

Lhx1 and *Lhx5* maintain the inhibitory-neurotransmitter status of interneurons in the dorsal spinal cord

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Lhx1 and *Lhx5* are co-expressed in multiple interneuron cell types in the developing spinal cord. These include early-born dI4 and dI6 inhibitory interneurons, as well as late-born inhibitory dIL_A neurons (dIL_A), all of which express the paired-domain transcription factor Pax2. Although it appears that *Lhx1* and *Lhx5* do not control the initial specification of the neuronal cell types in which they are expressed, we have found a cell-autonomous requirement for either *Lhx1* or *Lhx5* to maintain the expression of *Pax2*, *Pax5* and *Pax8* in dorsal inhibitory neurons at later developmental stages. *Lhx1*; *Lhx5* double-knockout mice exhibit a downregulation of *Gad1* and *Viaat* (*Slc32a1*) from E13.5 onwards that is closely associated with a decrease in *Pax2* expression. *Pax2* is a key factor for dorsal GABAergic identity, with the expression of *Pax5* and *Pax8* being differentially dependent on *Pax2* in the dorsal horn. In summary, our findings support a model in which the differentiation of GABAergic interneurons in the dorsal cord depends on *Pax2*, with *Lhx1* and *Lhx5* helping to activate and maintain *Pax2* expression in these cells. *Lhx1* and *Lhx5* therefore function together with *Pax2*, *Pax5* and *Pax8* to establish a GABAergic inhibitory-neurotransmitter program in dorsal horn interneurons.

KEY WORDS: *Lhx1*, *Lhx5*, *Pax2*, Inhibitory neurons, Spinal cord, Mouse

INTRODUCTION

Neural circuits throughout the nervous system use a combination of fast-excitatory and fast-inhibitory neurotransmitters to regulate neural activity. In the vertebrate nervous system, fast inhibitory transmission is primarily mediated by two transmitters – GABA and glycine. The neurons that release these neurotransmitters express a number of genes that encode components of the inhibitory-neurotransmitter machinery. These include the vesicular inhibitory amino acid transporter (VIAAT, also known as *Slc32a1* – Mouse Genome Informatics), which loads GABA and glycine into secretory vesicles (McIntire et al., 1997), and the glycine transporter, GlyT2, which is responsible for glycine reuptake and transport across the plasma membrane of glycinergic neurons (Liu et al., 1993). In addition, GABAergic neurons express two genes, *Gad1* and *Gad2*, that encode glutamic acid decarboxylase – the enzyme that converts glutamate to GABA (Erlander and Tobin, 1991). During embryogenesis, the expression of these genes is activated in subsets of differentiating neurons, thus imbuing them with the necessary cellular machinery for fast inhibitory neurotransmission.

The developmental programs that determine the neurotransmitter status of a neuron remain largely unknown. Whereas the pattern and levels of a particular neurotransmitter can be regulated under certain circumstances by neural activity and target-derived signals during development (Schotzinger and Landis, 1988; Borodinsky et al., 2004), a cell's acquisition of a particular neurotransmitter 'phenotype' appears to be closely linked to the gene regulatory events that determine neuronal subtype identity. In the embryonic spinal cord, developing neurons fall into three fast neurotransmitter

classes: cholinergic neurons, excitatory glutamatergic neurons and inhibitory neurons that use GABA and or glycine as their primary transmitters. Motor neurons are primarily cholinergic (Phelps et al., 1991), as are a small population of interneurons of unknown function that are located near the central canal (Barber et al., 1991). Glutamatergic excitatory interneurons include the early-born dI1–3, dI5, V2 and V3 neurons, as well as a population of late-born dorsal interneurons, the so-called dIL_B neurons. The dI4, dI6, V0 and V1 classes of interneuron that are generated during the first wave of neurogenesis are inhibitory (Saueressig et al., 1999; Wenner et al., 2000; Lanuza et al., 2004; Glasgow et al., 2005), as are the late-born dIL_A neurons that settle in the dorsal horn (Cheng et al., 2004; Cheng et al., 2005; Mizuguchi et al., 2006).

Studies in the dorsal horn have begun to delineate the transcriptional mechanisms that control the neurotransmitter phenotype of spinal-interneuron cell types. Inhibitory neurons in the dorsal spinal cord are derived exclusively from cells that express the homeodomain transcription factor *Lbx1* (Gross et al., 2002; Muller et al., 2002; Matise, 2002; Cheng et al., 2004). These cells are comprised of two early-born populations – dI4 and dI6 neurons – and late-born dIL_A neurons that are generated during the second wave of neurogenesis, which begins at E12 in the mouse. All three classes of neuron express the paired-domain transcription factor *Pax2* together with the LIM-homeodomain transcription factors *Lhx1* and *Lhx5* (Gross et al., 2002; Muller et al., 2002). A subset of *Lbx1*-expressing neurons, dI5 and dIL_B neurons, also differentiate as glutamatergic neurons. These cells express the homeodomain transcription factors *Tlx1*, *Tlx3* and *Lmx1b*. *Tlx1* and *Tlx3* function in a cell-autonomous manner to specify glutamatergic dI5- and dIL_B-sensory neurons (Cheng et al., 2004), in part by over-riding an inhibitory differentiation program that is *Lbx1*-dependent (Cheng et al., 2005). Inactivation of *Tlx1* and *Tlx3* results in the loss of glutamatergic cell types in the dorsal horn, along with the concomitant upregulation of *Pax2* and GABAergic markers, such as *Viaat*. Conversely, *Tlx3* overexpression induces a switch from GABAergic to glutamatergic cell fate (Cheng et al., 2004; Cheng et al., 2005; Mizuguchi et al., 2006). Interestingly, the loss of *Lmx1b* does not alter the neurotransmitter status of dI5 and dIL_B neurons (Ding et al., 2004).

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Pax2-expressing neurons in the hindbrain and spinal cord predominantly differentiate as inhibitory interneurons (Maricich and Herrup, 1999; Gross et al., 2002; Cheng et al., 2004; Glasgow et al., 2005; Mizuguchi et al., 2006; Wildner et al., 2006). In the *Pax2*-mutant cord, there is a marked loss of GABAergic markers in the dorsal horn, demonstrating that *Pax2* functions as an obligatory regulator of the inhibitory-neurotransmitter program in these cells (Cheng et al., 2004). These dorsal inhibitory interneurons, as well as the ventrally-derived V0 and V1 inhibitory interneurons, also express *Lhx1* and *Lhx5* (Burrill et al., 1997; Moran-Rivard et al., 2001; Gross et al., 2002; Muller et al., 2002) (this study). This has led to the suggestion that the co-expression of *Pax2* and *Lhx1* and/or *Lhx5* may provide a transcription factor code for inhibitory neurons in the hindbrain and spinal cord. Although roles for *Lhx1* and *Lhx5* have been demonstrated in head, kidney and motor neuron development (Kobayashi et al., 2005; Kobayashi et al., 2004; Zhao et al., 1999; Kania et al., 2000), their overlapping expression in spinal interneurons (Sheng et al., 1997), coupled with the early embryonic lethal phenotype of the *Lhx1* mutant (Shawlot and Behringer, 1995), has impeded analyzing their role(s) in spinal-interneuron development.

In this study, we set out to address three questions: (1) Do *Lhx1* and *Lhx5* play a role in the early establishment of spinal-interneuron subtypes in the spinal cord? (2) Do *Lhx1* and *Lhx5* function in combination with *Pax2* to establish inhibitory-neurotransmitter phenotypes in the developing spinal cord? (3) Do *Lhx1* and *Lhx5* have roles in maintaining the neurotransmitter status of inhibitory interneurons and, if so, how is this function executed? Our results show that, although *Lhx1* and/or *Lhx5* are not required for the specification of early-born interneurons that form at E10.5-E11, both genes are necessary for the proper development of late-born inhibitory dIL_A interneurons. Moreover, we find that a reciprocal regulatory relationship exists between *Lhx1* and/or *Lhx5* and *Pax2* genes in these cells. *Lhx1*;*Lhx5* double mutants exhibit a selective loss of *Pax2* protein expression in the dorsal horn that precedes the reduction in *Gad1* and *Viaat* expression. As a result of this, late-born *Pax2*⁺ GABAergic neurons that settle in the lateral dorsal horn fail to retain their GABAergic identity. *Pax2* is also required to maintain *Lhx1*, *Lhx5*, *Pax5* and *Pax8* expression in these cells, demonstrating a genetic interdependence between these two transcription factor classes in late-born dorsal inhibitory neurons.

MATERIALS AND METHODS

Generation of *Lhx1*;*Lhx5* double-knockout mice

Lhx5 mice, in which exons 2-4 of the targeted *Lhx5* gene were replaced with a neomycin-resistance gene, were obtained from H. Westphal (Zhao et al., 1999) (see Fig. 3A). *Lhx1* conditional-mutant mice (*Lhx1*^{loxP}) were kindly provided by R. Behringer (M. D. Anderson Cancer Center, Houston, USA). The *Lhx1* coding region is flanked by two loxP sites in the *Lhx1*^{loxP} conditional allele (Kwan and Behringer, 2002) (see Fig. 3A). Selective inactivation of *Lhx1* in neurons was achieved by crossing a *Nestin-Cre* (*Nes*^{Cre}) transgenic line in which Cre-recombinase is under the control of a nervous-system-specific enhancer present in the second intron of the rat nestin gene (Lendahl et al., 1990) into a *Lhx1*^{loxP/loxP} background. In this study, conditional *Lhx1*-mutant mice with the genotype *Lhx1*^{loxP/loxP};*Nes*^{Cre} are referred to as *Lhx1*^{-/-} mutants. Double mutants with the genotype *Lhx1*^{loxP/loxP};*Lhx5*^{-/-};*Nes*^{Cre} are referred to as *DKO* mice. *Lhx5*^{-/-} and *Lhx1*^{-/-}-mutant mice both die at birth. *Lhx1*^{loxP/loxP} homologous strains are, however, healthy and fertile, and so *DKO* mice were generated by crossing parental lines comprised of genetic combinations of *Lhx5*^{+/-};*Nes*^{Cre};*Lhx1*^{loxP/+} with *Lhx1*^{loxP/loxP};*Lhx5*^{+/-} mice. *Pax2*^{-/-} and *Pax8*^{-/-} embryos were provided by A. Mansouri (Mansouri et al., 1998). *Pax5*^{+/-} embryos were derived from the breedings of heterozygous *Pax5*^{+/-} mice (Urbanek et al., 1994). The primers for genotyping all mutant animals are identical to those described in the aforementioned references.

Immunohistochemistry and in situ hybridization

Mouse embryos were fixed for 1 hour in 4% paraformaldehyde in phosphate-saline buffer (PBS), cryoprotected in 25% sucrose, embedded in OCT (Tissue-Tec) and sectioned at 20 μm. Immunohistochemistry was performed on frozen sections as previously described (Burrill et al., 1997). The following antibodies were used in the study: monoclonal anti-*Lhx1* and anti-*Lhx5* (4F2-10, Developmental Hybridoma Studies Bank), monoclonal anti-NeuN (Chemicon International), rat anti-BrdU monoclonal antibody (Harlan), anti-*Lbx1* (Gross et al., 2002), polyclonal anti-*Pax2* (Zymed) and guinea-pig anti-*Lmx1b* (gift of T. Jessell, HHMI, Columbia University, NY, USA). Species-specific antibodies conjugated to Cy2, Cy3 or Cy5 were used (Jackson ImmunoResearch). In situ hybridizations were performed as previously described (Goulding et al., 1993). *Pax2* immuno-in situ double localization was performed according to Cheng et al. (Cheng et al., 2004). In situ hybridizations were performed using probes specific for mouse *Lhx1* (Bertuzzi et al., 1996), *Lhx5* (Zhao et al., 1999), *Viaat* and *VGluT2*, and rat *Gad1*, as described previously by Mizuguchi et al. (Mizuguchi et al., 2006).

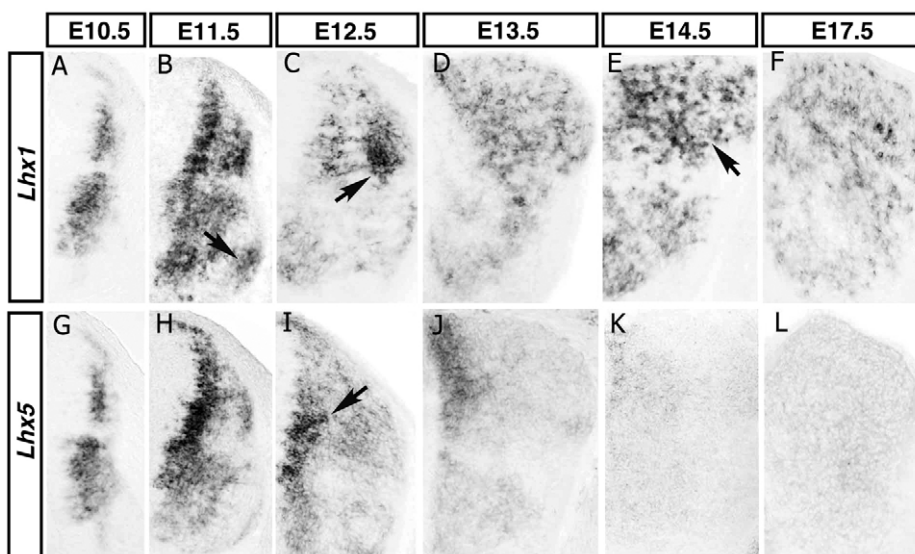


Fig. 1. *Lhx1* and *Lhx5* expression in the embryonic spinal cord. (A-F) In situ expression of *Lhx1*. (G-L) In situ expression of *Lhx5*. At E10.5 and E11.5, *Lhx1* and *Lhx5* are co-expressed in postmitotic neurons that correspond to dl2, dl4, dl6 and V0-V1 neurons (A,B,G,H). *Lhx1* is also present in a subset of the motor neurons ventrally (arrow in B). Expression of *Lhx1* and *Lhx5* begins to diverge at E12.5 (C,I). *Lhx1* is strongly expressed in the laterally located neurons (arrow in C), whereas *Lhx5* is more strongly expressed in the subventricular zone where newborn dIL_A cells are emerging (arrow in I). By E13.5, the expression of *Lhx1* and *Lhx5* in the dorsal horn is largely complementary (D,J). *Lhx1* persists in the dorsal horn (E,F; see arrow in E), whereas *Lhx5* is downregulated at E14.5 (K) and is completely absent at E17.5 (L).

Histology

Sections of 5 μm were cut from paraffin-embedded E17.5 spinal cords and stained with hematoxylin and Eosin, as described by Gross et al. (Gross et al., 2002). Cell counts were performed on three sections from three cords (i.e. nine sections each for wild-type and *DKO* embryos). For each section, cells in a single dorsal quadrant were counted twice in order to minimize counting errors. Statistical differences in cell counts between wild-type and mutant cords were determined using the Student's *t*-test.

Apoptotic cells in the developing spinal cord (E14.5-E17.5) were visualized by TUNEL labeling using the ApopTag-plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon International). Stainings were performed according to the manufacturer's instructions. Counts for apoptotic cells were tabulated for both the dorsal and ventral halves of the cord. Apoptotic cell counts for each sample represents the average of six sections (three sections from two cords).

BrdU labeling

Pregnant dams were injected intraperitoneally with 50 mg bromodeoxyuridine (50 $\mu\text{g}/\text{ml}$ dissolved in 0.9% saline) per gram of mouse bodyweight at E12.5. E14.5 embryos were collected and processed for immunohistochemistry sections, and stained with an antibody to Pax2 followed by anti-BrdU mouse monoclonal antibody.

Imaging

Antibody, TUNEL and BrdU staining was visualized on a Zeiss LSM 510 confocal microscope. In situ hybridization images were captured on a Zeiss Axiophot 2 microscope fitted with an Axiocam MRm camera. All figures were color-corrected and assembled using Photoshop and Canvas software.

RESULTS

Expression of the *Lhx1* and *Lhx5* genes in the developing spinal cord

As a first step towards analyzing the function of *Lhx1* and *Lhx5* in the embryonic spinal cord, we undertook a detailed analysis of the normal expression profiles of *Lhx1* and *Lhx5* during development. Although previous studies documented the early expression patterns of both genes in the nervous system (Sheng et al., 1997), these studies did not address the dynamic changes in the expression of *Lhx1* and *Lhx5* that occur at later developmental times. Previous studies by Gross et al. (Gross et al., 2002) using antibodies that recognize both Lhx1 and Lhx5 had indicated that both proteins are co-expressed in late-born dIL_A neurons that populate the dorsal horn. However, although *Lhx1* had been shown to persist dorsally at later stages by in situ hybridization (Muller et al., 2002), it was unclear whether *Lhx5* was also expressed at later developmental times.

To clarify this issue, we used in situ hybridization to compare the developmental expression profiles of *Lhx1* and *Lhx5* in the embryonic spinal cord. During the early phase of neurogenesis (E10.5-E11.5), *Lhx1* and *Lhx5* were found to be co-expressed in multiple spinal-interneuron populations, including three dorsal cell types – the dI2, dI4 and dI6 interneurons (Fig. 1A,B,G,H; also see Fig. S1 and Fig. S2 in the supplementary material). However, from E12.5 onwards, the expression patterns of these two genes began to diverge, leading to complementary patterns of expression in dorsal interneurons at later developmental times (Fig. 1C,I). In the E13.5 dorsal horn, the highest level of *Lhx5* transcripts was found medially, decreasing towards the lateral rim of the dorsal cord. *Lhx1* was expressed in an inverse gradient, with cells closest to the ventricular zone expressing low levels of *Lhx1* transcripts, while cells further away from the ventricular zone expressed higher levels (Fig. 1D,J). Whereas *Lhx1* continued to be expressed in a mosaic pattern in the dorsal horn neurons up to birth (Fig. 1E,F, and data not shown), little or no *Lhx5* expression was detected in the spinal cord from E14.5 to E17.5 (Fig. 1K,L).

The divergent expression patterns of *Lhx1* and *Lhx5* in the late-born dIL_A cells can be accounted for in two ways. First, the complementary expression patterns of *Lhx1* and *Lhx5* in late-born dIL_A cells may reflect high-level expression of *Lhx1* in the dIL_A cells that are born first. These cells would be expected to accumulate in the more lateral regions of the dorsal horn, whereas later-born dIL_A cells that are located more medially might exhibit high *Lhx5*-low *Lhx1* expression. Alternatively, differentiating dIL_A cells may downregulate *Lhx5* and upregulate *Lhx1* as they migrate from the subventricular zone into the dorsal horn. Support for the later possibility comes from the observation that *Lhx5* begins to be downregulated when dIL_A cells cease being generated at E13.5 (Gross et al., 2002). For this reason, we favor a model in which newborn dIL_A cells express *Lhx5* at high levels, while the more mature dIL_A neurons downregulate *Lhx5* and upregulate *Lhx1*.

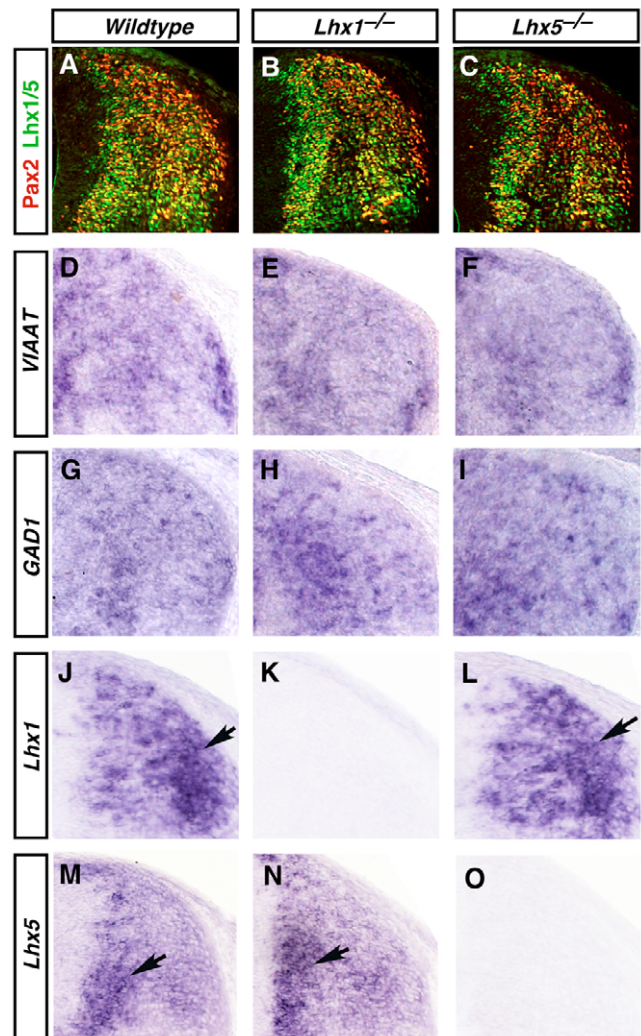


Fig. 2. Dorsal interneuron development in *Lhx1* and *Lhx5* single mutants. (A-C) Inactivation of either *Lhx1* or *Lhx5* does not alter the specification of dIL_A interneurons. dIL_A interneurons express Pax2 and Lhx5 in *Lhx1*^{-/-} embryos (B), or express Pax2 and Lhx1 in *Lhx5*^{-/-} embryos (C). (D-I) In both *Lhx1* and *Lhx5* single mutants, dIL_A cells retain their GABAergic identity, and express *Viaat* (D-F) and *Gad1* (G-I). (J-O) *Lhx1* expression is unchanged in the cord of *Lhx5*^{-/-} mutants. There is no upregulation of *Lhx1* mRNA at early (not shown) or later (arrows in J,L) stages. There is also no change in *Lhx5* expression in the cord of *Lhx1*^{-/-} mutants at E12.5 (arrows in M,N).

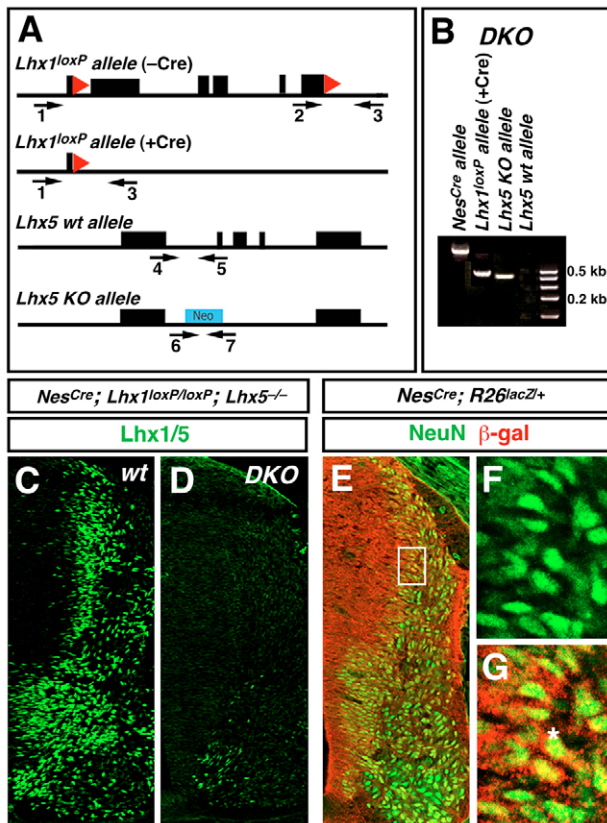


Fig. 3. Generation and testing of conditional *Lhx1*; *Lhx5* double-knockout animals. (A) Simplified schematic adapted from Kwan and Behringer (Kwan and Behringer, 2002) and Zhao et al. (Zhao et al., 1999) showing the targeted alleles of *Lhx1* and *Lhx5* that were used in this study. (B) PCR amplification of a DKO embryo carrying the *Nes^{Cre};Lhx1^{loxP}* and *Lhx5^{-/-}* alleles. The *Cre* transgene is detected as a 1 kb band. A 480-base-pair band is diagnostic for the *Cre*-deleted *Lhx1^{loxP}* allele. *Neo* is present in both *Lhx5* heterozygotes and *Lhx5* mutants. The absence of a wild-type *Lhx5* band of approximately 430 base pairs distinguishes *Lhx5* mutants from *Lhx5* heterozygous animals. This wild-type *Lhx5*-specific band is absent in DKO embryos. (C,D) *Lhx1*-protein expression is nearly completely abolished in the DKO cord at E12.5, with the exception of a few ventral *Lhx1⁺* escapees. (E) Expression of *lacZ* and *NeuN* in E11.5 spinal cords of *Nestin^{Cre};Rosa26^{lacZ/+}* animals. (F,G) High magnification of newly postmitotic *NeuN⁺* interneurons (green) in E (box) showing colocalization with β -gal (red; asterisk in G).

Spinal cord development is normal in *Lhx1* and *Lhx5* single mutants

Our observation that *Lhx1* and *Lhx5* are co-expressed at E10.5 and E11.5 suggested to us that *Lhx1* and *Lhx5* might function redundantly at these early developmental stages but adopt unique roles at later times when their expression patterns diverge. Consistent with this hypothesis, we did not observe any marked differences in the specification of early-born cells, including inhibitory cell types, in either of the single mutants (see Fig. S3 in the supplementary material). There were also no marked changes in the specification or differentiation of late-born dIL_A neurons in either of the single mutants. *Pax2*, which is selectively expressed in inhibitory dIL_A neurons showed a normal pattern of expression in cords taken from E12.5 *Lhx1^{loxP/loxP}* and *Lhx5^{-/-}* single mutants (Fig. 2A-C). There were also no marked changes in the expression

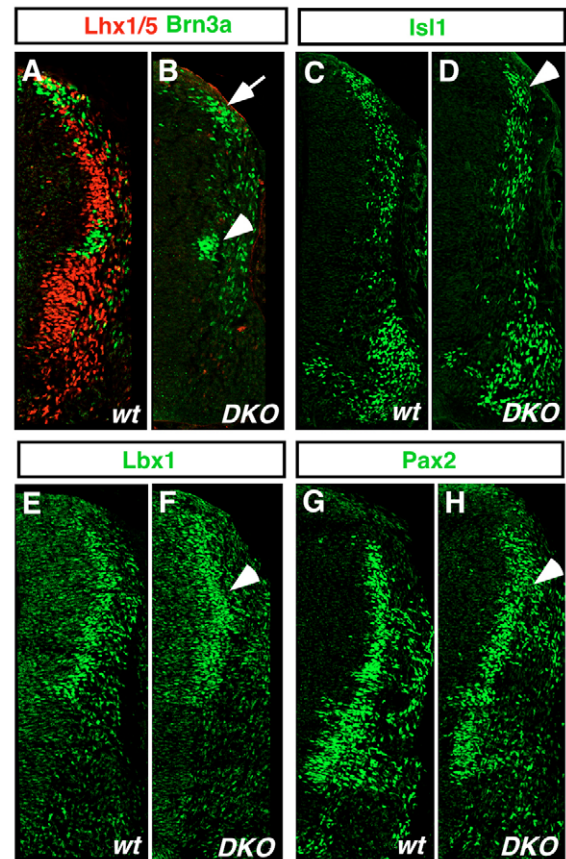


Fig. 4. Early inhibitory-interneuron cell types are correctly specified in the *Lhx1*; *Lhx5* double-knockout cord. (A-H) Cross sections through E11.5 wild-type (*wt*) and *Lhx1*; *Lhx5* mutant (*DKO*) cord at forelimb level. (A,B) In the *DKO* cord, expression of *Brn3a* is unchanged in d11-d13 (arrow in B) and in d15 (arrowhead) neurons. (C,D) *Isl1* expression in d13 neurons (arrowhead) is also unchanged. (E,F) *Lbx1* expression in d14-d16 neurons is normal in the *DKO* cord (F, arrowhead). (G,H) *Pax2*, which marks d14 inhibitory neurons (arrowhead) as well as the more ventrally located d16, V0 and V1 inhibitory neurons, is expressed in a normal pattern in the *DKO* cord (H). A small decrease in the level of *Pax2* expression was observed.

patterns of either *Lbx1* or *Lmx1b* in these late-born cells (data not shown). *Viaat* and *Gad1* continued to be expressed in late-born dIL_A neurons in each of the single mutants (Fig. 2E,F,H,I), demonstrating that these cells acquire their normal inhibitory-neurotransmitter phenotype.

In view of the normal development of late-born dIL_A neurons in the single mutants, we questioned whether *Lhx1* and *Lhx5* show compensatory changes to their expression when the other gene is lost. At E12.5, *Lhx5* is expressed prominently in a band of cells in the subventricular zone, whereas *Lhx1* shows a domain of high expression more laterally in the developing dorsal mantle zone. No change in *Lhx1* expression was observed in E12.5 *Lhx5^{-/-}* embryos (compare Fig. 2J with 2L), indicating that *Lhx1* is not upregulated in the absence of *Lhx5*. Likewise, in *Lhx1* single mutants there was no upregulation of *Lhx5* in the postmitotic dIL_A neurons in the dorsal

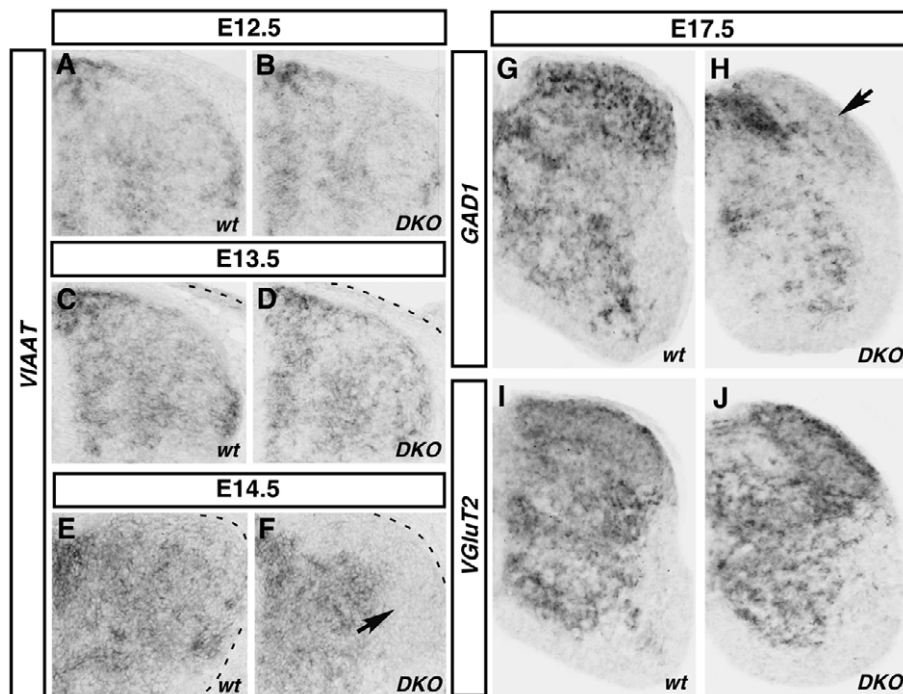


Fig. 5. *Lhx1*;*Lhx5* double-knockout mutants fail to maintain inhibitory-neurotransmitter gene expression in the dorsal horn. (A–D) Dorsal quadrants of the spinal cord at E12.5 and E13.5 showing the expression of *Viaat*. In the *DKO* cord, a normal pattern of *Viaat* expression in dL_A neurons is observed up to E13.5. (E,F) Beginning at E14.5, *Viaat* is downregulated in the most-dorsolateral cells in the *DKO* cord (arrow in F). (G,H) E17.5 *DKO* animals show a marked loss of *Gad1* expression in the dorsal regions (arrow in H). (I,J) *VGLUT2* expression is not altered in the *DKO* dorsal horn at E17.5.

mantle zone (Fig. 2M,N). In summary, *Lhx1* and *Lhx5* are regulated independently, and each alone is sufficient to specify late-born dL_A neurons.

Inactivation of *Lhx1* and *Lhx5* in the embryonic spinal cord

The observation that neuronal specification is largely normal in the spinal cord of *Lhx1* and *Lhx5* single mutants prompted us to examine the effects of deleting both genes on the specification of inhibitory neurons in the spinal cord. To do this, a conditional knockout allele of the *Lhx1* gene (Kwan and Behringer, 2002) was used in combination with a *Lhx5*-null allele (Zhao et al., 1999) to generate *Lhx1*;*Lhx5* double mutants. When *Lhx1*;*Lhx5* double knockout (*DKO*) mice were generated using a *Nestin*^{Cre} (*Nes*^{Cre}) transgene to selectively inactivate *Lhx1* in neural progenitors, we observed a dramatic abolition of *Lhx1* and *Lhx5* expression throughout the spinal cord (Fig. 3). Nonetheless, some cells that migrate towards the ventral midline continued to express the *Lhx1* protein (approximately 15–20 cells per E11.5 hemicord, Fig. 3D). These cells are likely to be a subset of V0 or dI6 interneurons.

To further assess the extent of *Nes*^{Cre}-mediated recombination in the embryonic spinal cord, a *ROSA26*-derived reporter line that conditionally expresses the *lacZ* gene (*R26*^{lacZ}) was used to identify cells that had undergone Cre-mediated recombination (Soriano, 1999). When mice carrying the *R26-lacZ* reporter gene were crossed with *Nes*^{Cre} mice, embryos carrying both alleles exhibited intense β -gal staining throughout the nervous system. We observed diffuse cytoplasmic β -gal immunofluorescence that overlapped extensively with the NeuN⁺ staining at all dorsoventral levels of the spinal cord, indicating widespread Cre-mediated recombination (Fig. 3E–G). Greater than 95% of the cells in the ventricular zone of E11.5 spinal cords were β -gal⁺ (Fig. 3E) and these NeuN⁺/ β -gal⁺ neurons were often in the process of migrating from the ventricular zone (Fig. 3F,G), demonstrating that the *Nes*^{Cre} transgene effectively inactivates the *Lhx1* gene in most spinal cord progenitors.

Lhx1 and *Lhx5* regulate late aspects of the inhibitory-neuron program in the dorsal horn

The cords of *Lhx1*;*Lhx5* *DKO* mice were examined at a number of ages up until birth. Cell-type specific markers were used to analyze the specification of early-born inhibitory interneurons at E11.5 (Fig. 4). At this stage, *Brn3a* and *Isl1*, which mark early-born excitatory dI1–dI3 and dI5 neurons (Gross et al., 2002), showed normal patterns of expression in the *DKO* cord (Fig. 4A–D). Furthermore there was no change in the expression of *Lbx1*, which marks all Class B neurons, including inhibitory dI4 and dI6 neurons (Fig. 4E,F). *Pax2*, which is expressed in dI4, dI6, V0 and V1 neurons also exhibited a normal pattern of distribution (Fig. 4G,H), indicating that all *Pax2*-expressing inhibitory-cell types are correctly specified in the absence of *Lhx1* and *Lhx5*.

At early developmental times (up to E13.5), the loss of *Lhx1* and *Lhx5* had no obvious effects on the expression of *Viaat* (Fig. 5A–D); however, a reduction in *Viaat* and *Gad1* mRNA levels in the most dorsal and lateral regions of the developing dorsal horn was noticed at E14.5 (Fig. 5E,F and see Fig. S4 in the supplementary material). This reduction in inhibitory-neurotransmitter gene expression was more pronounced at E17.5, with interneurons in the lateral dorsal horn exhibiting the greatest reduction in *Gad1* expression levels (Fig. 5G,H). The loss of *Gad1*-expressing neurons at E17.5 was confirmed by cell counts, which showed significantly fewer *Gad1*⁺ cells in the *DKO* dorsal horn compared with wild-type dorsal horns (*DKO* 113±12 s.d. cells versus wild-type 279±33 s.d. cells; *P*<0.0001).

Interestingly, the expression of *VGLUT2*, a marker of glutamatergic neurons, was largely unchanged in the *DKO* dorsal horn (Fig. 5I,J and see Fig. S4 in the supplementary material), thereby arguing that dorsal GABAergic neurons do not activate a glutamatergic-transmitter program in response to the loss of *Lhx1* and *Lhx5*. *Lmx1b* expression, which marks glutamatergic dL_B neurons, was not upregulated in the cord of *DKO* mutants, demonstrating that

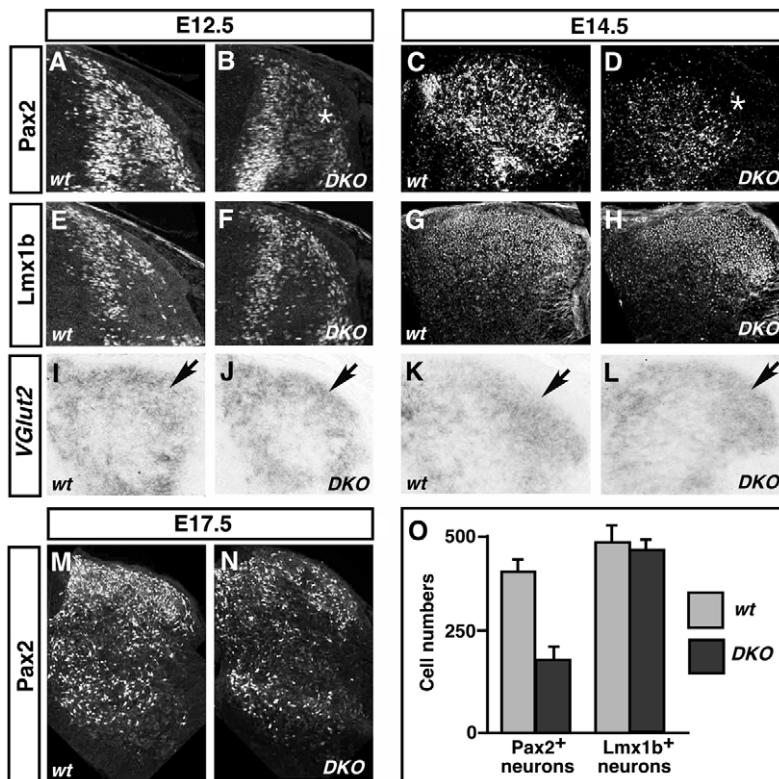


Fig. 6. *Lhx1* and *Lhx5* regulate late aspects of development in *Pax2* inhibitory interneurons.

(A-D) Dorsal horn quadrants showing the downregulation of *Pax2* in dIL_A neurons. This downregulation begins at E12.5 in *DKO* animals (asterisk, B) and is more pronounced at E14.5 (asterisk, D). (E-H) *Lmx1b*⁺ dIL_B neurons are specified normally and settle in the superficial dorsal horn in *DKO* animals at similar stages to wild type. (I-L) *VGlut2* is not upregulated in the superficial dorsal horn (arrows) indicating that GABAergic *Pax2*⁺ cells do not adopt a dIL_B glutamatergic phenotype in the *DKO* cord. (M,N) E17.5 *DKO* animals exhibit a loss of *Pax2* expression both dorsally and ventrally at E17.5. (O) Cell counts of *Pax2*⁺ and *Lmx1b*⁺ cells at E14.5 show a >60% reduction in the number of dorsal *Pax2*⁺ cells without any increase in the number of *Lmx1b*⁺ dIL_B cells.

dIL_A neurons do not acquire a dIL_B fate (see Fig. 6). The *DKO* phenotype thus resembles that seen in the *Pax2*-mutant spinal cord, where the selective loss of inhibitory markers in the dorsal horn is not accompanied by an upregulation of excitatory markers such as *VGlut2* (Cheng et al., 2004). Although the loss of *Gad1* was most pronounced in the dorsal horn, some loss of *Gad1* was noted in the ventral horn, suggesting that *Lhx1* and *Lhx5* also have a similar role in maintaining inhibitory-gene expression in ventral neurons.

***Lhx1* and *Lhx5* maintain *Pax2* expression in dorsal inhibitory interneurons**

In view of the parallels between the spinal cord phenotypes of the *Lhx1* and *Lhx5* *DKO* and *Pax2* mutants, we investigated whether the altered expression of *Viaat* and *Gad1* in the cord of *Lhx1* and *Lhx5* *DKO* mutants might be caused by a reduction in *Pax2* expression. At E12.5, a moderate reduction in the expression level *Pax2* in dIL neurons was seen in the *DKO* cord (Fig. 6A,B); by E14.5, this reduction was even more pronounced (Fig. 6C,D). This loss of *Pax2* expression was most prominent at the lateral margins of the dorsal horn, where *Gad1* and *Viaat* expression are reduced the most (Fig. 6C,D asterisk, and see Fig. S4 in the supplementary material). Interestingly, no significant accumulation of *Pax2*⁺ cells was noticed medially, which would have indicated a defect in dIL_A cell migration. Instead, it appears that the dIL_A neurons fail to maintain *Pax2* expression as they migrate and settle in the lateral dorsal horn. As noted previously, the dIL_A cells in the *DKO* dorsal horn do not switch to a glutamatergic *Lmx1b*⁺ dIL_B fate; there was no increase in *Lmx1b*⁺ cell numbers (Fig. 6E-H) or *VGlut2* expression (Fig. 6I-L) in the dorsal horn.

To further investigate the nature of the loss of *Pax2*-expressing cells in the *DKO* dorsal horn, we investigated whether a normal complement of dIL neurons are generated. Spinal cords were pulsed with BrdU at E12.5, when late-born dIL_A neurons are in the midst of being born (Gross et al., 2002), and these cords were analyzed at

E14.5. No difference in BrdU labeling in the dorsal spinal cord of *DKO* embryos compared to their wild-type counterparts was observed (Fig. 7A,B), nor was there any marked change in the distribution of these BrdU-labeled dIL neurons. Whereas the gross migration of late-born neurons in the *DKO* spinal cord appeared to be largely unaffected, some small differences in their settling patterns were noticed (Fig. 7C-F).

Cell counts at E17.5 revealed no significant difference in cell numbers in the dorsal horns of wild-type and *DKO* mice (Fig. 7C-F; wild type 1173±93 s.d. versus *DKO* 1153±77 s.d., *P*<0.001). TUNEL assays were also used to assess whether the *Pax2*-expressing neurons in the dorsal horn undergo premature programmed cell death. There was no increase in apoptotic cell numbers between E14.5 and E17.5 in the *DKO* cord (see Fig. S5 in the supplementary material), nor was there an increase in activated caspase-3 expression in the *DKO* cord, thereby arguing that the dIL neurons do not undergo programmed cell death when *Lhx1* and *Lhx5* are absent. These data demonstrate that the reduction in *Gad1* expression at E17.5 in the cord of *DKO* mutants is unlikely to arise from a loss of dIL_A neurons. Instead, our data support a model in which *Lhx1* and *Lhx5* are required to maintain the expression of *Pax2* and *Gad1* in late-born dIL_A neurons.

Reciprocal genetic interactions between *Lhx1* and *Lhx5* with *Pax2* in the developing spinal cord

The similarity in the deficits in inhibitory-neurotransmitter gene expression that occur in *Pax2*- and *Lhx1*/*Lhx5*-mutants led us to investigate whether there are genetic interactions between these two classes of genes. Whereas the expression of the *Lhx1* and *Lhx5* proteins in the cord of *Pax2*^{-/-} mutants was initially unchanged at early developmental times (E11.5-E12.5; Fig. 8A-D), by E14.5 there was a marked loss of *Lhx1* and *Lhx5* expression in the dorsal horn (Fig. 8E-H, arrows). In view of previous findings showing that *Gad1*

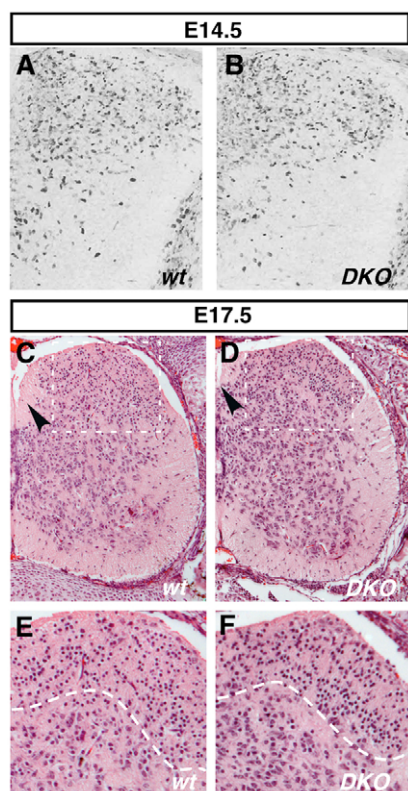


Fig. 7. BrdU pulse-chase and histological analysis of late neuron development in DKO spinal cord. (A,B) Late-born interneurons were pulsed with BrdU at E12.5, and were analyzed at E14.5 when the downregulation of *Gad1* is first observed in the dorsal horn. The distribution of BrdU⁺ cells in wild-type (wt) and DKO spinal cord is similar, suggesting that there are no major defects in cell birth or cell migration in the DKO cord. (C-F) Anatomical analysis indicates no marked loss of neurons in the superficial dorsal horn. The dorsal funiculus is reduced in size (arrowheads) in the lumbar spinal enlargement of the DKO cord. E and F: enlargements of the boxed regions in C and D.

expression is lost in the cord of *Pax2*^{-/-} mutants (Cheng et al., 2004), we analyzed in more detail the temporal changes in *Viaat* expression that occur when *Pax2* is absent. A reduction in *Viaat* expression levels in the dorsal spinal cord was seen as early as E12.5 (Fig. 8K,L), even though *Lhx1* and *Lhx5* continued to be expressed in the cord of *Pax2*^{-/-} mutants at these times (Fig. 8B,D). Consequently, the loss of *Viaat* expression in the *Pax2*^{-/-} mutant cord precedes that of *Lhx1* and *Lhx5*, indicating that the regulation of *Viaat* by *Pax2* at E12.5 is *Lhx1* and/or *Lhx5* independent. At E14.5, the loss of *Viaat* expression in the *Pax2*^{-/-} spinal cord was more apparent (Fig. 8M,N), which is consistent with what has previously been reported for *Gad1* expression (Cheng et al., 2004).

The preferential loss of inhibitory-neurotransmitter-specific gene expression in late-born dorsal neurons is a common feature of the *Lhx1*;*Lhx5* DKO and *Pax2*^{-/-} cords (see above). This raises the question as to why are early-born neurons, particularly those located in the ventral spinal cord, largely unaffected by the loss of these genes? One clue comes from the previous demonstration that *Pax2* together with *Pax5* and *Pax8* form a subfamily of highly homologous *Pax* genes (Walther et al., 1991), which are, in many instances, functionally equivalent (Bouchard et al., 2000). *Pax2*,

Pax5 and *Pax8* are expressed in the developing neural tube in overlapping domains (Nornes et al., 1990; Plachov et al., 1990; Asano and Gruss, 1992; Schwarz et al., 1997), with all three proteins being co-expressed with *Lhx1* and *Lhx5* in dI4 and dI6 neurons, and in dIL_A neurons (data not shown). We therefore investigated whether *Pax5* and *Pax8* might continue to be expressed in ventral but not dorsal regions of the *Pax2*^{-/-} mutant spinal cord, thus compensating for the loss of *Pax2* expression in neurons that continue to express inhibitory-neurotransmitter-specific genes, such as *Viaat*.

Sections from *Pax2*-mutant cords were stained using antibodies that recognize *Pax5* and *Pax8*. In the *Pax2*^{-/-} mutant cords, we observed a complete absence of *Pax5* in the dorsal spinal cord at early stages (E12.5; Fig. 9B) and at E17.5 (Fig. 9E). *Pax8* was transiently expressed up to E12.5, albeit at reduced levels (Fig. 9H). However, from E14.5 onwards, *Pax8* was also completely absent from the dorsal horn (Fig. 9K, data not shown). Because *Pax2*, *Pax5* and *Pax8* are likely to function redundantly, the pronounced loss of *Pax5* and *Pax8* in the dorsal cord of *Pax2*^{-/-} mutants could account for the reduced expression of *Viaat* and *Gad1* in this domain. Moreover, the continued expression of *Pax5* and *Pax8* ventrally at E12.5 may explain the persistence of ventral inhibitory neurons in the *Pax2*^{-/-} cord (Fig. 8N). There is also a population of GABAergic neurons in the ventral horn that do not express *Pax2*, *Pax5* or *Pax8* (G. Lanuza and M.G., unpublished), and these cells may contribute to the residual *Viaat* expression that is seen in the ventral *Pax2*^{-/-} cord.

By E17.5, few, if any, neurons in the *Pax2*^{-/-} spinal cord express *Pax5* and *Pax8*, with only a few ventral neurons continuing to express *Pax8* (Fig. 9E,K). This late reduction in *Pax5* and *Pax8* is consistent with the loss of *Viaat* and *Gad67* expression in ventral neurons that occurs at later times in the *Pax2*^{-/-} cord. Notably, the *Pax5* and *Pax8* single-mutant mice do not exhibit any inhibitory-neuron phenotype, nor is there a concomitant loss of *Pax2* expression in these animals. *Pax5* and *Pax8* are therefore epistatic to *Pax2* in the dorsal spinal cord.

The observation that *Pax2* is required for the continued expression of *Pax8* and *Pax5* prompted us to examine whether *Pax8* and *Pax5* are similarly dependent on *Lhx1* and/or *Lhx5* for their maintenance at E12.5. Although *Pax5* and *Pax8* expression in the dorsal spinal cord was markedly reduced at E12.5 in the DKO cord (Fig. 9C,I), which is in line with the reduction of *Pax2* at this time, expression of both proteins persisted up until E17.5 in some cells scattered throughout the ventral and dorsal horn (Fig. 9F,L). This residual expression of *Pax5* and *Pax8* in the dorsal horn might explain why some dorsal horn interneurons in the *Lhx1*;*Lhx5* DKO cord continue to express *Viaat* and *Gad1*. In summary, our analyses reveal that *Lhx1* and *Lhx5* play a crucial role in maintaining the expression of not only *Pax2*, but also that of *Pax5* and *Pax8* in the dorsal inhibitory neurons. The downregulation of *Pax5* and *Pax8* in the *Pax2*^{-/-} cord also suggests that the loss of *Pax5* and *Pax8* in the *Lhx1*;*Lhx5* DKO is mediated in part by the loss of *Pax2*.

DISCUSSION

This study demonstrates a key role for the LIM-homeodomain transcription factors *Lhx1* and *Lhx5* in inhibitory-neuron development in the dorsal spinal cord. *Lhx1* and *Lhx5* are co-expressed together with *Pax2* in the majority of differentiating inhibitory neurons in the spinal cord, where they function together to maintain *Pax2* expression in subsets of spinal inhibitory interneurons and establish a stable GABAergic differentiation program in these cells. Inactivating *Lhx1* and *Lhx5* in the embryonic cord results in the loss of *Pax2* expression in dorsal neurons, which is followed by the downregulation of the inhibitory neuronal markers

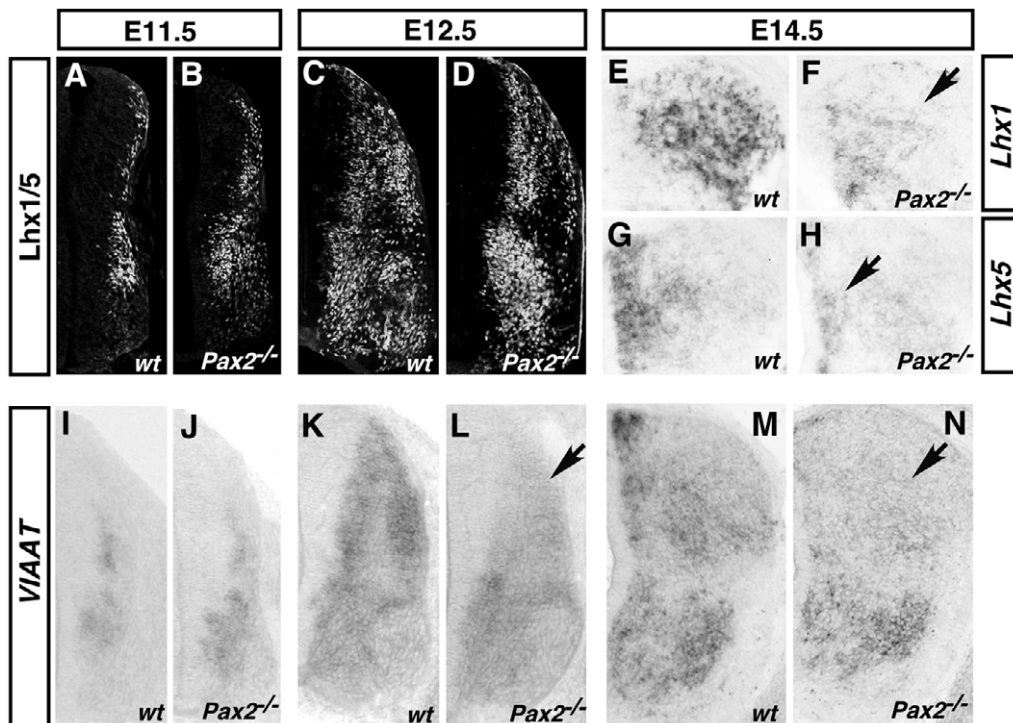


Fig. 8. Pax2 maintains *Lhx1* and *Lhx5* expression in dIL_A interneurons. (A–D) Expression of *Lhx1* and *Lhx5* in the *Pax2*^{-/-} cord. *Lhx1* and *Lhx5* antibody stainings showing *Lhx1* and *Lhx5* expression in the dorsal neurons in *Pax2*-independent at E11.5 (A,B) and E12.5 (C,D). (E–H) In situ hybridization analysis of *Lhx1* (E,F) and *Lhx5* (G,H) at E14.5 shows that both genes depend on *Pax2* for maintenance in the dIL_A population. (I–N) *Viaat* expression is correctly initiated in the *Pax2*^{-/-} mutant cord (I,J), but begins to be downregulated dorsally at E12.5. The dIL_A population shows a downregulation of *Viaat* at E12.5 (arrow in L). *Viaat* expression in the dorsal horn is largely missing by E14.5 (arrow in N) (see also Cheng et al., 2004). Notice that *Viaat* is downregulated dorsally, but remains largely intact in the ventral cord.

Gad1 and *Viaat*. Thus, *Lhx1* and *Lhx5*, together with *Pax2*, form part of a transcriptional network that generates and maintains the differentiated phenotype of inhibitory neurons in the dorsal spinal cord.

***Lhx1*, *Lhx5* and neuronal cell-type specification in the spinal cord**

Mash1 (also known as *Ascl1* – Mouse Genome Informatics), *Ptf1a*, *Lbx1* and *Pax2* all play crucial roles in the development of dorsal inhibitory neurons (Gross et al., 2002; Muller et al., 2002; Cheng et al., 2004; Glasgow et al., 2005; Mizuguchi et al., 2006). *Ptf1a* and *Mash1* are expressed in the precursors of dI4 and dIL_A neurons, and they are required for the initial specification of each cell type. *Lbx1* is expressed in postmitotic Class B neurons, where it functions upstream of *Pax2*, *Lhx1* and *Lhx5* in specifying dI4 and dIL_A inhibitory neurons. In analyzing the *Lbx1*-mutant phenotype, Gross et al. (Gross et al., 2002) proposed a model in which *Lhx1* and *Lhx5* would function in establishing the identity of dI2 and dI4 neurons. In testing this postulate with *Lhx1*;*Lhx5* DKO mice, we found no deficits in the initial specification of dI2 and dI4 neurons (Fig. 4), demonstrating that *Lhx1* and *Lhx5* do not confer subtype identity on either of these two dorsal cell types. Although *Lhx1* and *Lhx5* are largely dispensable for the specification of dorsally derived dI4 and dIL_A neurons, both genes may play roles in other aspects of dI4 and dIL_A development. Interestingly, we observed some loss of ventrally-derived V1 neurons in the DKO cord. The exact function of *Lhx1* and *Lhx5* in these cells is not clear and needs to be investigated further.

***Lhx1*, *Lhx5* and *Pax2* coordinately regulate GABAergic-interneuron development**

Although *Lhx1* and *Lhx5* do not regulate the initial choice between inhibitory dIL_A and excitatory dIL_B cell fates in the dorsal horn (Fig. 4), one or other gene is needed for dIL_A neurons to maintain their

differentiated inhibitory phenotype, and for the full induction of *Pax2* in newborn dIL_A neurons. The observation that some spinal neurons continue to express inhibitory-neurotransmitter markers when *Lhx1* and *Lhx5* are inactivated argues that both genes are not obligatory determinants for inhibitory neurotransmission, and that they are thus unlikely to directly control the transcription of inhibitory-neurotransmitter-specific genes, such as *Viaat*, *Gad1*, *Gad2* and *GlyT2*. This conclusion is also consistent with the gradual loss of *Viaat* and *Gad1* transcripts that occurs in the cord of *Lhx1*;*Lhx5* DKO mutants (Fig. 5).

Our study did not precisely define the time period when *Lhx1* and *Lhx5* are required for inhibitory-neuron differentiation; however, the reduced expression of *Pax2* at E12.5 in the DKO cord suggests that there may be a critical period up to E12.5 when either *Lhx1* or *Lhx5* is needed to consolidate *Pax2* expression and the inhibitory program. Further support for the idea that the *Lhx* genes are required at early rather than later times comes from the observation that *Lhx1* expression after E13.5 is apparently not necessary for continued *Pax2* expression, or for the maintenance of *Viaat* and *Gad1*, because all three inhibitory markers continue to be expressed in the *Lhx1*-mutant cord after E13.5.

In spite of the strong similarities in the spinal cord phenotypes of the *Lhx1*;*Lhx5* DKO and *Pax2*^{-/-} mutants, there are differences. Although these dissimilarities most likely reflect temporal differences in *Pax2* expression in the DKO versus *Pax2*^{-/-} cord, it is nonetheless possible that *Lhx1*, *Lhx5* and *Pax2* have distinct roles in GABAergic-neuron development. For instance, *Lhx1* and *Lhx5* might regulate inhibitory markers at later developmental times in a manner that is independent of its role in maintaining *Pax2*. Alternatively, the transient expression of *Pax2* that occurs at E10.5–E11.5 in the *Lhx1*;*Lhx5* DKO cord might be sufficient for the initiation of *Viaat* and *Gad1* expression, and for its persistence in some neurons even after *Pax2* is downregulated, thus accounting for any differences in *Viaat* and/or *Gad1* expression between the two mutants.

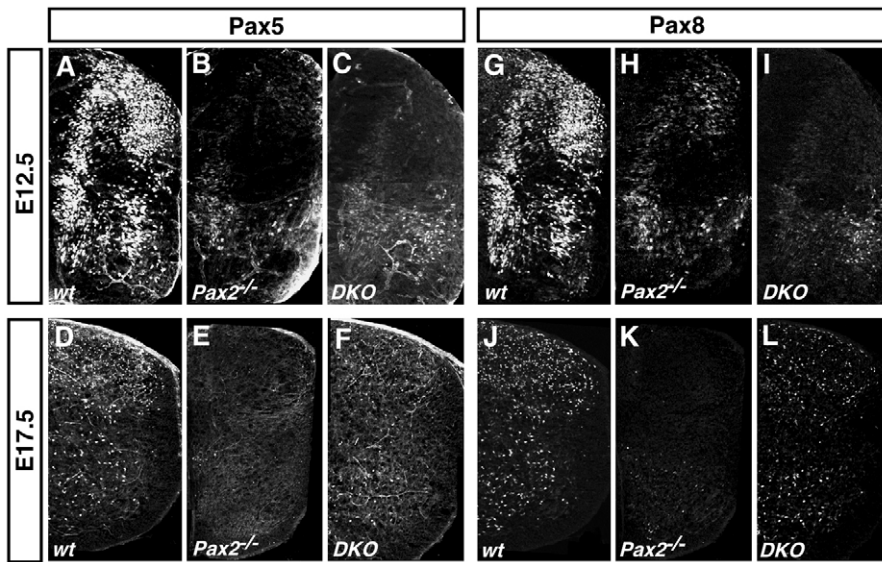


Fig. 9. Expression of Pax5 and Pax8 is downregulated in DKO and Pax2^{-/-} spinal cords. (A-C) Pax5 expression is lost in the Pax2^{-/-} and DKO cord at E12.5. Some ventral Pax5⁺ cells persist in the DKO cord. (D-F) At E17.5, Pax5 expression is partially lost in the DKO cord but, unlike Pax8, it is completely abolished in Pax2^{-/-} cord. (G-I) At E12.5, Pax8 expression is gradually downregulated dorsally but maintained ventrally at reduced levels in both the Pax2 and Lhx1; Lhx5 DKO-mutant cord. (J-L) At E17.5, there is a complete loss of Pax8 expression dorsally. Some Pax8 expression is retained ventrally in the Pax2^{-/-} cord. The DKO cord shows a partial loss of Pax8 expression in both dorsal and ventral regions.

Conservation of the Pax2-Pax5-Pax8 gene cassette in the spinal cord

Our studies also implicate Pax5 and Pax8 in the regulation of inhibitory-neurotransmitter cell identity in the spinal cord, because Pax5 and Pax8 are expressed together with Pax2 in many spinal inhibitory neurons. Studies in the kidney and midbrain and/or hindbrain have provided evidence that Pax2, Pax5 and Pax8 are functionally redundant in many contexts (Bouchard et al., 2002; Kobayashi et al., 2005). In the CNS, Pax2 and Pax5 have been shown to be functionally equivalent in the development of the mid- to hind-brain boundary (Bouchard et al., 2000), and Pax5 and Pax8 are epistatic to Pax2 at the midbrain-hindbrain junction (Pfeffer et al., 1998). Our results reveal that Pax5 and Pax8 expression in dorsal inhibitory neurons also depends on Pax2, with the loss of GABAergic cells in the DKO- and Pax2-mutant cords being closely correlated with the reduction in Pax5 and Pax8 expression (Fig. 9). By contrast, Pax5 and Pax8 are less dependent on Pax2 in the ventral spinal cord (Fig. 9). Consequently, the differential effects that losing Pax2 has on the expression of Pax5 and Pax8 in dorsal versus ventral neurons may be the major reason why inhibitory-neurotransmitter gene expression is preferentially depleted in dorsal interneurons. In summary, the close correlation between neurons that continue to express *Viaat* and *Gad1*, and those cells in which Pax5 and Pax8 protein expression perdures in the Pax2- and DKO-mutant cords, provides further evidence that Pax2, Pax5 and Pax8 may function redundantly to regulate inhibitory-neurotransmitter gene expression in the developing spinal cord.

Lhx1 and Lhx5 consolidate the inhibitory-neurotransmitter program

Our genetic analyses place Lhx1 and Lhx5 downstream of many of the known transcriptional determinants that control the generation of inhibitory neurons in the dorsal spinal cord. In the dorsal horn, *Mash1* and *Ptf1a* play early roles in specifying inhibitory neurons from *Gsh1/2⁺/Mash1⁺* progenitors. Ptf1a, a bHLH transcription factor whose expression is dependent upon *Mash1* in dIL_A cells, functionally antagonizes the activity of the Tlx1 and Tlx3 transcription factors in Lbx1⁺ dIL cells to promote a GABAergic fate (Glasgow et al., 2005; Mizuguchi et al., 2006; Wildner et al., 2006). This program is not universal, as forebrain inhibitory interneurons do not express *Ptf1a*, *Pax2*, *Pax5* or *Pax8*. Instead, it is the *Dlx* genes,

together with *Mash1*, that regulate the development of these GABAergic neurons (Yun et al., 2002). Furthermore, we have recently identified a population of inhibitory interneurons in the ventral spinal cord that do not express Pax2, Pax5 or Pax8 (G. Lanuza and M.G., unpublished). Taken together, these findings suggest that multiple developmental programs in the developing nervous system can specify an inhibitory-neurotransmitter fate. How these divergent transcriptional programs activate the genes required for fast inhibitory neurotransmission remains to be determined. Inhibitory-neuron determinants, such as *Ptf1a* and *Pax2*, rather than directly controlling genes such as *Viaat* and *Gad1*, could activate a set of 'core factors' that regulate their expression. Alternatively, these 'neurotransmitter' genes could contain multiple cis-regulatory elements that are recognized by the different combinations of cell-type-specific transcription factors such that their expression is activated in a context-dependent manner.

Although the initial expression of inhibitory-neurotransmitter-specific genes, such as *Viaat* and *Gad1*, are closely linked to the initial acquisition of particular cell fates, this study demonstrates that their continued expression in these neurons is dependent upon transcription factors such as Lhx1 and Lhx5 that act to consolidate the inhibitory differentiation program. Interestingly, it appears that the loss of Pax2 and/or Lhx1 and Lhx5 does not result in a cell-fate switch by these cells (Cheng et al., 2004) (this study). Rather, presumptive 'inhibitory' neurons simply downregulate many of the genes that are necessary for fast inhibitory neurotransmission. These findings are consistent with a model in which the initial choice of neurotransmitter phenotype is closely tied to neuronal-specification events, and it suggests that for certain neuronal subtypes, the choice of neurotransmitter phenotype, once made, is irrevocable. Nonetheless, some neurons are able to change their neurotransmitter expression in response to changes in neural activity (Borodinsky et al., 2004) or target-derived signals (Schotzinger and Landis, 1988), which argues that plasticity exists in the developmental programs that control the neurotransmitter status of a neuron.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/2/357/DC1>

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