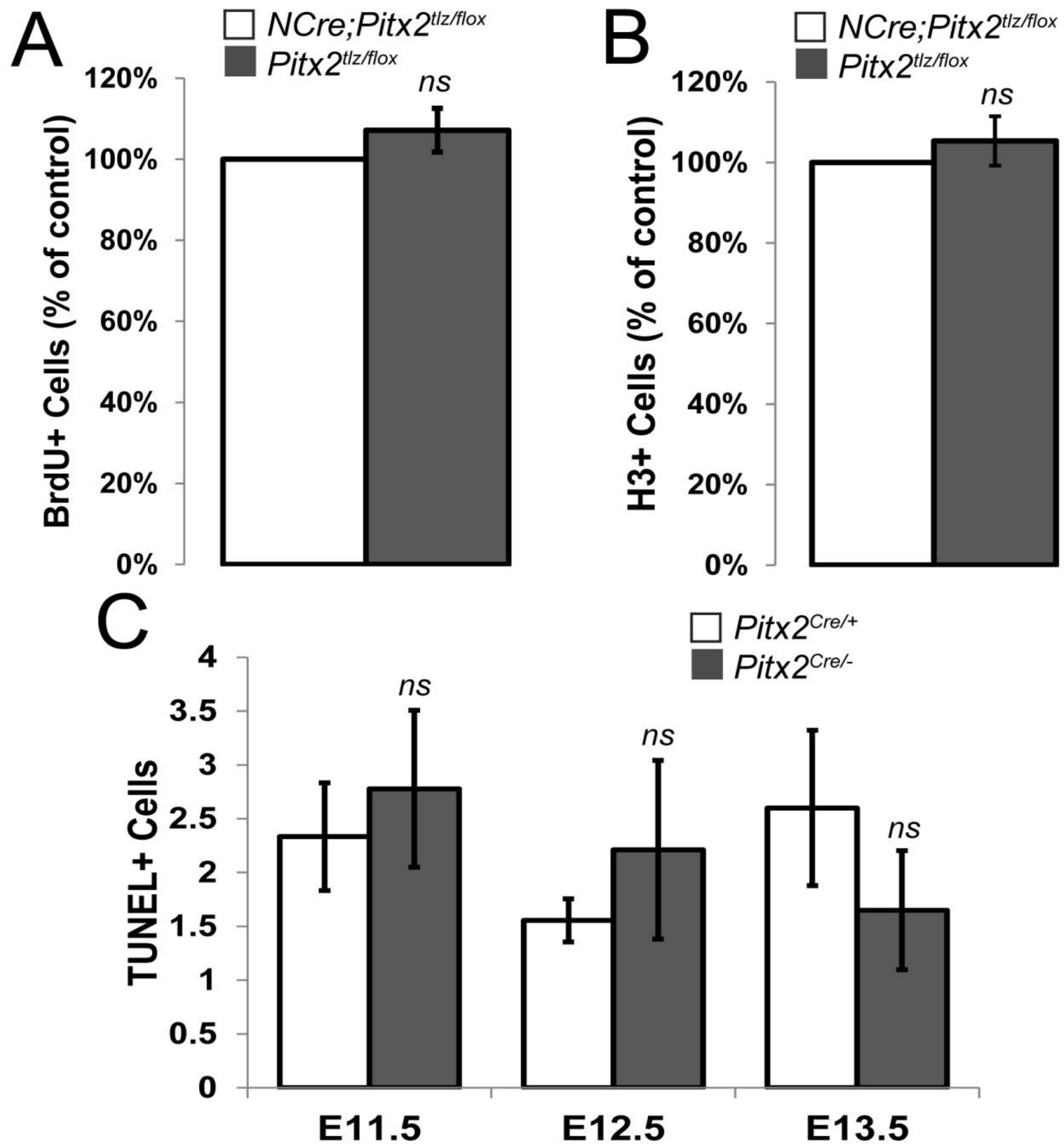

Figure A.3. ***Pitx2*** **positively regulates midbrain progenitor proliferation through non-tissue autonomous mechanisms.** The number of BrdU (A) and H3 (B) positive cells in dorsal midbrains of E14.5 *NestinCre;Pitx2*$^{tlz/flox}$ embryos is similar to *Pitx2*$^{tlz/flox}$ littermates (*N*=3). (C) TUNEL-positive cell counts in E11.5-E13.5 *Pitx2*$^{Cre/-}$ midbrains are not significantly different from *Pitx2*$^{Cre/+}$ littermates (*N*=3). *ns,* not significant.