Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn

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Summary

Mutations in the human and mouse *PTF1A/Ptf1a* genes result in permanent diabetes mellitus and cerebellar agenesis. We show that Ptf1a is present in precursors to GABAergic neurons in spinal cord dorsal horn as well as the cerebellum. A null mutation in *Ptf1a* reveals its requirement for the dorsal horn GABAergic neurons. Specifically, Ptf1a is required for the generation of early-born (dI4, E10.5) and late-born (dIL^A, E12.5) dorsal interneuron populations identified by homeodomain factors Lhx1/5 and Pax2. Furthermore, in the absence of Ptf1a, the dI4 dorsal interneurons trans-fate to dI5 (Lmx1b$^+$), and the dIL^A to dIL^B (Lmx1b$^+$;Tlx3$^+$). This mis-specification of neurons results in a complete loss of inhibitory GABAergic neurons and an increase in the excitatory glutamatergic neurons in the dorsal horn of the spinal cord by E16.5. Thus, Ptf1a function is essential for GABAergic over glutamatergic neuronal cell fates in the developing spinal cord, and provides an important genetic link between inhibitory and excitatory interneuron development.

Key words: Spinal cord development, Dorsal horn inhibitory neurons, BHLH transcription factor, Mouse

Development

Introduction

Truncation of the human *PTF1A* gene leads to permanent neonatal diabetes mellitus and cerebellar hypoplasia (Sellick et al., 2004), while a null mutation of *Ptf1a* (*Ptf1-p48*) in mouse results in neonatal lethality with pancreatic and cerebellar agenesis (Hoshino et al., 2005; Krapp et al., 1998; Rose et al., 2001; Sellick et al., 2004). *Ptf1a* encodes a basic helix-loop-helix (bHLH) transcription factor most closely related to the Twist subclass of bHLH genes (Obata et al., 2001). It was first identified as one of three subunits of the PTF1 transcription factor complex required for expression of pancreatic digestive enzyme genes (Cockell et al., 1989). As a bHLH factor, Ptf1a is notable in that it not only heterodimerizes with the E-protein, E47, but it also complexes with Rbpsuh (RBP-Jk, CBF1), an intercellular mediator of Notch signaling, and thus, may directly impact Notch signaling pathways (Beres et al., 2005; Obata et al., 2001). This is a unique characteristic for a bHLH factor, and provides added complexity to the molecular models proposed for neural specific bHLH factors and Rbpsuh function in nervous system development (Artavanis-Tsakonas et al., 1999; Bertrand et al., 2002; Bray and Furriols, 2001).

In addition to expression in the pancreas and developing cerebellum, Ptf1a is present in the dorsal neural tube of early stage embryos (Obata et al., 2001), an embryonic structure that gives rise to the dorsal horn of the spinal cord in the mature animal. The spinal cord dorsal horn largely consists of excitatory (glutamatergic) and inhibitory (GABAergic) neurons that modulate somatosensory inputs from the periphery, including pain, temperature and mechanoception. Distinct neuronal subtypes in the developing dorsal neural tube have been defined by the timing of their birth, and key differences in the expression of homeodomain (HD) transcription factors (Jessell, 2000). The requirement for bHLH transcription factors, Math1 (Atoh1 – Mouse Genome Informatics), Ngn1, Ngn2, Mash1 (Ascl1 – Mouse Genome Informatics) and Olig3, for the formation of specific neuronal subtypes defined by the HD factors has been demonstrated (Bermingham et al., 2001; Gowan et al., 2001; Helms et al., 2005; Müller et al., 2005), but their roles in specifying neurotransmitter identity have not been reported. Rather, the HD genes are involved in generating glutamatergic versus GABAergic neurons in the dorsal horn. *Tlx1* and *Tlx3* have been shown to be post-mitotic selector genes for the glutamatergic transmitter phenotype (Cheng et al., 2004). In the dorsal horn of *Tlx1/3* double mutants, glutamatergic neurons are reduced while GABAergic neurons are increased. By contrast, no selector gene has been described for the GABAergic phenotype, although HD factors Lbx1 and Pax2 play roles in generating GABAergic neurons in the dorsal horn (Cheng et al., 2005; Cheng et al., 2004; Gross et al., 2002; Müller et al., 2002).

Here we report that, in the neural tube, Ptf1a is required for

dorsal neural tube Pax2 expression and suppression of Tlx3, which leads to the formation of GABAergic neurons while suppressing the formation of glutamatergic neurons. Thus, Ptf1a and Tlx1/3 act as opposing switches for the generation of neurons with specific neurotransmitter phenotypes, revealing genetic interactions that link the development of these two classes of interneurons in the spinal cord. A disruption in the balance between inhibitory and excitatory neuronal activity of the magnitude that occurs in the absence of Ptf1a function has profound consequences for the organism, and may explain the respiratory difficulties and cerebral excitability seen in humans with a mutation in this gene (Hoveyda et al., 1999; Sellick et al., 2004).

Materials and methods

Animals

The *Ptf1a*^{Cre} mutant mice have been previously described; these mice have Cre-recombinase in place of the *Ptf1a* protein-coding region (Kawaguchi et al., 2002). The reporter strains *R26R-stop-YFP* (Srinivas et al., 2001) and *R26R-stop-LacZ* (Soriano, 1999) (Gt(ROSA)26Sor^{tmlSor}) were used to visualize cells and their progeny that were exposed to Cre recombinase activity. Genotyping was done using PCR with primers 5′-AACCAGGCCCAGAAGGTTAT-3′ and 5′-TCAAAGGGTGGTTCGTTCTC-3′ for wild-type *Ptf1a* locus, and with 5′-GCATAACCAGTGAAACAGCATTGCTG-3′ and 5′-GGA-CATGTTCAGGGATCGCCAGGCG-3′ for *Cre* in the mutant allele. R26R mice were genotyped as described (Soriano, 1999).

X-gal staining

Embryos were fixed for 1 hour in 4% formaldehyde in 0.1 mol/l sodium phosphate buffer pH 7.4 at room temperature and washed three times in 0.1 mol/l sodium phosphate buffer pH 7.4 for 30 minutes each. Embryos were incubated at 30°C for 24 hours in X-gal staining solution (PBS/5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 2 mmol/l $MgCl_2$, 1 mg/ml X-gal), washed three times in 0.1 mol/l sodium phosphate buffer pH 7.4 and whole-mount images were taken; then the embryos were sunk in 30% sucrose in 0.1 mol/l sodium phosphate buffer pH 7.4 overnight at 4°C, embedded in OCT, and cryosectioned at 40 μm. Adult mice were perfused with 4% formaldehyde in 0.1 mol/l sodium phosphate buffer pH 7.4 under standard conditions. Brains and spinal cords were fixed for an additional 2 hours at 4°C and processed as above for cryosection at 20 μm. Sections were incubated in X-gal solution, rinsed and counterstained with Nuclear Fast Red or Nissl.

Immunofluorescence and mRNA in-situ hybridization

Appropriately staged embryos were dissected in ice-cold 0.1 mol/l sodium phosphate buffer pH 7.4 and fixed for 2 hours at 4°C in 4% formaldehyde in 0.1 mol/l sodium phosphate buffer pH 7.4. Embryos were processed as above for cryosection at 30 μm.

Immunofluorescence was performed using the following primary antibodies: Mouse anti-BrdU (Becton Dickinson), guinea pig anti-VGLUT2 (Chemicon), mouse anti-Lhx1/5 (4F2) (Developmental Studies Hybridoma Bank), rabbit anti-Ptf1a (Li and Edlund, 2001), mouse anti-Mash1 (Lo et al., 1991), rabbit anti-GFP (Molecular Probes), chicken anti-GFP (Chemicon), guinea pig anti-Lmx1b (Müller et al., 2002), mouse anti-GAD67 (Sigma), rabbit anti-Islet1/2 (Tsuchida et al., 1994), rabbit anti-Pax2 (Zymed), rabbit anti-Tlx3 (gift from T. Müller and C. Birchmeier), and mouse anti-GABA (Sigma). For BrdU experiments, BrdU (200 μg/g body weight) was injected into pregnant mothers for 1 hour before sacrifice. Double immunofluorescence of Ptf1a and BrdU was performed sequentially with Ptf1a antibodies followed by treatment of the sections with 2N HCl for 15 minutes, 0.1 mol/l sodium borate pH 8.5 for 20 minutes, and incubation with primary BrdU. Specific neuronal cell types were counted using confocal (Bio-Rad MRC 1024) images from a minimum of three different animals on three or more sections. mRNA in-situ hybridization was carried out as described (Birren et al., 1993; Cheng et al., 2004). Antisense probes were made from plasmids provided by Q. Ma (Cheng et al., 2004). All sections shown are from the forelimb level.

Results

Ptf1a is present in the developing nervous system

We used recombination-based lineage tracing in vivo to characterize regions of the nervous system derived from *Ptf1a*-expressing cells. The mouse strain *Ptf1a*$^{Cre/+}$, which has the *Ptf1a* protein-coding region replaced by that of Cre recombinase (Kawaguchi et al., 2002), was crossed with the Cre reporter strain *R26R-stop-lacZ*$^{+/-}$. The *R26R-stop-lacZ*$^{+/-}$ allele provides a permanent lineage marker for the cells with Cre recombinase activity (Soriano, 1999). β-Gal staining in a *Ptf1a*$^{Cre/+}$;*R26R-stop-lacZ*$^{+/-}$ embryo at embryonic day (E) 11.5, and dissected brain and spinal cord at E16.5, illustrate that before these stages *Ptf1a* is expressed in the neural tube, and the *Ptf1a* lineage extends from the hindbrain caudally to the tail (Fig. 1A,B). β-Gal staining was also detected in the pancreas at E11.5 (Fig. 1A, arrow). Within the neural tube, β-gal staining was restricted to dorsal regions (Fig. 1A, inset; B, arrowhead), and in restricted regions in the ventral diencephalon (Fig. 1B, inset) that include the preoptic nucleus and ventral hypothalamus. To identify the cell types in the adult nervous system that are derived from *Ptf1a*-expressing cells, we examined β-gal staining in adult brains and spinal cords from these mice. A majority of stained cells in the spinal cord reside in the dorsal horn primarily in laminae I-IV, with a few scattered cells found in ventral laminae (Fig. 1C,D). In the cerebellum, *Ptf1a*-expressing cells gave rise to multiple GABAergic cell types, including at least a subset of Purkinje, stellate, basket and Golgi cells (Hoshino et al., 2005) (Fig. 1E). A population of cells in the ventral hypothalamus was also detected (Fig. 1F). The requirement for Ptf1a for the development of GABAergic neurons in the cerebellum has recently been reported (Hoshino et al., 2005).

Ptf1a is restricted to post-mitotic cells within the ventricular zone of the dorsal neural tube in an overlapping pattern with Mash1

The β-gal staining in the dorsal neural tube resembled the expression pattern of another bHLH transcription factor, Mash1 (Gowan et al., 2001; Helms et al., 2005). To characterize the expression pattern of *Ptf1a* in more detail in this region, we used double label immunofluorescence with rabbit anti-Ptf1a (Li and Edlund, 2001) and mouse anti-Mash1 (Lo et al., 1991), or mouse anti-BrdU in BrdU-pulsed embryos. At E10.5, Ptf1a was detected within the central portion of the dorsal Mash1 domain (Fig. 1G). In this region, Mash1 levels were low relative to levels in adjacent dorsal and ventral regions (Fig. 1G′). Because Mash1 is present in ventricular zone cells adjacent to dI3-dI5 neurons (Helms et al., 2005), this pattern suggests that Ptf1a may be in the dI4 precursor domain. Within the domain common to both factors, Ptf1a and Mash1 co-labeled a subset of cells located on the ventricular side (Fig. 1G, inset). The pattern of Ptf1a is dynamic, and by E11.5 the

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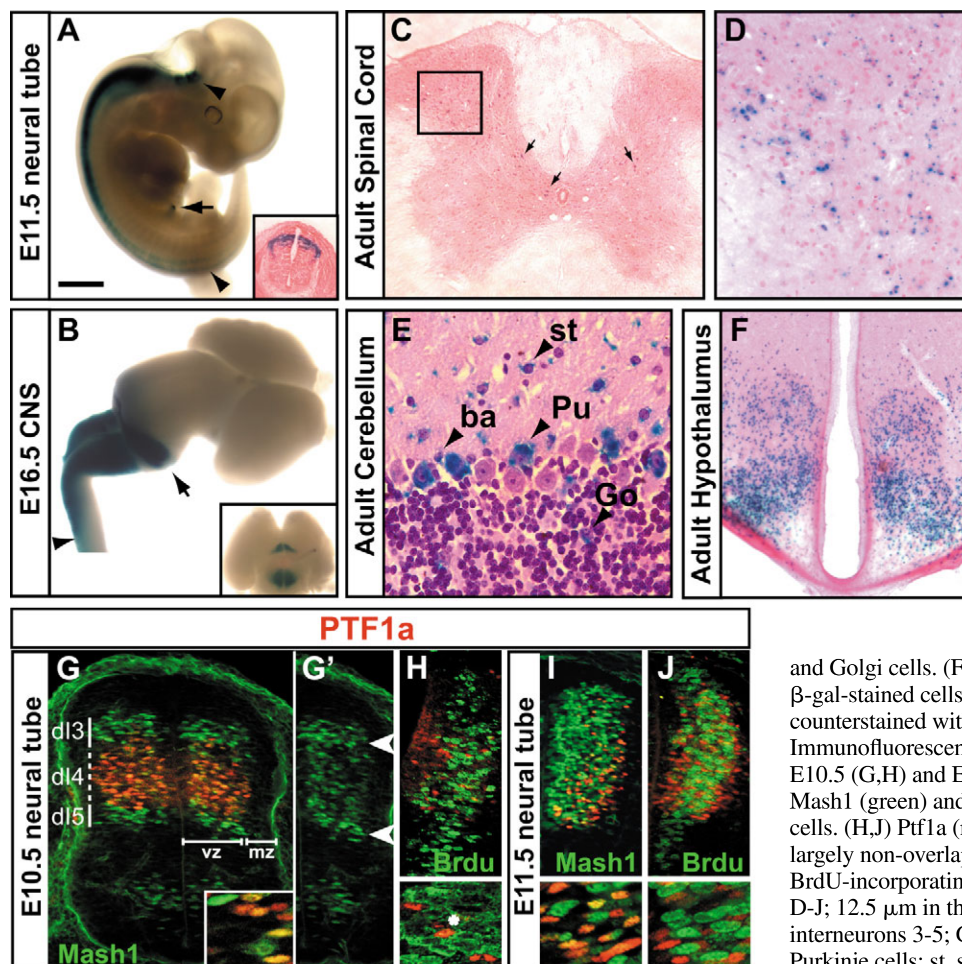

Fig. 1. Ptf1a is in precursors to multiple neural tissues and overlaps with Mash1 in the dorsal neural tube. The Ptf1a lineage was visualized by β-gal staining of *Ptf1a*$^{Cre/+}$*;R26R-stop-lacZ*$^{+/-}$ mice. (A) E11.5 whole mount, showing β-gal staining in the dorsal neural tube (inset) from the mid-hindbrain to the tail (arrowheads). Staining in the pancreas is indicated by the arrow. (B) brain and spinal cord dissected from an E16.5 embryo, stained for β-gal, showing expression in cerebellum (arrow) and dorsal spinal cord (arrowhead). Ventral diencephalon also reveals cells from the Ptf1a lineage (inset). (C,D) Adult cervical spinal cord, showing β-gal stained cells primarily in laminae I-IV. The boxed area in C is shown at higher magnification in D. Arrows (C) highlight labeled cells found ventral to boxed area. (E) Adult cerebellum with β-gal-stained Purkinje, stellate, basket and Golgi cells. (F) Coronal section of the adult brain reveals β-gal-stained cells in the ventral hypothalamus. Tissue is counterstained with Nuclear Fast Red (C,D,F) and Nissl (E). Immunofluorescence on transverse neural tube sections of E10.5 (G,H) and E11.5 (I,J) wild-type mice. (G,I) Ptf1a (red), Mash1 (green) and their co-labeling (yellow) in a subset of cells. (H,J) Ptf1a (red) and BrdU incorporation (green) are largely non-overlapping. The asterisk in H indicates a rare BrdU-incorporating Ptf1a cell. Scale bar: 1 mm in A; 50 μm in D-J; 12.5 μm in the insets. ba, basket cells; dI3-dI5, dorsal interneurons 3-5; Go, Golgi cells; MZ, marginal zone; Pu, Purkinje cells; st, stellate cells; VZ, ventricular zone.

dorsal and ventral boundaries of Ptf1a became identical with those of Mash1 (Fig. 1I). At this stage of neural tube development, the dorsal ventricular zone gives rise to the two late-born neurons, dIL^{A} and dIL^{B}. Ptf1a marks cells that appear to have exited the cell cycle, as they rarely co-label with BrdU incorporation at either E10.5 or 11.5 (Fig. 1H,J). This cell-cycle status of Ptf1a cells contrasts to the Mash1 population, in which a significant proportion of cells are still dividing (Helms et al., 2005). Taken together, Ptf1a is largely restricted to post-mitotic cells in the ventricular zone of the dorsal neural tube in a pattern that suggests it may be in the precursors to dI4, dIL^{A} and/or dIL^{B} neurons.

Ptf1a is required to generate dI4 and to suppress dI5 dorsal interneurons

Embryos null for Ptf1a (*Ptf1a*$^{Cre/Cre}$) were examined at E10.5 for markers that distinguish interneuron populations dI2-dI6. These early neuronal populations are defined by several criteria that include birth date (before E11) and the expression of HD transcription factors (Helms and Johnson, 2003) (Fig. 2I). In the absence of Ptf1a (*Ptf1a*$^{Cre/Cre}$), the dI4 population was absent, as demonstrated by the loss of Pax2;Lhx1/5 double positive cells (Fig. 2A,B; arrow). Conversely, dI5 neurons, marked by Lmx1b, expanded dorsally into the domain normally containing dI4 neurons (Fig. 2A-D; arrow). No change in cell number was observed in the dI2 (Lhx1/5) and dI3 (Isl1) populations (Fig. 2B,D; see counts in Fig. 2J). No change in cell death was observed using TUNEL or Caspase3 immunocytochemistry at this stage (data not shown). Thus, Ptf1a is essential for the generation of dI4 interneurons and the suppression of dI5 (Fig. 2K). This is by contrast to recent studies showing Mash1 is required for dI5 while suppressing dI4 neurons (Helms et al., 2005).

In the *Ptf1a* null mutant embryos, the number of cells increased in the dI5 cells complemented the number of dI4 neurons lost (Fig. 2J). These results suggest that in the absence of Ptf1a, the dI4 cells trans-fate into dI5. To test this directly, we crossed the *Ptf1a*$^{Cre/+}$ mice to the *R26R-stop-YFP* Cre reporter strain, which will express YFP in cells with Cre recombinase and in all descendants of these cells (Srinivas et al., 2001). In *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/-}$ mice, the dI4 marker Lhx1/5 co-localized with YFP, demonstrating that dI4 neurons are derived from Ptf1a precursor cells, and thus, the loss of dI4 neurons in the Ptf1a null embryos is cell-autonomous (Fig. 2E,F). In these embryos, YFP did not co-label with the dI5 marker Lmx1b (Fig. 2G). By contrast, in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/-}$ embryos, the dI4 neurons were lost and YFP now co-localized with Lmx1b (dI5) (Fig. 2G,H). Thus, in the absence of Ptf1a, the cell generates a dI5 neuron rather than a dI4.

At all stages examined, YFP expression was higher in the *Ptf1a* mutant than in the *Ptf1a* heterozygous embryos. This is

at least partly due to the presence of two Cre alleles in *Ptf1a*$^{Cre/Cre}$ versus one allele in *Ptf1a*$^{Cre/+}$. However, it could also reflect a component of a negative feedback loop in the regulation of the *Pft1a* locus.

Ptf1a is required to generate late-born dIL^{A} and to suppress dIL^{B} interneurons

A second round of neurogenesis occurs in the developing spinal cord between E11 and E13 to form the dIL^{A} and dIL^{B} populations of dorsal interneurons (Gross et al., 2002; Müller et al., 2002). The *Ptf1a* mutants were examined at E12.5 with Pax2 and Lhx1/5, which mark dIL^{A}, and Lmx1b and Tlx3, which mark dIL^{B}, to determine if these two late-born populations require Ptf1a. In the absence of Ptf1a, Pax2 was completely lost and Lhx1/5 was dramatically reduced, specifically in the dorsal half of the neural tube, revealing a loss of dIL^{A} neurons (Fig. 3A,B). The number of cells expressing Lmx1b or Tlx3 (dIL^{B}) was significantly increased in the absence of Ptf1a, while cells expressing Isl1 were unaffected (Fig. 3C,D,I,J; see Fig. 3K for cell counts). No increase in cell death was detected using TUNEL or Caspase3 immunocytochemistry at this stage (data not shown). These results demonstrate that Ptf1a is required for the formation of dIL^{A} neurons and normally suppresses the formation of dIL^{B} neurons.

Although not as complete as the dI4/dI5 switch at E10.5, there was a concomitant increase in dIL^{B} neurons when dIL^{A} neurons were lost, suggesting that there is a fate switch from dIL^{A} to dIL^{B} in *Ptf1a* mutant embryos. To visualize the switch in cell fate, we again utilized *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. In *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/–}$ embryos, Lhx1/5 (dIL^{A}) co-localized with YFP, demonstrating that the loss of dIL^{A} in the *Ptf1a* null is a cell-autonomous effect (Fig. 3E,E′,F,F′). The dIL^{B} markers Lmx1b and Tlx3 were not co-expressed with YFP in embryos heterozygous for *Ptf1a* (Fig. 3G,G′,I). However, in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/–}$ embryos, there was a dramatic increase in the number of YFP/Lmx1b and YFP/Tlx3 double positive cells, consistent with a cell fate switch from dIL^{A} to dIL^{B} (Fig. 3H,H′,J). Interpretation of this result is tempered by the increase in YFP expression in the *Ptf1a* mutant (Fig. 3F,H), as stated earlier. Also, the increase in the markers for dIL^{B} in the mutant cannot completely account for the number of dIL^{A} cells lost. This could reflect differences in temporal characteristics or detection efficiency of the individual markers. Regardless, the overlap in YFP and dIL^{B} markers in the mutant, combined with Ptf1a being largely restricted to post-mitotic cells, the increase in the number of dIL^{B}, and the lack of apoptosis, together suggest that Ptf1a

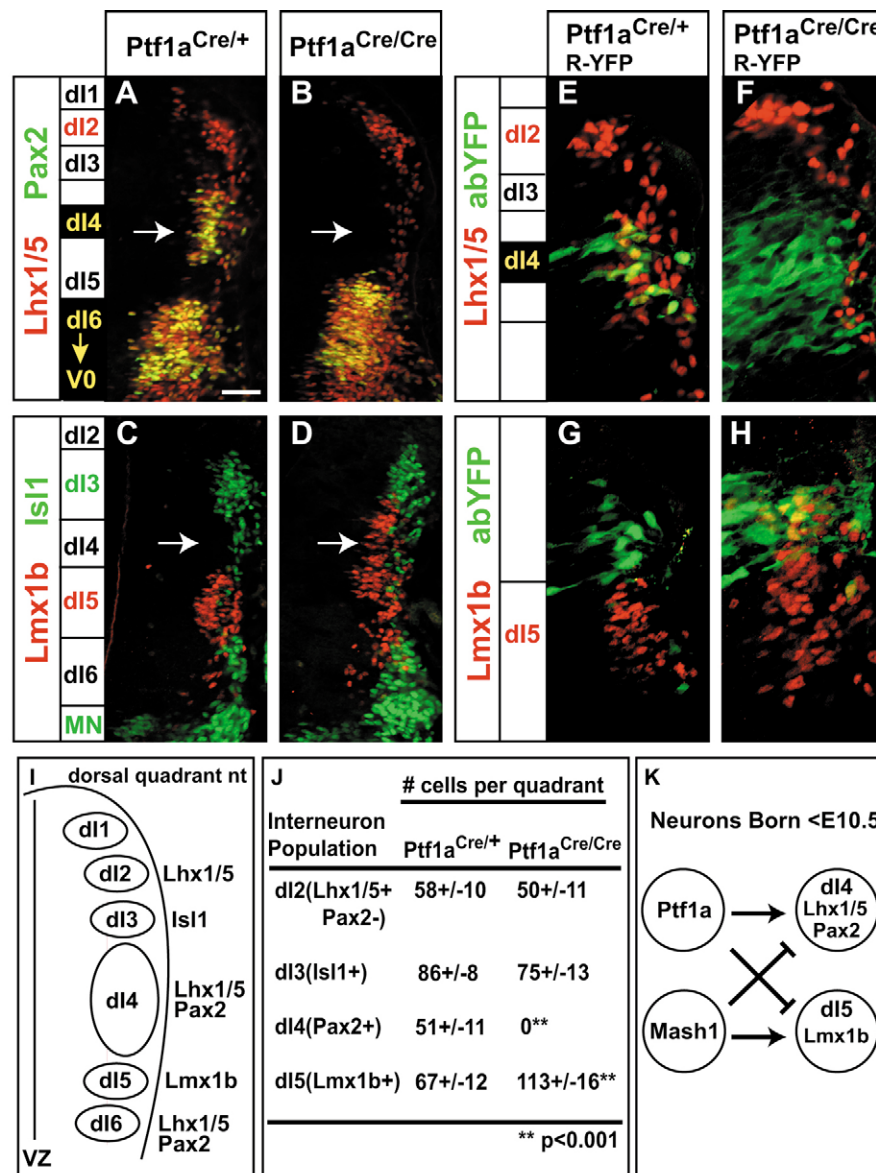

Fig. 2. dI4 are trans-fated to dI5 interneurons in Ptf1a-deficient embryos. Immunofluorescence on neural tube transverse sections of *Ptf1a*$^{Cre/+}$ (A,C) and *Ptf1a*$^{cre/cre}$ (B,D) mouse E10.5 embryos. (A,B) dI4 neurons marked in yellow by co-labeling of Lhx1/5 (red) and Pax2 (green) are lost in the *Ptf1a* null (arrows). (C,D) Lmx1b (red) labeling dI5 is expanded in *Ptf1a* null embryos, while Isl1 (green) labeling dI3 are not affected. (E-H) Anti-GFP antibody was used to detect YFP in E10.5 *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. YFP (green) co-localizes with the Lhx1/5 (dI4;red), but fails to do so in *Ptf1* null embryos (compare E and F). (G) Lmx1b (dI5;red) does not co-localize with YFP in the presence of Ptf1a, but they do co-localize in the null (H). The ventricle is to the left in all panels, as shown in a schematic representation of the markers used to distinguish individual neuronal populations (I). (J) Cell counts for each neuronal population. (K) Summary diagram showing that Ptf1a is required for dI4 and suppression of dI5 interneurons (see Results). Scale bar: 50 μm in A-D; 25 μm in E-H.

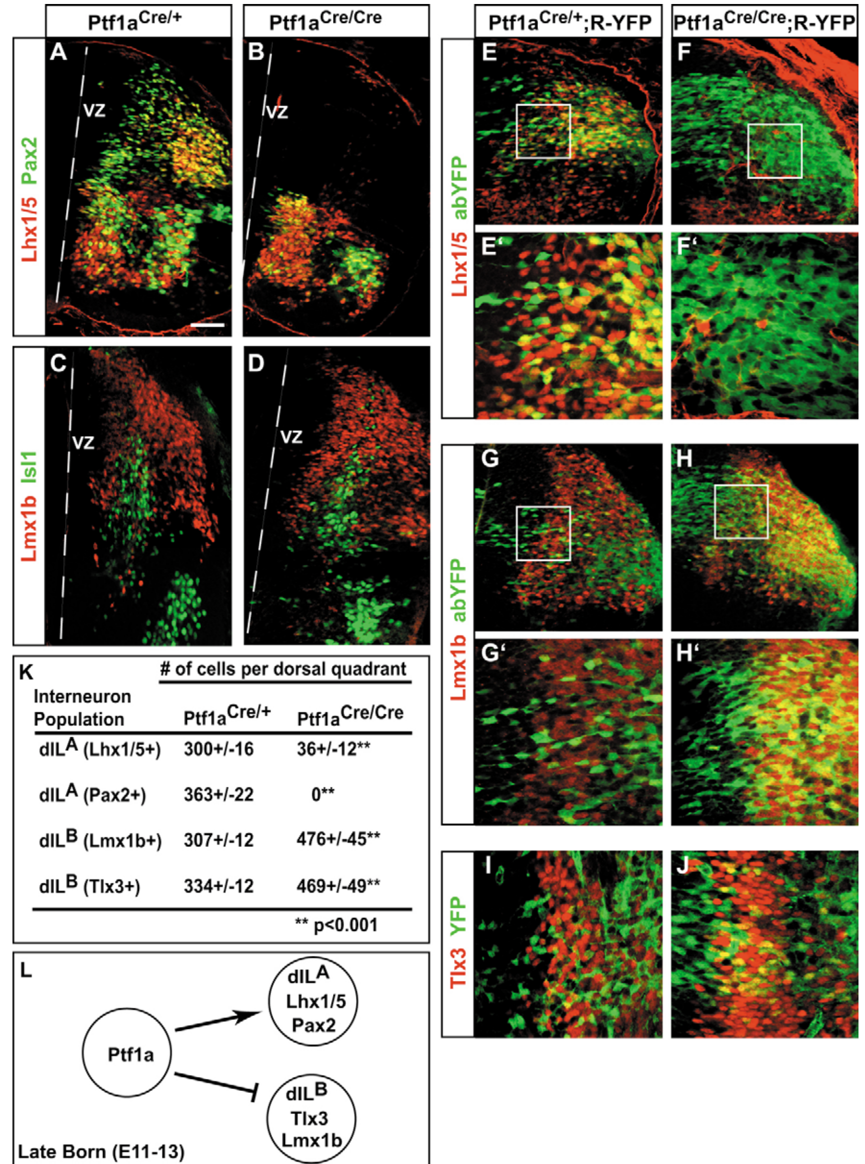

Fig. 3. dIL^A trans-fate to dIL^B interneurons in Ptf1a null embryos. Immunofluorescence on neural tube transverse sections of *Ptf1a*$^{Cre/+}$ (A,C) and *Ptf1a*$^{Cre/Cre}$ (B,D) mouse E12.5 embryos. (A,B) dIL^A neurons, marked by Lhx1/5 (red) and Pax2 (green), are lost specifically in the dorsal neural tube, but not the ventral neural tube in the *Ptf1a* null (dashed line indicates the position of the ventricle). (C,D) dIL^B neurons, marked by Lmx1b (red) increase in the *Ptf1a* null, but there is no change in the number of Isl1 (green) cells. (E-J) Anti-GFP antibody was used to detect YFP at E12.5 in *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. (E-F′) In the presence of Ptf1a, YFP (green) co-localizes (yellow) with the dIL^A marker, Lhx1/5 (red), but it does not do so in *Ptf1a* null embryos. (G-H′) Lmx1b (dIL^B; red) and (I-J) Tlx3 (dIL^B; red) rarely co-localizes with YFP in embryos with Ptf1a, while substantially more Lmx1b/YFP and Tlx3/YFP double positive cells appear in *Ptf1a*-deficient embryos (yellow). The boxed regions in E-H are shown at higher magnification in E′-H′. (K) Cell counts of specific neuronal populations. (L) Summary diagram showing that Ptf1a is required for the dIL^A interneuron cell fate, while suppressing dIL^B. Scale bar: 50 μm in A-H; 25 μm in E′-H′,I,J.

serves as a switch between the dIL^A and dIL^B interneuron subtypes (Fig. 3L).

Ptf1a is required for the formation of GABAergic neurons in the dorsal horn

In the absence of Pax2, expression of the GABAergic marker gene *Gad1* (encoding glutamic acid decarboxylase, GAD67) is lost in the dorsal horn of E13 mouse embryos (Cheng et al., 2004). As the *Ptf1a* null embryos completely lose expression of Pax2 in the dorsal neural tube, we predicted that the absence of Ptf1a would also result in loss of GABAergic neurons. To test this hypothesis, *Ptf1a* mutants were analyzed at E16.5 for expression of GABAergic markers. In-situ hybridization with a *Gad1* probe demonstrated that there was a complete loss of *Gad1* in the dorsal horn in the absence of Ptf1a, while the most ventral expression appeared largely unaffected (Fig. 4A,B). The protein encoded by *Gad1*, GAD67, was also absent in the dorsal horn of Ptf1a-deficient embryos (Fig. 4F), as was GABA, the neurotransmitter itself that defines a GABAergic interneuron (Fig. 4H).

Although the level of Ptf1a decreased by E16.5, we were able to use *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos to map the fate of *Ptf1a*-expressing cells into E16.5 dorsal horns to verify that the loss of GABAergic neurons is cell-autonomous. The vast majority of YFP in *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/–}$ embryos was restricted to the dorsal spinal cord at E16.5 (Fig. 4C). Co-localization of YFP with GAD67 and GABA indicated that the loss of GABAergic neurons in Ptf1a-deficient embryos is at least in part a cell-autonomous effect as expected (Fig. 4E,G).

These co-localization experiments are not as clear as the analysis with the transcription factor markers at earlier stages, because the neurotransmitter proteins tend to localize in the distal processes of the neurons, while the YFP is mainly cytoplasmic with some signal reaching distal processes. In Fig. 4E, arrows indicate regions outside the cell body where YFP and GAD67 co-localized. GABA is easier to detect in cell bodies, and thus the overlap with YFP is clearer (Fig. 4G, arrows). It is also important to note that the in-vivo recombination system used here to trace the lineage of Ptf1a cells is not 100% efficient and is not expected to indicate every Ptf1a descendent. Regardless, this analysis together with experiments described in Fig. 3 demonstrate that Ptf1a is in cells fated to become GABAergic neurons of the dorsal horn and that it is essential for this neuronal subtype to form.

YFP in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/–}$ embryos, which are deficient in Ptf1a, was also largely restricted to the dorsal horn, but it was detected in more cells and encompassed a broader medial-lateral area compared with heterozygous embryos (Fig.

4C,D). Besides suggesting that Ptf1a may normally be in a negative autoregulatory loop, the aberrant location of YFP-labeled cells on the lateral edges that appear to stream ventrally are consistent with mis-specification of neuronal subtype in the mutant.

Ptf1a suppresses glutamatergic neuronal differentiation in the dorsal horn

In the absence of Ptf1a there was a neuronal subtype switch from $dI4/dIL^A$ to $dI5/dIL^B$ (Figs 2, 3). dIL^B neurons form the glutamatergic neurons in the dorsal horn (Cheng et al., 2004). To determine if the aberrantly formed dIL^B neurons continue to mature with glutamatergic characteristics, we examined *Ptf1a* mutant embryos for vesicular glutamate transporter2 (VGLUT2) and glutamate receptor (GluR2/3). mRNA in-situ hybridization and immunofluorescence demonstrated an increase of VGLUT2 and GluR2/3 in the dorsal horn of *Ptf1a*$^{Cre/Cre}$ embryos at E16.5, when compared with *Ptf1a*$^{Cre/+}$ embryos (Fig. 5A-D, and data not shown). This increase was clearly indicated by an increase in VGLUT2 in the more superficial laminae, as indicated by the arrows in Fig. 5C,D. Likewise, an increase in the density of Tlx3-expressing cells was also seen at E16.5 (Fig. 5G,H), consistent with the importance of Tlx1/3 in the generation of dorsal horn glutamatergic neurons (Cheng et al., 2004). By contrast, no increase was detected in the glycinergic neuronal marker GlyT2 by mRNA in-situ hybridization (data not shown).

Just as at the earlier embryonic stages, we examined *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/–}$ and *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/–}$ embryos for co-localization of VGLUT2 with YFP to address the question of a neurotransmitter fate switch of the *Ptf1a* mutant cells. Significant co-expression of VGLUT2 with YFP was observed only in the *Ptf1a* null embryos (Fig. 5E,F). As with the GABAergic markers, the co-localization of VGLUT2 with YFP has caveats due to the enrichment of VGLUT2 in distal processes. However, taken together with the loss of Pax2 expression and increase in Tlx3, these results demonstrate that Ptf1a functions as a switch; it is required for the generation of GABAergic neurons and suppresses generation of glutamatergic neurons in the dorsal horn of the spinal cord (Fig. 5I).

Discussion

GABAergic neurons in the dorsal horn produce presynaptic inhibition of primary sensory afferents, and thus represent a major gatekeeper for the strength of sensory input to the spinal cord (Rudomin and Schmidt, 1999). The imbalance of inhibitory and excitatory neuronal activity can result in sensory disorders such as hyper- or hypo-algesia; thus, how these different types of neurons form is fundamental to understanding the development of neuronal circuits that modulate responses to sensory input. The functional importance of these local circuit inhibitory neurons is exemplified in disorders such as those seen in peripheral nerve injury, in which unregulated pain responses (allodynia) are associated with the loss of inhibitory activity of GABAergic neurons (Ibuki et al., 1997; Wiesenfeld-Hallin et al., 1997). We demonstrate the requirement of the bHLH transcription factor, Ptf1a, for the overwhelming majority of GABAergic neurons present in the dorsal spinal cord.

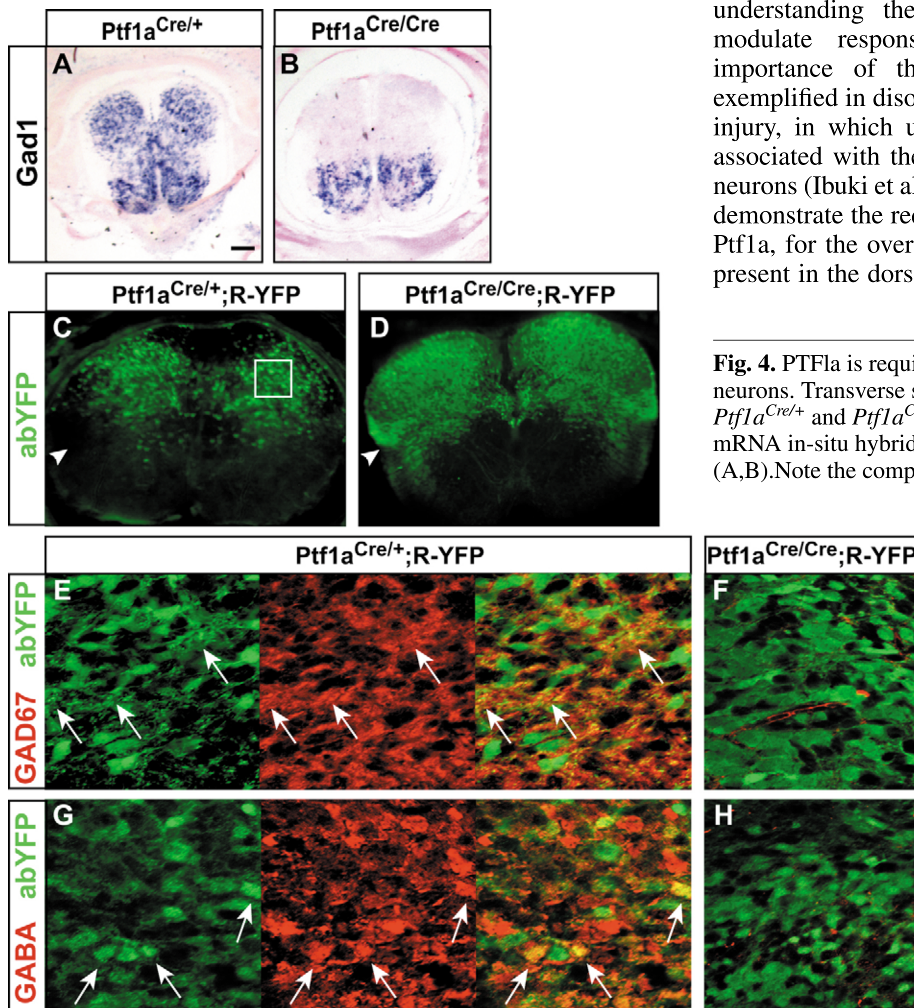

Fig. 4. PTFla is required for generation of dorsal horn GABAergic neurons. Transverse sections through spinal cord cervical regions of *Ptf1a*$^{Cre/+}$ and *Ptf1a*$^{Cre/Cre}$ mouse E16.5 embryos were processed for mRNA in-situ hybridization with the GABAergic marker gene Gad1 (A,B).Note the complete absence of Gad1 in the dorsal regions in the absence of Ptf1a. Loss of ventral Gad1 was not consistently observed. (C-H) Anti-GFP antibody was used to detect YFP in *Ptf1a*^{Cre}*;R26R-stop-YFP* E16.5 embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. (C) The lineage reporter YFP is detected largely in the dorsal regions in wild type. (D) By contrast, in the mutant, YFP is detected at higher levels in more cells and with a different organization (arrowheads). The boxed area in C is the approximate area shown in E-H. Immunofluorescence for GAD67 (E) and the neurotransmitter GABA (G) co-localize with YFP in embryos heterozygous for *Ptf1a* (arrows) but not in embryos lacking *Ptf1a* (F,H). Panels E-H are all from dorsal horn regions. Scale bar: 110 μm in A,B; 13 μm in E-H.

GABAergic and glutamatergic neurons appear to be alternative fate choices in the dorsal neural tube, and their development is genetically linked through the function of transcription factors such as Ptf1a, Tlx1/3, Lbx1 and Pax2 (Cheng et al., 2005; Cheng et al., 2004; Gross et al., 2002; Müller et al., 2002). Recently it was proposed that the HD transcription factors Tlx1 and Tlx3 are selector proteins biasing choice of glutamatergic over GABAergic cell fates (Cheng et al., 2004). This may at least be in part due to their inhibition of *Pax2* expression, as Pax2 is required for the formation of GABAergic neurons in the dorsal horn, and in the Tlx1/3 double mutant, Pax2 is dramatically increased (Cheng et al., 2004). Pax2 does not appear to have selector function, as there is no concomitant increase in glutamatergic neurons in *Pax2* null embryos (Cheng et al., 2004). Another HD factor, Lbx1, is also required for generating the correct numbers of GABAergic neurons (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002). Recently, the suppression of Lbx1 activity by Tlx3 has been suggested as a mechanism for selecting dorsal horn glutamatergic cell fate (Cheng et al., 2005). Results presented here demonstrate that Ptf1a is largely in post-mitotic cells in the dorsal neural tube. Therefore, Ptf1a has selector function opposite to Tlx1/3; it is required for the generation of dI4 and dIL^A fates, which mature into GABAergic neurons, and it suppresses the alternative fates, dI5 and dIL^B, which form glutamatergic neurons (Fig. 5I). The function of Ptf1a in switching cell fates in the dorsal spinal cord is similar to the role attributed to Ptf1a in pancreatic development. Inactivation of Ptf1a switches progenitor cells from pancreatic lineages to duodenal lineages (Kawaguchi et al., 2002).

Efforts to unravel the transcription factor code for the specification of spinal cord neurons have revealed a complex interplay of spatial and temporal control between bHLH and HD transcriptions factors (Caspary and Anderson, 2003; Lee and Pfaff, 2001). bHLH transcription factors present in the ventricular zone form the basis for the code for dI1 to dI5 in the dorsal neural tube. Math1 and Ngn1/2 specify the two most dorsal interneuron populations, dI1 and dI2 (Bermingham et al., 2001; Gowan et al., 2001). Mash1 specifies dI3 and dI5 neurons (Helms et al., 2005), while Ptf1a is required for dI4 (Fig. 2). The function of Mash1 and Ptf1a in dI3-dI5 is consistent with their expression patterns. Ptf1a is present in the central region of the Mash1 domain, a region that contains cells with the lowest Mash1 expression. Indeed, using an in-vivo recombination-based lineage tracing paradigm, Mash1 efficiently traces to dI3 and dI5, but not to dI4 neurons (Helms et al., 2005), while Ptf1a traced to dI4 (Fig. 2). Further refinement of the code is seen with co-expression of bHLH factors such as Mash1 and Olig3 (Müller et al., 2005), which specify the dI3 fate, while Mash1 alone, or with another factor such as the HD factor Lbx1, specify dI5 (Helms et al., 2005). The fate switch of the dI4 to dI5 rather than dI3 in the Ptf1a null may reflect the presence of Mash1 and Lbx1 in these cells, as the presence of Lbx1 suppresses the dI3 marker Isl1 (Gross

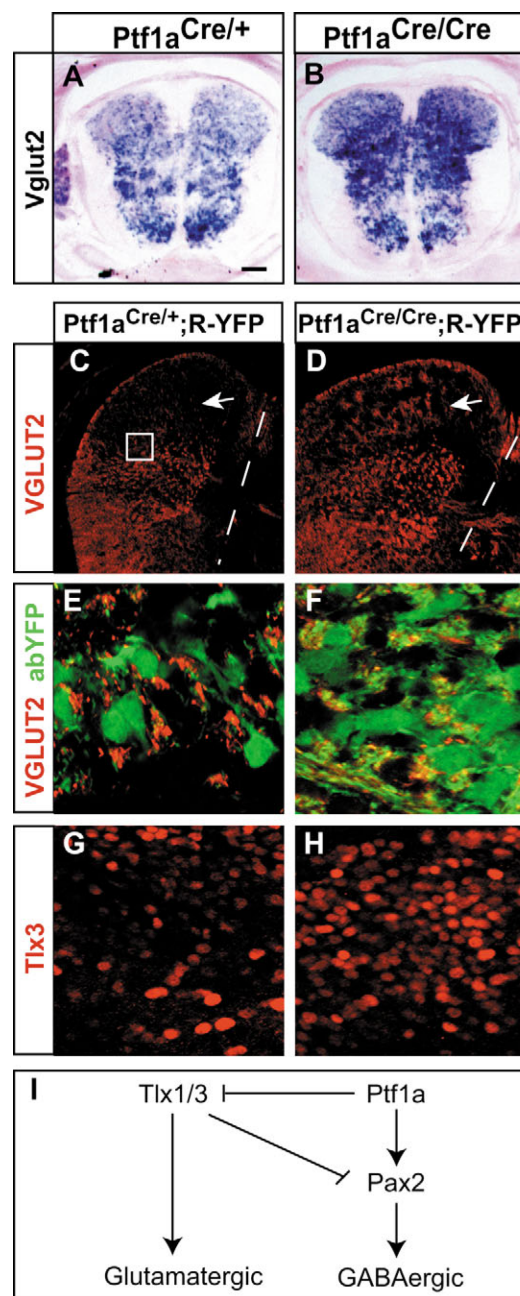

Fig. 5. PTFla is required for suppression of dorsal horn glutamatergic neurons. Transverse sections through spinal cord cervical regions of $Ptf1a^{Cre/+}$ and $Ptf1a^{Cre/Cre}$ mouse E16.5 embryos were processed for mRNA in-situ hybridization for the glutamatergic marker Vglut2 gene (A,B) or for immunofluorescence for the protein VGLUT2 (C,D). Note the increase in Vglut2 specifically in the dorsal regions in the absence of Ptf1a. The arrows in C,D indicate superficial laminae that have substantial increase in VGLUT2 in the mutant. The dashed line in C,D indicates the midline. (E,F) Anti-GFP antibody was used to detect YFP in $Ptf1a^{Cre}$*;R26R-stop-YFP* E16.5 embryos. VGLUT2 and YFP seldom co-localize in embryos containing Ptf1a (E); however, co-localization of VGLUT2 and YFP is detected in distal processes in the *Ptf1a* null (F). The density of Tlx3+ cells is increased in dorsal regions in the mutant (G,H). (E-H) are all from dorsal horn regions indicated by the box in C. (I) A model for the role of Ptf1a in GABAergic and glutamatergic neurons in the dorsal horn. Ptf1a acts in two ways: determination of GABAergic neurons by inducing Pax2, which is required for the expression of the GABAergic phenotype in these cells, and suppressing Tlx3, which is required for specifying glutamatergic neurons by inducing glutamatergic specific genes and suppressing Pax2 (Cheng et al., 2004). Scale bar: 110 μm in A,B; 50 μm in C,D; 13 μm in E-H.

et al., 2002; Müller et al., 2002). HD factors in addition to Lbx1, including Msx3 and Gsh1/2, also influence the transcription factor code, probably through regulation of bHLH expression (Kriks et al., 2005; Liu et al., 2004). Taken together, it is clear that neural-specific bHLH factors, combined with HD factors, play an important role in obtaining the neuronal diversity seen in the mature spinal cord.

Ptf1a is a Twist-like bHLH factor that can heterodimerize with E-proteins such as E47, and bind e-box containing DNA (Beres et al., 2005; Obata et al., 2001). However, it is unique in the bHLH family in that it also directly interacts with Rbpsuh, a transcriptional effector of the Notch signaling pathway, to bind a combined e-box;T/C box containing DNA sequence (Beres et al., 2005; Obata et al., 2001). Evidence from Beres et al. (Beres et al., 2005) suggests that the interaction site of Rbpsuh with Ptf1a overlaps that of Rbpsuh with Notch intracellular domain. Thus, the timer, Ptf1a;E-protein:Rbpsuh, reveals a Notch-independent Rbpsuh mechanism of action. These different protein-protein and protein-DNA interactions suggest a variety of mechanisms of action for Ptf1a, including many of those mechanisms previously described for selector gene function (Mann and Carroll, 2002). For example, similar to Twist function, heterodimerization with E-proteins could sequester this shared partner from other class A bHLH factors such as Mash1. Depending on the transcriptional activity of these different heterodimers, the consequence could be either an increase or decrease in specific target expression (Obata et al., 2001). Furthermore, by forming a trimer with Rbpsuh and E-protein (Beres et al., 2005), this complex not only has altered DNA target recognition, but may also directly impact Notch signaling by competing for use of the Rbpsuh subunit. Combining these different protein-protein interactions together with complex arrangement of cis-elements on transcriptional targets, makes Ptf1a a crucial component for the intricate interactions required for generating the appropriate number of specific neuronal cell-types.

Overexpression of Ptf1a, or a DNA-binding mutant of Ptf1a, in the chick neural tube resulted in excess Lhx1/5$^+$ cells (dI2-like) (data not shown). This phenotype is probably due to sequestering E-protein, as co-expressing E47 with Ptf1a suppressed this phenotype (data not shown). The possible competing roles for Ptf1a in different complexes, such as the heterodimer with E-proteins versus the heterotrimer with E-protein and RbpsuH, make overexpression paradigms difficult to interpret. Even so, mis-expression of Ptf1a in the dorsal telencephalon conferred a GABAergic phenotype to the newly forming neurons (Hoshino et al., 2005), supporting a role for Ptf1a in determining GABAergic neuronal cell fates.

Ptf1a is not required for all GABAergic neurons in the nervous system. Its expression is restricted to a subset of cells in the developing diencephalon, hindbrain and spinal cord. In *Ptf1a* mutant embryos, cells expressing GABAergic markers remain, as is seen in the ventral spinal cord (Fig. 4B). Two other bHLH transcription factors, Mash1 and Heslike (also known as Helt), have been shown to mediate GABAergic fate in the telencephalon (Fode et al., 2000; Miyoshi et al., 2004). Mash1 has overlapping expression with Ptf1a in the dorsal neural tube but it is Ptf1a and not Mash1 that is required for the GABAergic phenotype in the spinal cord. Thus, different bHLH factors are required for GABAergic neurons in different regions of the nervous system. It remains to be determined whether Ptf1a codes for shared characteristics in GABAergic neurons in the dorsal horn of the spinal cord and in the cerebellum, in addition to the neurotransmitter phenotype, that are distinct from those in GABAergic neurons derived from Mash1-expressing cells in the telencephalon.

Dramatic phenotypes have been detected in the development of the pancreas (Kawaguchi et al., 2002), the cerebellum (Sellick et al., 2004) and the dorsal spinal cord (this manuscript). In pancreas, there is almost a complete loss of islet and exocrine tissue. The requirement for Ptf1a for generation of multiple GABAergic interneurons in the cerebellum and deep cerebellar nuclei, including Purkinje, stellate, basket and Golgi cells, is responsible for the cerebellar agenesis (Hoshino et al., 2005). Phenotypes in these two tissues also occur in humans with a truncation in the *PTF1A* gene (Sellick et al., 2004). As shown here, the *Ptf1a* null mouse develops a dorsal spinal cord with nearly a complete loss of inhibitory GABAergic neurons with an increase in glutamatergic neurons. This excess in excitatory neurons unopposed by inhibitory neurons may explain the irregular respiratory patterns and increased cerebral excitability in human patients with mutations in *Ptf1a* (Hoveyda et al., 1999). Given the absolute requirement for Ptf1a for the formation of the cell types studied so far, it is likely that additional neurons in the brainstem and the ventral hypothalamus, two other domains of *Ptf1a* expression, are mis-specified in the mutant as well.

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