TECHNOLOGY REPORT

A Novel *TaulacZ* Allele Reveals a Requirement for *Pitx2* in Formation of the Mammillothalamic Tract

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Summary: The hypothalamic mammillary region is critical for spatial memory and vestibular processing. *Pitx2* encodes a paired-like transcription factor that is highly expressed in the developing mammillary region and is required for subthalamic nucleus formation. Here we analyzed a loss of function *Pitx2-TaulacZ* knock-in allele to study the effects of *Pitx2* deficiency on neuronal projections in the embryonic mammillary region. *Pitx2*-expressing neurons contribute axons to principal mammillary, mammillotegmental and mammillotectal tracts. Embryos with *Pitx2* deficiency exhibit axonal fibers in the principal mammillary tract that are improperly bundled and disorganized, yet project caudally toward the tectum and tegmentum. Embryos with *Nestin-Cre* mediated conditional *Pitx2* deficiency exhibit truncated mammillothalamic tracts (mtt) that fail to elongate, and reduced *Pax6*-positive cells at the branching point of the principal mammillary and mtt. These data suggest that *Pitx2* mediates cell-autonomous and nonautonomous guidance cues necessary for mammillary collaterals destined to project to the anterior thalamus. genesis 50:67–73, 2012. © 2011 Wiley Periodicals, Inc.

Key words: migration; differentiation; transcription factor; neuronal projections; hypothalamus

Neuronal migration and connectivity in the mammalian brain are highly transcriptionally regulated processes, yet detailed understanding of the molecular mechanism regulating these processes in the hypothalamus is lacking (Nobrega-Pereira and Marin, 2009). The mammillary bodies, located in the caudal hypothalamus, participate in the Papez limbic circuit and have critical functions in spatial memory and vestibular processing (Valverde *et al.*, 2000; Vann and Aggleton, 2004). The principal mammillary tract (pmt) first appears at E10.0 and is one the earliest visible neuronal projections in the developing mouse brain (Easter *et al.*, 1993). The pmt gives rise to mammillotegmental (mtg) and mammillotectal (mtc) tracts which are visible by E14.0 (Szabo *et al.*, 2011). Between E17 and E18 in mouse, collaterals of the pmt branch off and project rostrally toward the anterior thalamus, giving rise to the mammillothalamic tracts (mtt) (Valverde, 1998; Valverde *et al.*, 2000).

Analysis of mouse mutants has revealed a small number of transcription factors that regulate formation of principal mammillary, mammillothalamic, and mtg projections: FOXB1 (Alvarez-Bolado *et al.*, 2000), PAX6 (Szabo *et al.*, 2011; Valverde *et al.*, 2000), and SIM1/SIM2 (Marion *et al.*, 2005). Collaterals from the mtg projections form in the mouse around E17.5 and give rise
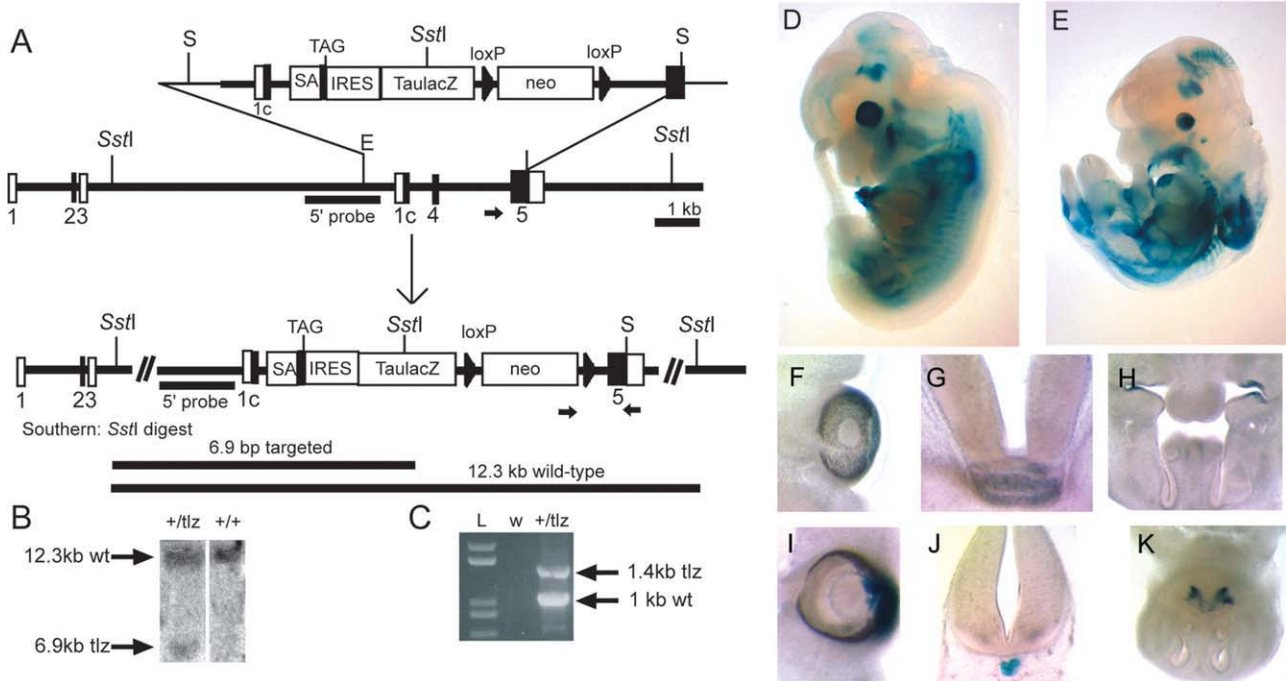
FIG. 1. Generation and characterization of the *Pitx2*^*TaulacZ*^ allele. (**a**) The mouse *Pitx2* gene contains 6 exons including the homeodomain-encoding exon 4. A *SalI* (S) fragment of the targeting vector was used for electroporation in ES cells. (**b**) A 5′ Southern probe was used to detect 6.9 kb targeted vs. 12.3 kb wild type bands in ES cells and targeted mice. (**c**) Genotyping primers corresponding to intron 4–5 sequence, the neo cassette, and exon 5 (arrows in a) yield 1.4 kb *Pitx2*^*TaulacZ*^ (*tlz*) and 1 kb wild type (wt) bands. (**d**) E12.5 embryos of genotype *Pitx2*^*tlz/+*^ (d) and *Pitx2*^*tlz/null*^ (**e**) were labeled for β-galactosidase activity. *Pitx2*^*tlz/null*^ embryos exhibit cardiac and abdominal defects similar to *Pitx2*^*null/null*^ embryos. X-gal stained vibratome sections of E14.5 *Pitx2*^*tlz/+*^ (**f–h**) and *Pitx2*^*tlz/null*^ (**i–k**) embryos show high β-galactosidase label and defects in tissues known to be disrupted by *Pitx2* deficiency, including medial displacement of the eyes (f vs. i), pituitary hypoplasia (g vs. j), and severe tooth defects (h vs. k).

to the mtt, through a process that depends on PAX6 and FOXB1-mediated signaling events at the border between dorsal and ventral thalamus (Alvarez-Bolado *et al.*, 2000; Szabo *et al.*, 2011; Valverde *et al.*, 2000). *Pax6* and *Foxb1* mutants both have abnormalities in the mtt but have intact mtg projections, whereas *Sim1/Sim2* double mutants lack both the mtt and mtg tracts (Alvarez-Bolado *et al.*, 2000; Marion *et al.*, 2005; Valverde *et al.*, 2000).

Pitx2, a paired-like homeodomain transcription factor, is highly expressed in the developing mammillary and retromammillary regions (Martin *et al.*, 2002; Mucchielli *et al.*, 1996; Skidmore *et al.*, 2008). Loss of *Pitx2* function in mice leads to medially shifted rostral projections in the tectum, absence of subthalamo-tegmental projections, and arrested migration of subthalamic neurons from the retromammillary region and mammillary neurons from the mammillary region (Martin *et al.*, 2004). It is not known, however, whether mammillary projections or neural projections in other brain regions are sensitive to reduced *Pitx2* function.

Here we tested whether *Pitx2*-expressing neurons exhibit brain region-specific requirements for proper positioning and axon formation in the developing mouse brain, with a focus on the mammillary region. We ana-

lyzed a newly generated *TaulacZ* (*tlz*) knock-in allele in combination with *Pitx2* null and *Nestin-Cre* (*NCre*)-mediated conditional alleles, to distinguish early versus later effects on neuronal migration and projection formation. Previous studies from our laboratory utilized a different *Nestin-Cre* mouse line which expresses Cre in a mosaic pattern in developing brain tissues (Sclafani *et al.*, 2006). The *Nestin-Cre* line used in this report is widely expressed throughout the neural tube as early as E10.5 (Tronche *et al.*, 1999).

To directly label neuronal projections in *Pitx2*-expressing cells, we generated a *Pitx2-TaulacZ* mouse allele which expresses β-galactosidase under the control of the regulatory sequences of the Tau bovine neurofilament gene (Fig. 1). The targeting vector, containing an IRES-*TaulacZ* (*tlz*) cassette (Mombaerts *et al.*, 1996) was generated and introduced into the mouse *Pitx2* locus using homologous recombination in ES cells. The targeting vector was designed to remove the homeodomain-encoding exon 4 (Fig. 1a) that is common to all three mouse PITX2 isoforms (a, b, and c) (Cox *et al.*, 2002), thereby inactivating PITX2. *Pitx2*^*tlz/+*^ mice were viable and fertile. In addition, *Pitx2*^*tlz/tlz*^ and *Pitx2*^*tlz/null*^ embryos exhibited similar defects of the heart, abdominal viscera, and distal turning (Fig. 1e)
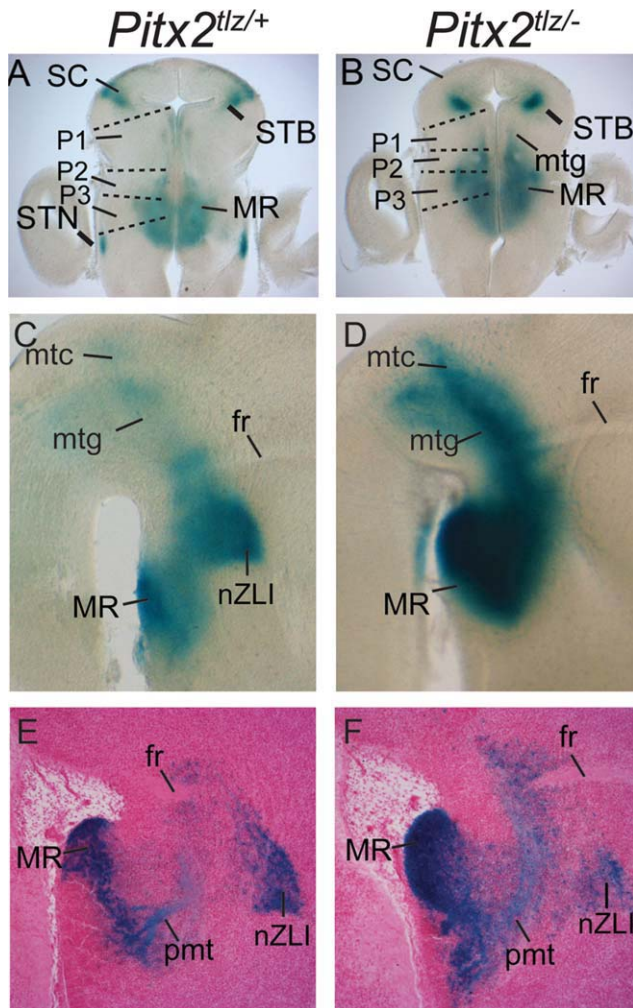
FIG. 2. Hypothalamic and midbrain neuronal populations are disrupted in *Pitx2^{tlz/null}* mice. β-galactosidase stained E14.5 (100 μm) vibratome coronal sections at the level of the subthalamic nucleus (with dorsal toward the top) (**a**, **b**) or sagittal sections through parasagittal hypothalamus (with dorsal to the right) (**c**, **d**). (a) *Pitx2^{tlz/+}* embryos have abundant staining in the superior colliculus (SC), subtectal band (STB), subthalamic nucleus (STN), and mammillary region (MR). (b) *Pitx2^{tlz/null}* embryos exhibit increased label in the STB and MR and no label in the STN or superficial SC. (c, d) β-galactosidase activity is widespread in the *Pitx2^{tlz/+}* mammillary region and is increased in the *Pitx2^{tlz/null}* mutant. (**e**, **f**) Cryosectioned and eosin counterstained sagittal sections of *Pitx2^{tlz/+}* and *Pitx2^{tlz/null}* E14.5 embryos show dense β-gal activity in the MR, pmt, and nucleus of the zona limitans (nZLI). Label is expanded in *Pitx2^{tlz/null}* mutants. Dorsal is to the right. fr, fasciculus retroflexus.

as those reported previously in *Pitx2^{Cre/null}* (Skidmore *et al.*, 2008) and *Pitx2^{null/null}* embryos (Gage *et al.*, 1999; Kitamura *et al.*, 1999; Lin *et al.*, 1999; Liu *et al.*, 2002; Lu *et al.*, 1999). Finally, PITX2 immunofluorescence was negative in *Pitx2^{tlz/tlz}* embryos, confirming that *Pitx2^{tlz}* is a null allele (data not shown). To define the expression of β-galactosidase from the *Pitx2^{tlz}* allele, whole E12.5 *Pitx2^{tlz/+}* and *Pitx2^{tlz/null}* embryos

(both containing only one copy of the *tlz* allele) were analyzed for β-galactosidase activity (Fig. 1d,e). β-galactosidase activity was detected in the embryonic *Pitx2^{tlz/+}* and *Pitx2^{tlz/null}* eye, pituitary, teeth, heart, craniofacial regions, and in a restricted pattern in the neural tube, similar to areas of endogenous *Pitx2* mRNA and protein expression (Fig. 1f–k).

Analysis of β-galactosidase staining in vibratome coronal and sagittal sections of E14.5 *Pitx2^{tlz/+}* brains showed high levels of β-galactosidase in the developing subthalamic nucleus, mammillary region, and superior colliculus (Fig. 2a,c,e). β-galactosidase activity in the *Pitx2^{tlz/+}* embryonic hypothalamus and midbrain was similar to previously published *Pitx2* mRNA and protein expression patterns (Martin *et al.*, 2002; Mucchielli *et al.*, 1996). In addition, β-galactosidase activity was diffuse throughout the basal midbrain, suggesting that it labels mtg and mtc projections that pass through the brainstem tegmentum and tectum (Fig. 2a,c). β-galactosidase staining was increased in the mammillary/retromammillary region in *Pitx2^{tlz/null}* mutants, consistent with lack of migration of subthalamic neurons and recruitment of their projections into an expanded mtg (Fig. 2b,d). Together, these data support roles for *Pitx2* in hypothalamic and midbrain neuronal migration (Skidmore *et al.*, 2008), and suggest that *Pitx2* may also be important for axon guidance. In the midbrain, *Pitx2^{tlz/null}* embryos exhibited inwardly shifted (toward the ventricle) β-galactosidase activity in the superior colliculus, consistent with lack of migration of local subtectal *Pitx2*-expressing neurons, which normally translocate into the superficial superior colliculus (Fig. 2b) (Martin *et al.*, 2004; Waite *et al.*, 2011).

Vibratome sectioned tissues provided critical information about brain region specific differences in β-galactosidase staining, but individual axons and terminal projections could not be readily distinguished from cell bodies. To address this, we prepared 30 μm sagittal sections from frozen embryos and stained for β-galactosidase activity (Fig. 2e,f). Analysis of serial sections through the basal hypothalamus of E14.5 *Pitx2^{tlz/+}* embryos confirmed abundant β-gal label in the mammillary region, the nucleus of the zona limitans, and in fibers of the pmt (Fig. 2e). In *Pitx2^{tlz/null}* embryos, β-gal label was dramatically increased throughout the mammillary region and in the diencephalic tegmentum around the enlarged pmt, with relatively less label in the nucleus of the zona limitans (nZLI; Fig. 2f), suggesting that cell bodies normally destined for the dorsally migrated subthalamic and nZLI nuclei fail to migrate and aggregate near their respective tegmental origins. These medially shifted *Pitx2*-expressing mutant neurons retain their ability to project axons caudally from the mammillary area.

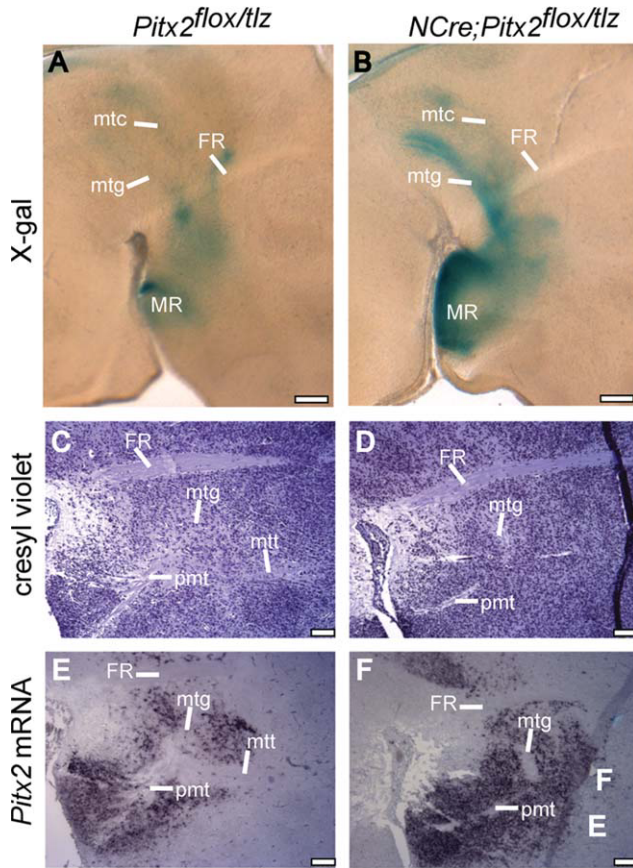Similar to previously reported *Nestin-Cre;Pitx2* conditional mutants (Sclafani *et al.*, 2006), *NCre;Pitx2^{tlz/flox}*

FIG. 3. Late gestation *Pitx2* deficient embryos exhibit altered mammillary trajectories. (**a**, **b**) Sagittal vibratome (150 μm) sections of β-galactosidase-stained E18.5 *Pitx2^{tlz/flox}* (a) and *NCre;Pitx2^{tlz/flox}* (b) embryos show abundant label in the basal hypothalamus and midbrain, which is increased in the *NCre;Pitx2^{tlz/flox}* embryos. (**c**, **d**) Cresyl violet staining sagittal sections adjacent to (e, f) confirm visible principal mammillary tract (pmt), mtg, and mammillothalamic tracts (mtt) in control embryos; the mtt appears hypoplastic in *NCre;Pitx2^{flox/tlz}* mutants. (**e**, **f**) *Pitx2* mRNA in sagittal sections of E18.5 embryos shows high *Pitx2* expression throughout the mammillary region, with increased label in the *NCre;Pitx2^{tlz/flox}*. In all panels, dorsal is toward the right. Scale bars are 100 μm. FR, fasciculus retroflexus.

conditional mice died in the early postnatal period, of unclear causes (data not shown). There were no external abnormalities in these pups to suggest craniofacial defects as a cause of death. *Pitx2^{tlz/flox}* E18.5 embryos exhibited β-gal staining in the mammillary hypothalamus, diencephalic tegmentum, and mtg and mtc tracts (Fig. 3a). To test whether β-gal activity from the *tlz* allele accurately reflects endogenous *Pitx2* expression, we characterized *Pitx2* mRNA expression in both control (*Pitx2^{tlz/flox}*) and conditional mutant E18.5 embryos. We used an antisense probe against the 3′ UTR of *Pitx2* mRNA that recognizes both wild type and null (recombined) alleles (see probe location in Fig. 1) (Martin *et al.*, 2004). In control embryos, *Pitx2* mRNA was high in the diencephalic tegmentum and mammillary area (Fig. 3e).

In conditional mutant (*NCre;Pitx2^{tlz/flox}*) embryos, both β-gal staining and *Pitx2* mRNA were expanded medially in the mammillary/retromammillary region (Fig. 3b,f), consistent with earlier observations that brain-specific loss of *Pitx2* results in persistent abnormalities in neuronal migration in the hypothalamus (Skidmore *et al.*, 2008; Waite *et al.*, 2011). Cresyl violet staining of sections adjacent to those processed for *Pitx2* mRNA showed intact pmt and mtg fibers, but absent mtt projections in the conditional mutant (Fig. 3c,d).

Increased β-gal in the mtc and mtg tracts of *NCre;-Pitx2^{tlz/flox}* embryos suggested generalized defects in mammillary projections beyond those previously reported from subthalamic nucleus to the tegmentum (Martin *et al.*, 2004). We used DiI in E18.5 control (*NCre; Pitx2^{flox/+}* and *Pitx2^{flox/+}*) and conditional mutant embryos to investigate the effects of *Pitx2* deficiency on mammillary projections. Control embryos exhibited intact principal mammillary, mtt, and mtg projections that were easily visualized in sagittal vibratome sections of DiI injected brains (Fig. 4a,c). In contrast, mtt fibers were truncated or severely diminished in *Pitx2* conditional mutants (Fig. 4b,d). Immunofluorescence with antineurofilament on paraffin-embedded sections also revealed intact principal mammillary and mtg projections in both control and conditional mutant embryos, and reduced mtt in conditional mutants (Fig. 4e–h). These results provide further evidence that mammillothalamic fibers require *Pitx2* for proper extension from an intact pmt.

Pax6 is normally highly expressed near the bifurcation of the pmt and mtt. *Pax6* mutant embryos selectively lack mtt (Tsuchiya *et al.*, 2009; Valverde *et al.*, 2000) and a specific group of *Pax6*-expressing cells derived from the prethalamus is essential to elicit the pmt-mtt axonal branching (Szabo *et al.*, 2011). We tested whether PAX6-positive cells are altered in *Pitx2* mutants, using immunofluorescence on paraffin embedded sagittal sections of control versus conditional mutant E18.5 embryos. Double immunofluorescence with neurofilament and PAX6 showed abundant PAX6+ cells at the pmt-mtt bifurcation and along the nascent mtt, as previously described (Fig. 4g) (Valverde *et al.*, 2000). However, PAX6+ cells at the pmt-mtt bifurcation were reduced in the conditional mutants, despite normal appearing numbers of cells along the purported route of the absent mtt (Fig. 4h).

METHODS AND MATERIALS

Generation of *Pitx2^{tlz/+}* Embryonic Stem Cells and Mice

A previously generated *Pitx2* targeting vector (Gage *et al.*, 1999) was modified to remove *loxP* sites and a *neomycin* cassette by *BamHI* partial digest and inverse
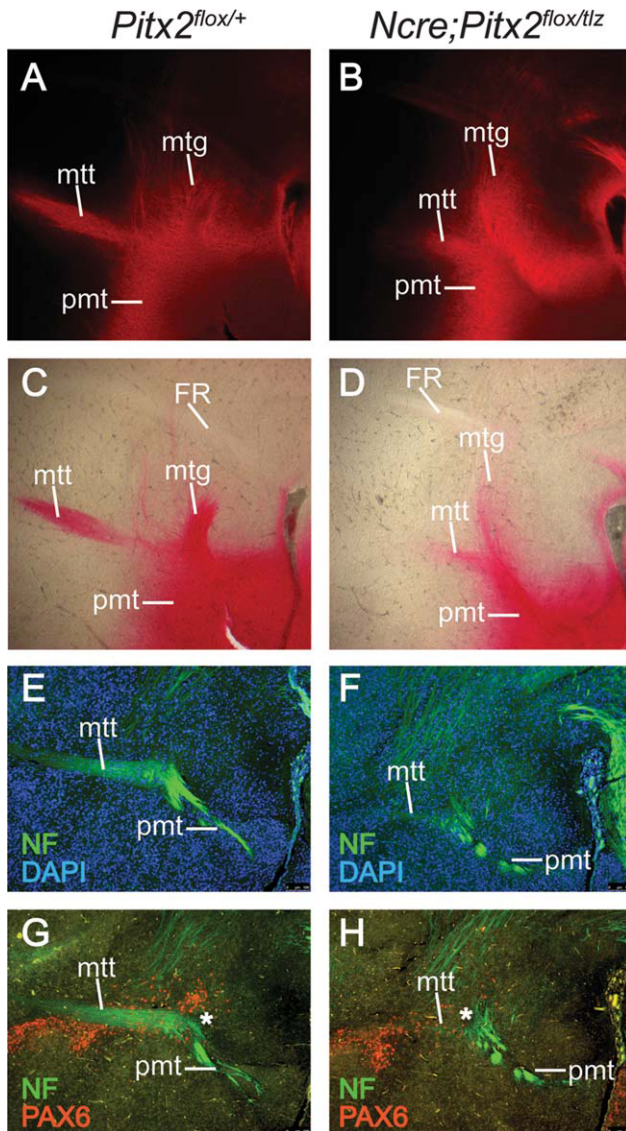
FIG. 4. Mammillothalamic tracts and PAX6-expressing cells are disrupted in *Pitx2* conditional mutants. E18.5 *Pitx2*^{tlz/flox} (**a**, **c**) and *Nestin-Cre;Pitx2*^{tlz/flox} (**b**, **d**) embryos were exposed to DiI for 6 weeks then vibratome sectioned in the sagittal plane. Fluorescent (a, b) and brightfield (c, d) images of the mammillary region show the principal mammillary tract (pmt), mammillothalamic tract (mtt), and mammillotegmental tract (mtg). The mtt is severely hypoplastic in the *Nestin-Cre;Pitx2*^{tlz/flox} (b, d) embryos. Antineurofilament immunofluorescence on E18.5 *Pitx2*^{tlz/flox} (**e**) and *Nestin-Cre;Pitx2*^{tlz/flox} (**f**) embryos confirms severely reduced mtt fibers in the *Nestin-Cre;Pitx2*^{tlz/flox} mutant. Blue label in e and f shows DAPI-stained cell nuclei. (**g**, **h**) Double immunofluoresence with antineurofilament and anti-PAX6 shows reduced PAX6+ cells at the pmt-mtt bifurcation in *Nestin-Cre;Pitx2*^{tlz/flox} mutants. In all images, dorsal is toward the left.

PCR of the resulting 8.4 kb fragment, which engineered a *PacI* site. The resulting PCR product was concentrated using a QiaQuick PCR Purification kit (Qiagen), digested with *PacI*, and religated. A 6.8 kb IRES-*TaulacZ* fusion construct (provided by Dr. Peter Mombaerts,

Max Planck Institute of Biophysics, Frankfurt, Germany) (Mombaerts *et al.*, 1996) was subcloned as a *PacI* fragment into the newly generated targeting vector, and confirmed by restriction mapping and sequencing.

The final *Pitx2*^{tlz} targeting vector consisted of a 1.7 kb 5′ homology fragment and a 3.2 kb 3′ homology fragment, an endogenous *Pitx2* splice acceptor and 8 bp of 5′ exon 4 sequence, followed by an engineered stop codon (TAG), and the undisrupted, endogenous *Pitx2* nuclear localization signal [immediately 5′ of exon 4 (Gage and Camper, 1997)], which allows for translation of TaulacZ protein from a bicistronic mRNA. The targeting vector was digested with *SalI* and gel purified, and the resulting 12.46 kb fragment was electroporated into E14Tg2A embryonic stem (ES) cells [originally derived from mouse strain 129 (Nagy *et al.*, 1993)]. Transfected cells were selected for using G418. Six clones (3.125% targeting efficiency) were identified using 3′ genotyping PCR. These clones were further confirmed by Southern blotting with a 5′ probe (Fig. 1), and by PCR of the 5′ homology arm. Three clones were expanded for ES cell injection into donor blastocysts (from a mixed C57BL/6J X DBA/2J genetic background). Resultant chimeric males were bred to C57BL/6J females to establish germline transmission of the *Pitx2*^{TaulacZ} (*Pitx2*^{tlz}) allele. The *Pitx2*^{tlz} allele is a null *Pitx2* allele, since the homeodomain and downstream sequences are replaced by the *TaulacZ* fusion construct. The neomycin cassette contained in the *TaulacZ* cassette was removed by mating *Pitx2*^{tlz/+} mice with EIIa-Cre homozygyous mice (Xu *et al.*, 1999). *Pitx2*^{tlz/+} mice were genotyped to ensure absence of the EIIa-Cre transgene, then back-crossed to generation N8 with C57BL6/J. All experiments and procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals (UCUCA).

Mice and Genotyping

Mice were PCR genotyped for the presence of *Pitx2*^{tlz} using Chromo Taq (Denville) with primers 5′GGTGGGGTGGGGGTGTCTGTAAAA3′, 5′GCTAGGCGCGAAGGTTCTCCAGTG3′, and 5′CGCAGGTAGCAGAGCGGGTAAACT3′ using an initial denaturation at 94°C for 2 min, followed by 35 cycles of 95°C × 30 s, 59°C × 30 s, 72°C × 2 min 30 s, and a 10 min incubation at 72°C. *Pitx2*^{null/+} mice were bred and genotyped as previously described (Gage *et al.*, 1999), and were maintained, along with *Pitx2*^{null/+} to generation N19 on a C57BL/6J background. *Nestin-Cre* (Tronche *et al.*, 1999) transgenic mice (Jackson Laboratory, Bar Harbor, Maine, stock# 003771) were bred to *Pitx2*^{tlz/+} mice, and *Nestin-Cre;Pitx2*^{tlz/+} mice were bred to *Pitx2*^{flox/flox} (Gage *et al.*, 1999) mice to generate litters for analysis.

Embryo Preparation and β-galactosidase Assay

For β-galactosidase staining, timed pregnancies were established, and the morning of plug identification was designated as E0.5. Embryos were removed from pregnant mice after cervical dislocation and hysterectomy. Amniotic sacs, tails, or limbs were used for genotyping. Litters of E12.5–E18.5 embryos were dissected and fixed in 4% formaldehyde for 30 min to 1 h, depending on age, then washed in PBS and X-gal wash buffer, as described earlier (Sclafani *et al.*, 2006). Embryos were subsequently postfixed in 4% formaldehyde and brains were dissected, embedded in 4% agarose, and vibratome sectioned at 100–150 µm. Embryos processed for frozen sectioning were fixed, cryosectioned on a Leica CM1950 cryostat at 30 µm, then X-gal stained and counterstained with eosin as described (Sclafani *et al.*, 2006). For each result shown, at least three different embryo pairs were analyzed, and representative images presented. For whole-mount analysis, embryos were cleared in a glycerol gradient. X-gal stained embryos were visualized on a Leica MZ10F dissecting microscope; sections were visualized on a Leica DM500B upright microscope. Images were digitally captured and processed in Photoshop CS3 (Adobe).

In situ Hybridization and Immunofluorescence

E14.5 or E18.5 embryos were collected, fixed in 4% paraformaldehyde and processed for *Pitx2 in situ* hybridization or immunofluorescence as previously described (Martin *et al.*, 2002). *Pitx2* antisense probe for *in situ* hybridization was against the $3'$UTR (Martin *et al.*, 2002), and was labeled using digoxigenin (Roche, Indianapolis, IN) and NBT/BCIP (Roche) with hybridization at 55°C. Immunofluorescence was performed on paraffin-embedded tissues sectioned at 9 µm with antineurofilament (1:100, #2H3 from Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-PAX6 [1:500, Covance (Princeton, NJ)] using Alexa-488 or Alexa-555 conjugated secondary goat antimouse or goat antirabbit antibodies from Invitrogen (Carlsbad, CA). Tissues were counterstained with DAPI (Invitrogen) (1:50,000) in PBS for 5 min. Tissues were imaged on a Leica upright DM500B fluorescence microscope and processed using Photoshop CS3 (Adobe, San Jose, CA).

DiI Injections

For axonal tracing, E18.5 embryos were harvested and fixed in 4% paraformaldehyde. Brains were removed from the skull, and crystals of 1,1V-dioctadecyl-3,3,3V,3V-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen) inserted into the ventral aspect of the cephalic flexure. Embryos were incubated at 37°C for 6 weeks, then embedded in 4% low-melt agarose and vibratome sectioned (Leica Vibratome 1500, Wetzlar, Germany) at 100 µm. Images were captured by brightfield and fluorescence microscopy using a dsRed filter with a Leica MZ10F dissecting microscope and processed in Photoshop CS3 (Adobe).

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