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 dIL4A neurons (dIL4), 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 Viat1 (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 (Schottzinger and Landis, 1988; Borodinsky et al., 2004), a cell 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 (Phipps 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 dILB 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 dILB 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 dIL4A 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 dILB 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 dILB-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 Viat1. 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 dILB 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 dILA 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 Viat 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 (Lhx1flox/llox) 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 Lhx1flox/llox conditional allele (Kwan and Behringer, 2002) (see Fig. 3A). Selective inactivation of Lhx1 in neurons was achieved by crossing a Nestin-Cre (Nestin-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 Lhx1flox/llox background. In this study, conditional Lhx1-mutant mice with the genotype Lhx1flox/llox;NesCre are referred to as Lhx1c−/− mice. Double mutants with the genotype Lhx1flox/llox;Lhx5flox/flox;NesCre are referred to as DKO mice. Lhx5c−/− and Lhx1c−/−-mutant mice both die at birth. Lhx1flox/llox;NesCre homozygous strains are, however, healthy and fertile, and so DKO mice were generated by crossing parental lines comprised of genetic combinations of Lhx5flox/flox;Lhx1flox/llox;NesCre with Lhx1flox/llox;Lhx5flox/flox 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), polyclonal anti-Pax2 (Zymed) and 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 preformed using probes specific for mouse Lhx1 (Bertuzzi et al., 1996), Lhx5 (Zhao et al., 1999), Viat 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 dII2, dI4, dI6 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 dIIc cells are emerging (arrow in I). By E13.5, the expression of Lhx1 and Lhx5 in the dorsal horn is largely complementary (D,I). 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 represent the average of six sections (three sections from two cords).

BrdU labeling
Pregnant dams were injected intraperitoneally with 50 mg bromodeoxyuridine (50 μg/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 bodyweight at E12.5. E14.5 embryos were collected and processed for (three sections from two cords).

Apoptotic cell counts for each sample represents the average of six sections

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 dILA neurons that populate the dorsal horn. However, although Lhx1 had been shown to persist dorsally at later stages by in situ hybridization (Mulher 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,J). 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 dILA cells can be accounted for in two ways. First, the complementary expression patterns of Lhx1 and Lhx5 in late-born dILA cells may reflect high-level expression of Lhx1 in the dILA cells that are born first. These cells would be expected to accumulate in the more lateral regions of the dorsal horn, whereas later-born dILA cells that are located more medially might exhibit high Lhx5-low Lhx1 expression. Alternatively, differentiating dILA 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 dILA cells cease being generated at E13.5 (Gross et al., 2002). For this reason, we favor a model in which newborn dILA cells express Lhx5 at high levels, while the more mature dILA 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 dILA interneurons. dILA 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, dILA 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).
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 dILA neurons in either of the single mutants. Pax2, which is selectively expressed in inhibitory dILA and neurons showed a normal pattern of expression in cords taken from E12.5 Lhx1loxP/loxP and Lhx5−/− single mutants (Fig. 2A-C). There were also no marked changes in the expression patterns of either Lbx1 or Lmx1b in these late-born cells (data not shown).

In view of the normal development of late-born dILA 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 dILA neurons in the dorsal
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 NestinCre (NesCre) 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 NesCre-mediated recombination in the embryonic spinal cord, a ROSA26-derived reporter line that conditionally expresses the lacZ gene (R26lacZ) was used to identify cells that had undergone Cre-mediated recombination (Soriano, 1999). When mice carrying the R26-lacZ reporter gene were crossed with NesCre 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. 3G), demonstrating that the NesCre 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 Is1, 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 Lhx1, 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. 5LJ 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 dILx neurons, was not upregulated in the cord of DKO mutants, demonstrating that
dILA neurons do not acquire a dILB 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 dILA cell migration. Instead, it appears that the dILA neurons fail to maintain Pax2 expression as they migrate and settle in the lateral dorsal horn. As noted previously, the dILA cells in the DKO dorsal horn do not switch to a glutamatergic Lmx1b+ dILB fate; there was no increase in Lmx1b+ cell numbers (Fig. 6F-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 dILA 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 dILA 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 dILA 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 dILA 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 dILA 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...
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 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., 1992; Schwarz et al., 1997), with all three proteins being co-expressed with Lhx1 and Lhx5 in dl4 and dl6 neurons, and in dl7 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.
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*, *Lhx1* 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 dILA neurons, and they are required for the initial specification of each cell type. *Lhx1* is expressed in postmitotic Class B neurons, where it functions upstream of *Pax2*, *Lhx1* and *Lhx5* in specifying dI4 and dILA inhibitory neurons. In analyzing the *Lhx1*-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 dILA neurons, both genes may play roles in other aspects of dI4 and dILA 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 dILA and excitatory dILB cell fates in the dorsal horn (Fig. 4), one or other gene is needed for dILA neurons to maintain their differentiated inhibitory phenotype, and for the full induction of Pax2 in newborn dILA 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.
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 dILa cells, functionally antagonizes the activity of the Tlx1 and Tlx3 transcription factors in Lhx1+ 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

References


