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

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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 mice, 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 neuronal development are not known. Here we report the onset of *Pitx2ab* and *Pitx2c* isoform-specific expression 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.

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Keywords

migration; transcription factor; midbrain; isoform; differentiation; axon

1. 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 gene regulation, wherein a single gene gives rise to multiple protein isoforms with distinct functions, greatly increasing functional capacity. 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), utilize gene splicing (Modrek et al., 2001; Yeo et al., 2004), nonetheless, there are few detailed studies of protein isoform functions 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 for *Pitx2* deficiency exhibit ocular, tooth, and brain phenotypes similar to humans with *PITX2* mutations, but the underlying molecular mechanisms of these defects are only partially understood (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Liu et al., 2003; Lu et al., 1999; 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 for modulation of gene expression and exhibit dosage and tissue-specific requirements (Kioussi et al., 2002; Simard et al., 2009). In mouse, PITX2C (but not PITX2AB) 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). Conversely, PITX2A is the only isoform expressed in and required for

heart development in zebrafish (Essner et al., 2000). *In vitro,* PITX2C is necessary for retention of myoblasts in an undifferentiated state and for continued proliferation (Martinez-Fernandez et al., 2006), whereas PITX2A regulates actin-myosin changes in HeLa cells to promote cell spreading and migration (Wei and Adelstein, 2002). Interestingly, no unique *in vivo* requirements for PITX2A or PITX2B have been identified in the mouse, although PITX2AB appears to be sufficient for tooth development (Liu et al., 2003).

All three *Pitx2* isoforms appear to be equally expressed 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.

2. Materials and Methods

2.1 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 as previously described (Liu et al., 2001; Liu et al., 2002). *Pitx2c-lacZ* transgenic mice were created by Hiroshi Hamada and express *lacZ* under the control of 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;*$Pitx2^{tlz/flox}$ embryos.

2.2 Tissue Preparation

The morning of plug identification was designated as E0.5. Pregnant females underwent cervical dislocation and hysterectomy and embryos were dissected into PBS. 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 tail tissue was used for genotyping. All procedures were approved by the University Committee on Use and Care for Animals at the University of Michigan.

2.3 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.

2.4 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.*

2.5 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 $MgCl_2$ overnight, and frozen in O.C.T. embedding medium (Tissue Tek, Torrance, CA). Frozen sections were postfixed 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.

2.6 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, wells were photographed in brightfield on a Leica MZ10F dissecting microscope. Digital images were processed with Adobe Photoshop CS3 software.

2.7 RNA isolation and real-time PCR

The midbrain of E14.5 and hypothalamus of E14.5 and E18.5 littermate mice were microdissected and RNA was isolated using the RNAqueous-Micro RNA Isolation Kit (Ambion, Austin, TX, USA). Isolated RNA was treated with DNase I prior to cDNA synthesis. cDNA was generated using the Superscript First-Strand cDNA Synthesis system for quantitative real-time PCR with random primers (Invitrogen, Carlsbad, CA, USA). Relative gene expression levels were assayed using TaqMan Gene Expression Master Mix and TaqMan probes (Applied Biosystems, Foster City, CA, USA) for *Gapdh*, *Pitx2abc*,

Pitx2a, *Pitx2b* and *Pitx2c*. Each sample was run in triplicate using an Applied Biosystems StepOne-Plus Real-Time qPCR System. The gene expression level of *Gapdh* was used as an internal, positive control. The difference in threshold cycle (C_T) between the assayed gene and *Gapdh* for any given sample was defined as the change in threshold cycle (ΔC_T). The difference in ΔC_T between two samples was defined as $\Delta\Delta C_T$ which represents a relative difference in expression of the assayed gene. Fold change of *Pitx2a*, *Pitx2b*, or *Pitx2c* relative to total *Pitx2abc* was defined as $2^{-\Delta\Delta CT}$(Livak and Schmittgen, 2001).

3. Results

3.1 *Pitx2* isoforms and alleles

The mouse *Pitx2* gene is composed of two promoters and six exons (Fig. 1A). Alternative splicing and promoter usage generates three different *Pitx2* isoforms, PITX2A, PITX2B, and PITX2C (Fig. 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. 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).

3.2 *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. 2A). All three *Pitx2* isoforms were expressed in the midbrain at E14.5 (N=3 embryos) (Fig. 2A). *Pitx2b* cDNA was present at half the level of *Pitx2a* and *Pitx2c*. *In situ* hybridization using a cRNA probe that detects all three isoforms (Fig. 1A) showed that *Pitx2* mRNA is present at E8.5 in the branchial arches, but not in the neuroepithelium (Fig. 2B). Consistent with this, X-gal staining of E8.5 $Pitx2^{\Delta ab/+}$ tissues (in which βgal is expressed as a knock-in 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. 2C). Therefore, *Pitx2* is expressed very early in the neuroepithelium, and by E14.5 all isoforms are expressed, albeit at different levels.

Moreover, the expression level of *Pitx2c* decreases by 21% in midbrain and increases by 9% in hypothalamus with loss of one *Pitx2ab* allele, suggesting there may be mild dosage compensation by the remaining isoforms that influence our findings (Supplemental Fig. 1).

To determine the onset and isoform specificity of *Pitx2* expression in the brain, we performed X-gal staining on E9.25–E10.5 $Pitx2^{\Delta ab/+}$ (knock-in) and *Pitx2c-lacZ* transgenic embryos. At E9.25, occasional βgal-positive *Pitx2ab*-positive cells were observed in the ventral midbrain (Fig. 2D–D"); at E9.5, numerous βgal-positive cells were detected (Fig. 2E–E"). At E10.5, βgal-positive cells in $Pitx2^{\Delta ab/+}$ embryos were visible throughout the forebrain and midbrain (Fig. 2F–F"). In *Pitx2c-lacZ* embryos, βgal-positive cells were absent from the ventral midbrain at E9.25 (Fig. 2G') but present at E9.5–E10.5 (Fig. 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 outer regions of the ventral 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.

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

To determine which *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. Given that the immunofluorescence was performed using tyramide signal amplification, it was not possible to assign significance to apparent differences in staining intensity between the various embryos. However, some conclusions were drawn based on presence or absence of positively labeled cells. At E14.5, PITX2 protein in wild type mice localized to cells at the collicular surface and to a ventromedial (VM) neuronal population (Fig. 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. 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. 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. 3B); however, ventromedial PITX2 was not present in embryos lacking PITX2AB ($Pitx2^{\Delta ab/-}$ and $Pitx2^{\Delta ab/\Delta ab}$) and appeared reduced in embryos lacking *Pitx2c* (Fig. 3C, E,F). This suggests that *Pitx2c* is either not expressed in the VM population and/or that *Pitx2ab* is required for the formation of the VM population. Further analyses are necessary to distinguish between these possibilities. Additionally, medial mislocalization of *Pitx2*-expressing neurons in $Pitx2^{\Delta ab/-}$ and $Pitx2^{\Delta ab/\Delta ab}$ embryos (Fig. 3E,F and Fig. 4), suggests roles for *Pitx2ab* in neuronal migration.

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

Previous studies showed that *Pitx2* is necessary for superior colliculus 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 collicular surface (*N*=7 embryos) (Fig. 4A,D), whereas loss of all *Pitx2* isoforms (in $Pitx2^{tlz/-}$ embryos) resulted in medial or deep mislocalization of *Pitx2*-expressing cells (Fig. 4B). $Pitx2^{tlz/\Delta c}$ embryos, which have no functional *Pitx2c* and a single allele of *Pitx2ab*, exhibited normal collicular neuron localization (Fig. 4C). Thus, a single allele of *Pitx2ab* appears sufficient for superior collicular neuronal migration, whereas *Pitx2c* has a minor role. 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. 3E and 4E to Fig. 3D, 4A, and 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. 4F and Fig. 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. 5A–I), although several βgal-positive neurons were present in deeper layers in $Pitx2^{\Delta ab/\Delta ab}$ and $Pitx2^{\Delta ab/-}$ embryos (*) (Fig. 5 D–I). To determine the migrational phenotype of E18.5 midbrains in the absence of all *Pitx2* isoforms, $NCre;Pitx2^{tlz/flox}$ embryos were analyzed by *Pitx2 in situ* hybridization instead of βgal staining due to faint βgal staining from the $Pitx2^{tlz}$ allele at this stage. In $NCre;Pitx2^{tlz/flox}$ E18.5 conditional mutants, most *Pitx2*-expressing neurons were mislocalized to the deep BRN3A-positive layer (*), although a few neurons were properly localized between BRN3A-positive layers (Fig. 5J–L). Thus, complete loss of *Pitx2* leads to severely disrupted collicular neuron localization, whereas isoform-specific deletion results in milder phenotypes.

3.5 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 collicular surface (Fig. 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. 6B). The lack of βgal-expressing cells at more superficial locations in the *wild type;*$Pitx2^{tlz/-}$ colliculus argues against non-cell autonomous functions for *Pitx2*.

3.6 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 identity (Fig. 7B,B'). To determine whether specific *Pitx2* isoforms were required for GABAergic differentiation, *Pitx2* isoform-specific knockout embryos were analyzed for midbrain GABAergic differentiation. 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. 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 co-localization, suggesting a single allele of *Pitx2c* is sufficient for GABAergic differentiation of PITX2-positive collicular neurons (Fig. 7E–F'). Because $Pitx2^{\Delta ab/-}$ and $Pitx2^{\Delta c/tlz}$ *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 $Pitx2^{\Delta c/\Delta c}$ βgal-positive neurons are GABAergic, neither isoform is individually necessary.

3.7 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. 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. As in the midbrain, *Pitx2b* mRNA was less abundant than *Pitx2a* or *Pitx2c* in the E18.5 hypothalamus (Fig. 8B) . Embryos that were heterozygous or null for *Pitx2c* ($Pitx2^{\Delta c/+}$,$Pitx2^{\Delta c/\Delta c}$ or $Pitx2^{\Delta c/-}$) or null for *Pitx2ab* ($Pitx2^{\Delta ab/\Delta ab}$) displayed normal MTTs (Fig. 8C–J). However, embryos with only a single allele of *Pitx2c* ($Pitx2^{\Delta ab/-}$) failed to form the MTT (Fig. 8K–L), similar to embryos with *Nestin-Cre*-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*.

4. Discussion

4.1 Conclusion

Ours is the first study to identify unique *Pitx2* 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.* We also demonstrate that a subpopulation of collicular neurons requires *Pitx2ab* for proper migration, and a single allele of *Pitx2ab* or *Pitx2c* for GABAergic differentiation. Finally, we show that formation of the mammillothalamic tract requires a combination of two isoform-specific *Pitx2* alleles.

4.2 Pitx2 isoforms exhibit unique dosage effects during brain development

Pitx2 isoforms exhibit differential dosages in the developing midbrain and hypothalamus, suggesting they 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 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 also be partially functionally redundant, there may be isoform-specific gene regulation, or there may be a threshold level of *Pitx2* isoform necessary for neuronal development. This situation is reminiscent to that in the developing branchial arches, where *Pitx2* isoforms are interchangeable and contribute distinct dosages which translate into unique developmental functions (Liu et al., 2003).

4.3 Pitx2a and Pitx2b are dominant PITX2 isoforms during mouse brain development

The embryonic brain may be unique from other organs in its requirement for *Pitx2* isoforms. Loss of *Pitx2a* and *Pitx2b* results in more severe phenotypes than loss of *Pitx2c.* To date, there are no studies which have identified a requirement for *Pitx2ab* in tissue-specific development, and prior reports indicate 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) reminiscent of ocular defects observed in $Pitx2^{-/-}$ mice (Evans and Gage, 2005; Gage et al., 1999). The neuroepithelial origin of neural crest-derived ocular *Pitx2*-expressing cells may partly explain their sensitivity to reduced *Pitx2ab* function (Echelard et al., 1994; Gage et al., 2005), although the exact *Pitx2* isoform-specific requirements for eye development are unknown.

In contrast to *Pitx2* expression in the mouse brain, *Pitx2c* is the only *Pitx2* isoform expressed in the zebrafish brain. Interestingly, *Pitx2c* exhibits asymmetric expression in the left dorsal diencephalon (pineal gland), although its function in this region is unknown (Essner et al., 2000; Liang et al., 2000). In *vitro* studies on PITX2A and PITX2B functions have provided some 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 improved 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.

4.4 Pitx2c may be redundant during mouse brain development

In both midbrain and hypothalamus, the *Pitx2ab* allele appears necessary and sufficient for proper migration of superior colliculus neurons and for extension of the MTT. In contrast, the *Pitx2c* allele is neither necessary nor sufficient for either process. One potential explanation for these findings is that the *Pitx2ab* mutant allele disrupts both PITX2A and PITX2B, whereas the *Pitx2c* mutant allele disrupts only PITX2C, such that it is the overall amount of *Pitx2* isoforms present that is necessary for neuronal differentiation. If true, then loss of similar amounts of *Pitx2* isoforms should lead to similar phenotypes. This does not appear to hold true in the midbrain, where preservation of both *Pitx2a* and *Pitx2b* is sufficient for proper neuronal migration (Fig. 4C) whereas preservation of both *Pitx2c* copies (Fig. 4F) is not. We interpret these findings to suggest that the differing *Pitx2* isoforms exhibit unique properties and dosage sensitivities, and may have unique downstream targets.

In the brain, *Pitx2ab* appears to be more important than *Pitx2c*. This contrasts with other organs such as the heart, lungs, and gut, where *Pitx2c* is essential for normal development (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 heart and lung morphogenesis and for looping of the gut (Liu et al., 2001; Liu et al., 2002). Later in development, *Pitx2c* induces expression of atrial natriuretic factor *(ANF)* and *Plod1* and the cardiac transcription factors *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 downstream 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 in the heart through adulthood appears to be required for cardiac fitness, as loss of *Pitx2c* in the cardiac atrium results in susceptibility to atrial fibrillations (Chinchilla et al., 2011; Kirchhof et al., 2011; Wang et al., 2010). Thus, *Pitx2c* appears to have unique requirements in mediastinal organs; its precise role in the brain remains unclear.

In addition to organ-specific functions, the various *Pitx2* isoforms may exhibit unique transcriptional auto-regulation. *Pitx2a* has been shown to induce expression of *Pitx2c* (Guioli and Lovell-Badge, 2007; Kala et al., 2009), and in all tissues examined thus far, all three isoforms are expressed (Gage and Camper, 1997; Kieusseian et al., 2006; Liu et al., 2001; Liu et al., 2003). In our experiments, there were mild (9–21%) changes in *Pitx2c* mRNA levels with reduced *Pitx2ab*, but it is not clear whether this leads to changes in the amount of protein present.

4.5 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,* (glutamate decarboxylase), 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.

4.6 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 stabilizing *Ngn2* to promote differentiation (Nguyen et al., 2006). Different termini of the $p27^{Kip1}$ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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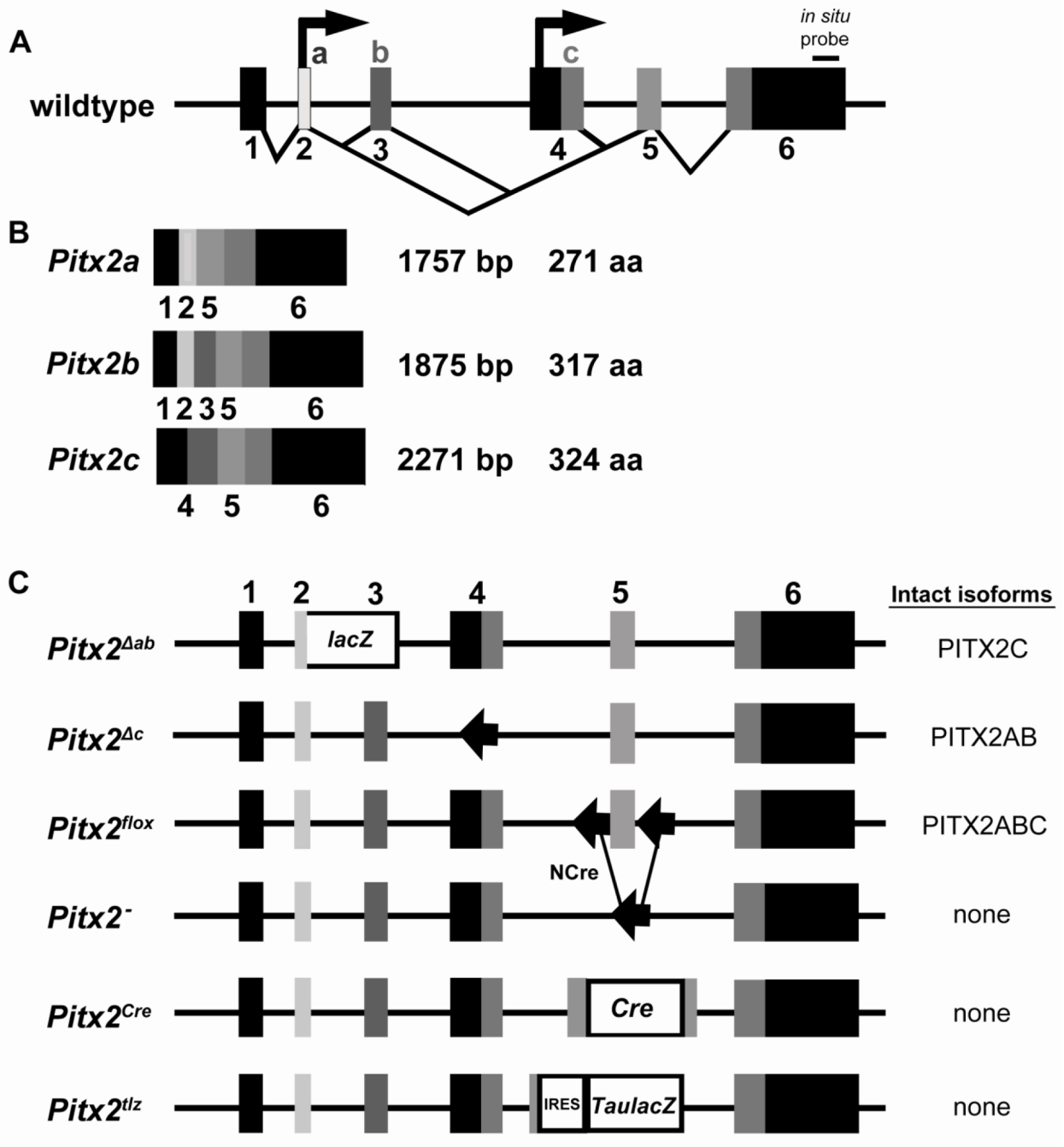

Figure 1.

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. *Pitx2* isoforms that remain intact are listed on the right.

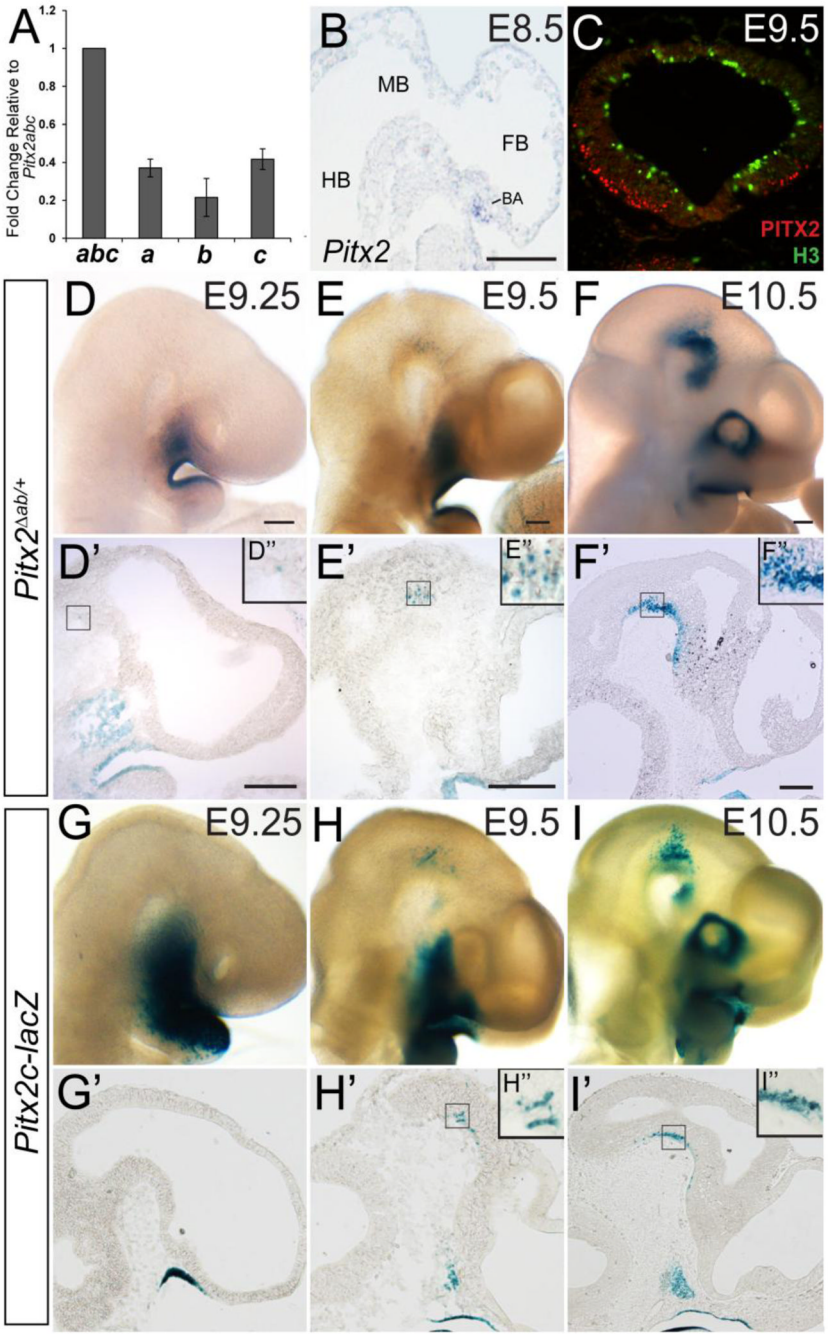

Figure 2.

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 *Pitx2c* are more abundant than *Pitx2b*. (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'.

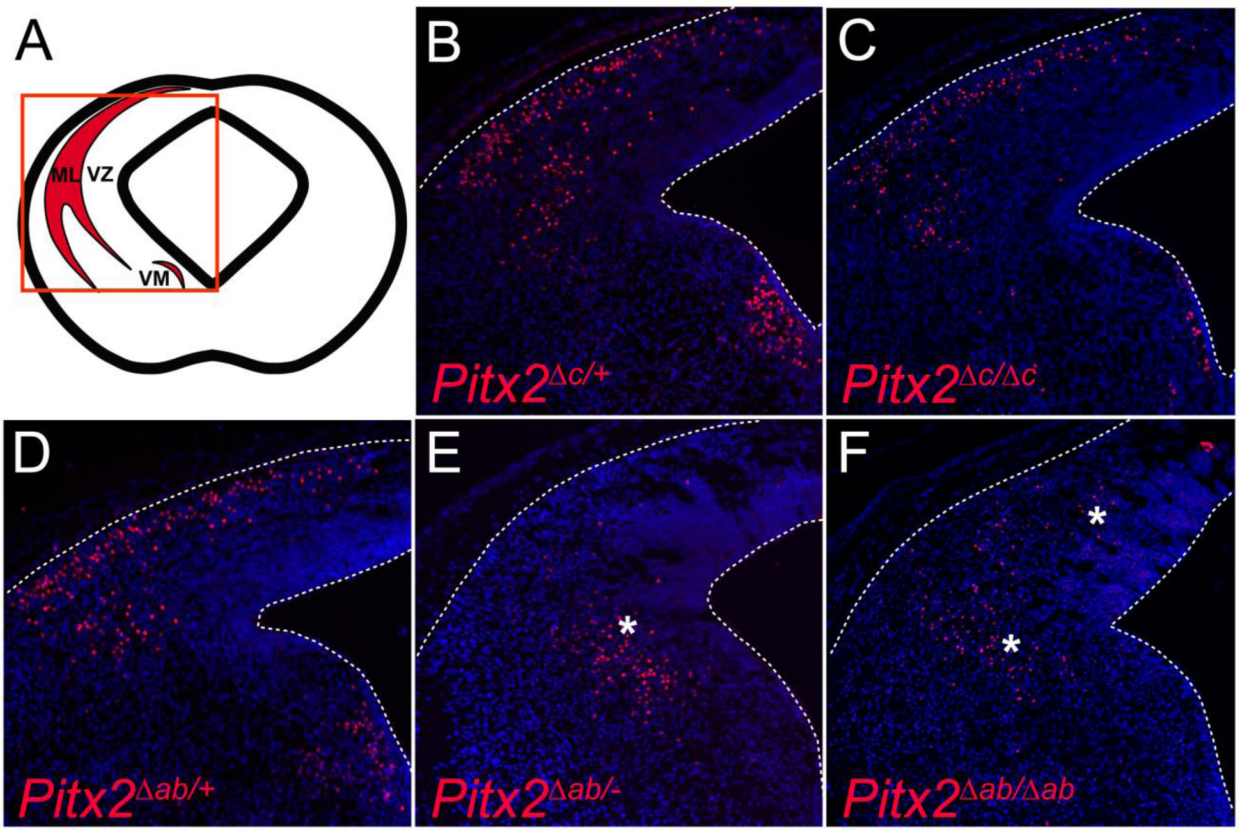

Figure 3.

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 (*).

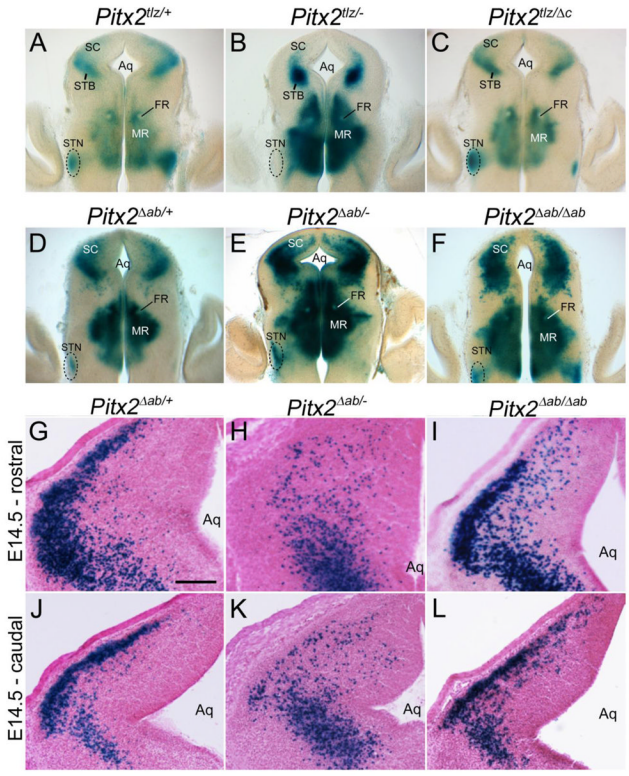

Figure 4.

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-positive neuron localization 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 *Pitx2*$^{\Delta ab/+}$ (G,J), *Pitx2*$^{\Delta ab/-}$ (H,K), or *Pitx2*$^{\Delta ab/\Delta ab}$ (I,L) 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.

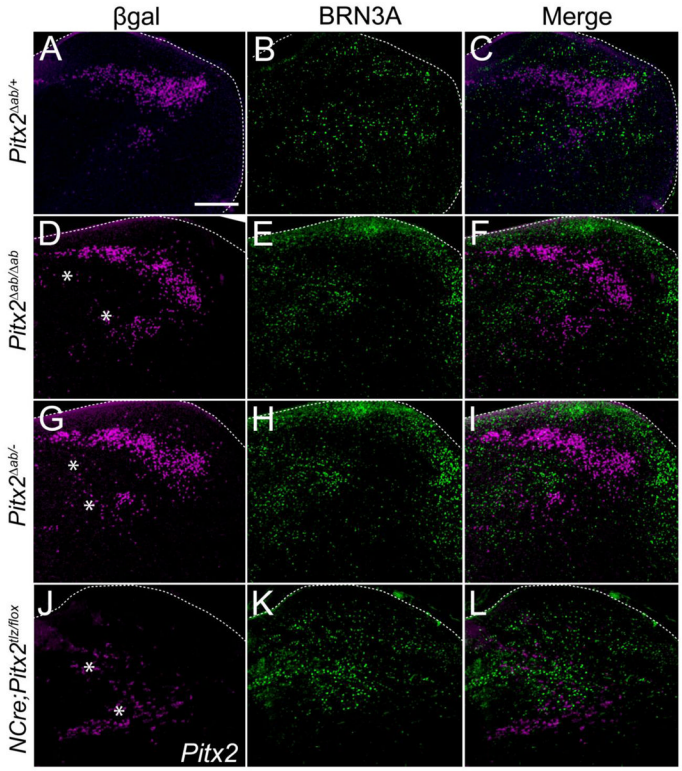

Figure 5.

Pitx2ab regulates the timing of midbrain neuronal migration. (A–L) Pseudocolored and merged images of neighboring coronal midbrain cryosections (orientation similar to panel A in figure 3) of E18.5 *Pitx2*$^{\Delta ab/+}$, *Pitx2*$^{\Delta ab/-}$, *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 βgal 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.

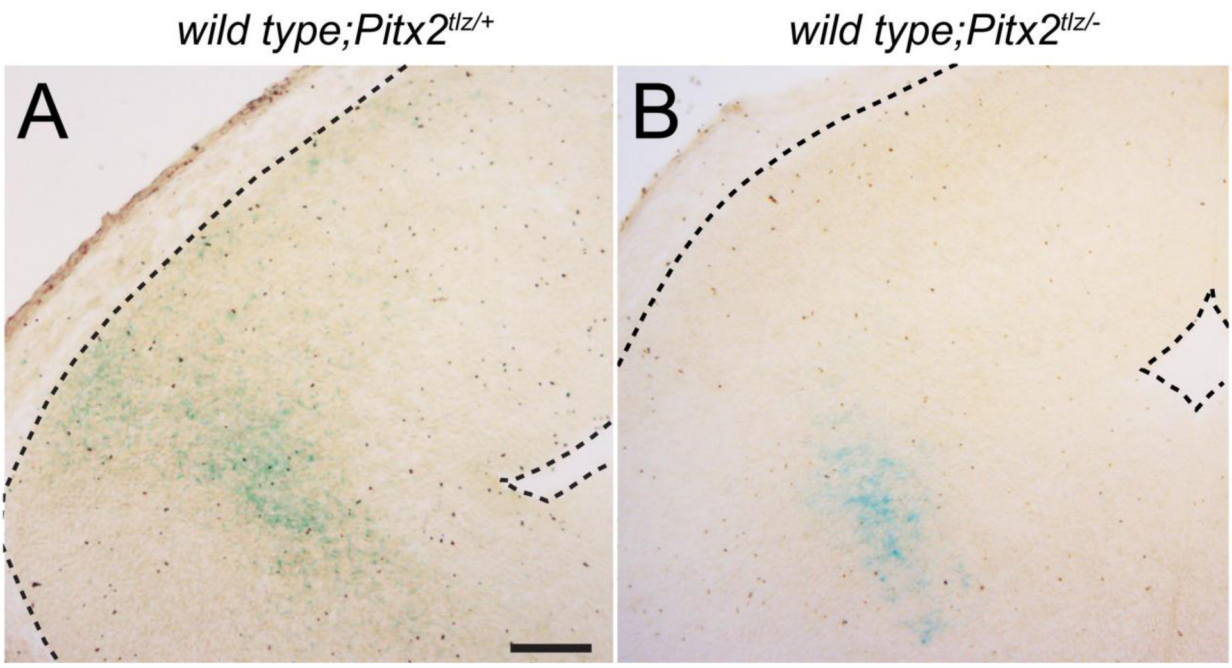

Figure 6.

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 βgal-positive 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.

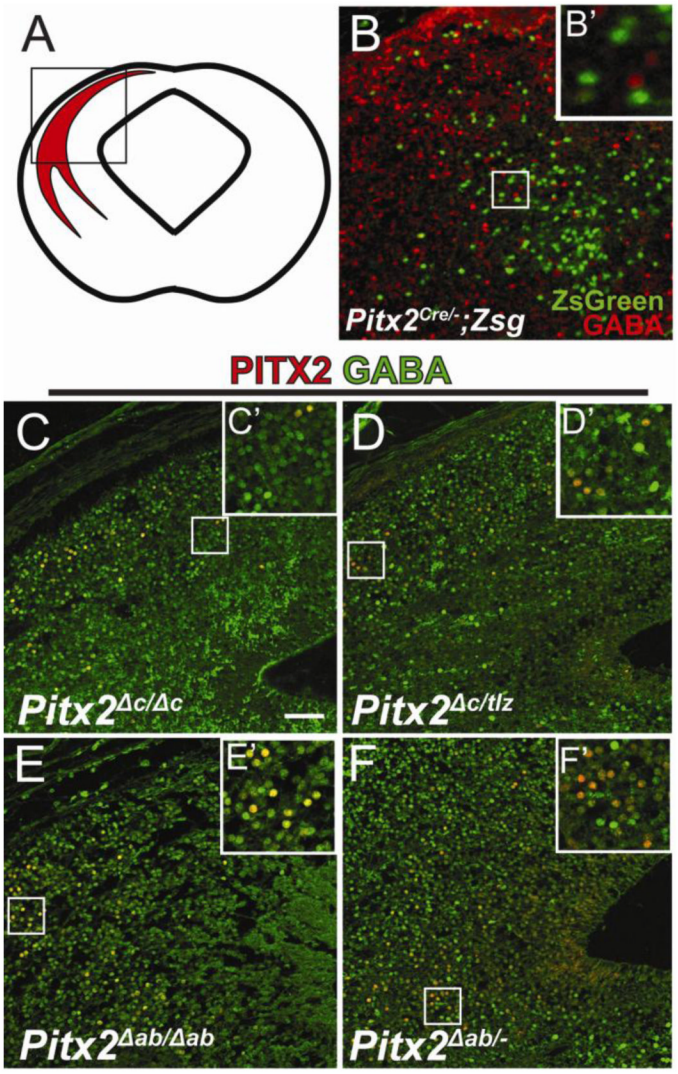

Figure 7.

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.

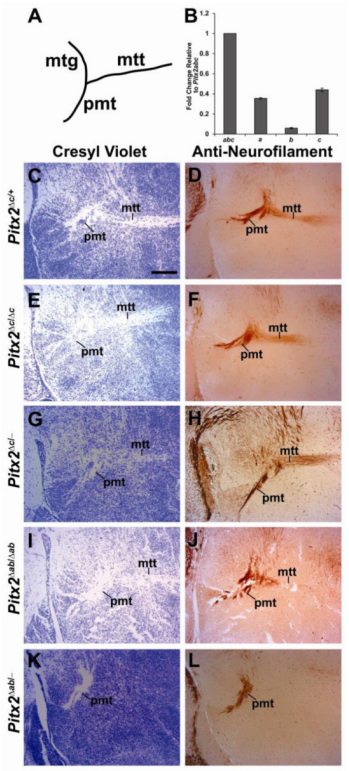

Figure 8.

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 E18.5 hypothalamus RNA shows that *Pitx2b* is more abundant than *Pitx2a* and *Pitx2c*. E18.5 sagittal brain sections were processed 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.

Table 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.

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