Gsh2 is required for the repression of Ngn1 and specification of dorsal interneuron fate in the spinal cord

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Summary

The molecular programs that specify progenitors in the dorsal spinal cord remain poorly defined. The homeodomain transcription factor Gsh2 is expressed in the progenitors of three dorsal interneuron subtypes, dI3, dI4 and dI5 neurons, whereas Gsh1 is only expressed in dI4 and dI5 progenitors. Mice lacking *Gsh2* exhibit a selective loss of dI3 interneurons that is accompanied by an expansion of the dI2 progenitor domain. In *Gsh2* mutant embryos, expression of the proneural bHLH protein Mash1 is downregulated in dI3 neural progenitors, with *Mash1* mutants exhibiting a concordant reduction in dI3 neurons. Conversely, overexpression of Gsh2 and Mash1 leads to the ectopic production of dI3 neurons and a concomitant repression of Ngn1 expression. Our results provide evidence that genetic interactions involving repression of *Ngn1* by *Gsh2* promote the differentiation of dI3 neurons from class A progenitors.

Key words: Spinal cord, Gsh2, Gsh1, Mash1, Ngn1, Interneurons

Introduction

Neurogenesis in the embryonic spinal cord is marked by the emergence of distinct classes of neurons at sharply delineated positions along the dorsoventral (DV) axis of the neural tube (Jessell, 2000). This developmental patterning is controlled by the complex interplay between spatially graded extracellular signals and the cell-autonomous transcriptional programs that they activate in restricted populations of neural tube progenitors (Liem et al., 1997; Briscoe et al., 2000; Goulding and Lamar, 2000; Jessell, 2000). In the ventral neural tube, sonic hedgehog (Shh) functions as a morphogen to establish different ventral cell fates along the DV axis of the neural tube (reviewed by Jessell, 2000; Shirasaki and Pfaff, 2002). DV patterning by Shh is mediated by two classes of homeodomain transcription factors that are denoted Class I/II factors, which are differentially expressed in the ventral ventricular zone (Briscoe et al., 2000; Muhr et al., 2001; Novitch et al., 2001). These factors partition the ventricular zone into five distinct progenitor domains by recruiting members of the groucho class of co-repressors in order to selectively repress the transcription of their cognate Class I or Class II partner (Briscoe et al., 2000; Goulding and Lamar, 2000). In addition to having essential functions in establishing each progenitor domain and the boundaries that form between them, these transcription factors also function as cell-autonomous determinants of neuronal identity (Burrill et al., 1997; Ericson et al., 1997; Briscoe et al., 1999; Jessell, 2000; Sander et al., 2000; Vallstedt et al., 2001; Shirasaki and Pfaff, 2002).

Much less is known about the patterning and specification of neuronal cell types that emerge from the dorsal neural tube. Inductive signals from the ectoderm and dorsal midline play crucial roles in generating three dorsal interneuron cell types (Liem et al., 1995; Liem et al., 1997; Lee and Jessell, 1999), which are denoted Class A neurons (Gross et al., 2002; Muller et al., 2002). Both TGFβ-dependent and Wnt-dependent signaling pathways are necessary for generating Class A cell types (Lee and Jessell, 1999; Muller et al., 2002; Muroyama et al., 2002), whereas dI4-dI6 Class B neurons develop in a TGFβ-independent manner. To date, efforts to understand how progenitors in the alar plate are specified have focused primarily on the roles of the atonal-like and achaete scute-like bHLH proteins, which are expressed in restricted populations of dorsal progenitors (Bermingham et al., 2001; Gowan et al., 2001; Caspary and Anderson, 2003). Math1, for instance, is expressed in dI1 progenitors, where it functions as an obligate determinant of dI1 identity (Bermingham et al., 2001). Math1 additionally represses the expression of *Ngn1* and *Ngn2*, which function as proneural factors for the dI2 neuron differentiation program (Gowan et al., 2001).

Although the above studies demonstrate that the *Math1* and *Ngn1/Ngn2* bHLH proneural genes function as determinants of neuronal identity, it is unlikely that these proneural bHLH proteins function as the initial transcriptional determinants of dorsal patterning, as they are expressed later than dorsal patterning genes such as *Pax3/Pax7*, *Msx1*, *Gsh1/Gsh2* and *Dbx2* (Bang et al., 1997; Houzelstein et al., 1997) (M.G., unpublished). More importantly, the proneural bHLH proteins are typically expressed in a mosaic expression pattern in

differentiating progenitors (Guillemot et al., 1993; Ma et al., 1996; Fode et al., 1998; Ma et al., 1998), which is more consistent with their functioning as neural determination factors than as early DV patterning factors. It is not known, for instance, what roles homeodomain factors such as Pax3/Pax7, Msx1/Msx2/Msx3 and Gsh1/Gsh2 play in restricting the expression, and thus activity, of these proneural bHLH genes.

In this study, we examined the function of the Gsh genes in patterning dorsal alar plate progenitors. We show that the Gsh1 and Gsh2 homeodomain transcription factors are differentially expressed in the progenitors for dI3, dI4 and dI5 neurons. Furthermore, we provide evidence that *Gsh2* and the proneural bHLH gene *Mash1* function sequentially to determine dI3 identity, and that *Gsh2* activates the Mash1-dependent differentiation of dI3 neurons by suppressing the expression of *Ngn1* and *Ngn2*. We propose that *Gsh2*, acting in combination with other dorsal determinants downstream of TGFβ signaling, subdivides the Class A progenitor domain to generate a population of *Mash1*$^+$ dorsal progenitors that give rise to dI3 interneurons.

Materials and methods

Animals

Gsh1 (Li et al., 1996) and *Gsh2* (Szucsik et al., 1997) heterozygous mice were obtained from Steve Potter and Kenny Campbell (Children's Medical Research Foundation, Cincinnati, USA). *Mash1* heterozygous mice (Guillemot et al., 1993) were kindly provided by François Guillemot (National Institute for Medical Research, London, UK). Wild-type, heterozygous and mutant embryos were obtained from timed pregnancies, with E0.5 being the detection date of the vaginal plug. *Ngn1/Ngn2* double-mutant embryos were a kind gift from Qiufu Ma (Dana Farber Cancer Institute, Boston, USA). Genotyping of mice was performed by PCR using the following oligonucleotide primers. All PCR reactions were run for 30 cycles (1 minute 94°C, 1 minute 65°C, 1 minute 72°C), preceded by a 2-minute denaturing step at 94°C. *Mash1* and *Gsh2* mutants were genotyped using *neo*-specific primers to detect the mutated allele.

Gsh1 WT, GCACCGCAAGGCTGCAAGTGCTCTT and ATACCATGTGAGACAGTTCTCTCTGCTAGG;

Gsh1 KO, AGCGTCGTGATTAGCGATGATGAACCA and TCCAGTTTCACTAATGACACAAACGT;

Gsh2 WT, CAAGGGTTGTCAAGTAGAGTGG and CTTCACGCGACGGTTCTGAAAC;

Neo, CAAGATGGATTGCACGCAGG and CGATGTTTCGCTTGGTGGTC; and

Mash1 WT, CTCCGGGAGCATGTCCCCAA and CCAGGACTCAATACGCAGGG.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Gross et al., 2002; Moran-Rivard et al., 2001). The following antibodies were used in this study: anti-Gsh1/2 (rabbit polyclonal, S. Kriks), anti-Gsh2 (rabbit polyclonal, K. Campbell), anti-Mash1 (mouse monoclonal, D. Anderson), anti-Mash1 (rabbit polyclonal, Babco), anti-Isl1/2 (mouse monoclonal 40.2.D6, DHSB), anti-Brn3a (guinea pig polyclonal, E. Turner), anti-Foxd3 (rabbit polyclonal) (Dottori et al., 2001), anti-Lhx1/5 (mouse monoclonal 4F2-10, DHSB), anti-NeuN (mouse monoclonal, Chemicon), anti-Lbx1 (rabbit polyclonal) (Gross et al., 2002), anti-Ngn1 (rabbit polyclonal, J. Johnson), Pax7 (mouse monoclonal, DSHB), and Pax2 (rabbit polyclonal, Babco). For BrdU-labeling experiments, E10.5 mouse embryos were pulsed for 1.5 hours in utero with bromodeoxyuridine [5 mg/ml, 0.1 ml/10 g body weight, injected intraperitoneally (i.p.)]. Previous to incubation with anti-BrdU (rat, ImmunologicalsDirect), sections were treated with 2N HCl for 20 minutes, and 0.1 M borate buffer (pH 8.5) for 20 minutes. Species-specific secondary antibodies conjugated to Cy2, Cy3 and Cy5 were used to detect primary antibodies (Jackson ImmunoResearch).

Generation of antibodies to Gsh1/2

Antibodies that specifically recognize Gsh1 and Gsh2 together were generated by immunizing rabbits with a fusion protein containing the C-terminal fragment of the Gsh1 protein, which included the homeodomain of the mouse Gsh1 protein fused to glutathione-S-transferase pGEX (Pharmacia).

In ovo electroporation

Full-length cDNA for mouse *Gsh2* and mouse *Ngn1* was amplified from total cDNA generated from E11.5 neural tube total RNA. Full-length sequences were cloned into a pIRES-EGFP expression vector (Invitrogen, modified by M. Dottori) that contains the chick beta-actin promotor and a CMV enhancer. A full-length mouse *Mash1* cDNA was cloned into the pCAGGS expression vector.

White Leghorn eggs were incubated in a force-draft, humidified incubator at 38°C and electroporations were performed at E3. Stage HH11-13 chick embryos were electroporated with the constructs mentioned above at a concentration of 2.5 μg/ μl, as previously described (Muramatsu et al., 1997). Briefly, the DNA was injected into the lumen of the spinal cord using a picospritzer, and then electroporated into one side of the neural tube using a square wave BTX electroporator (six 50-msecond pulses at 25 mV). Embryos were incubated for a further 24 or 48 hours, before being processed for immunohistochemistry or in situ hybridization. GFP expression was used to assess electroporation efficiency. For both the *Gsh2*-EGFP and *Ngn1*-EGFP constructs, expression was confirmed using polyclonal antibodies that recognize Gsh1/2 (this study) and Ngn1 (obtained from J. Johnson), respectively.

In situ hybridization

In situ hybridization was performed as previously described (Goulding et al., 1993; Dottori et al., 2001). The in situ probes used were mouse *Gsh1* (Li et al., 1996), mouse *Ngn1* and *Ngn2* (Fode et al., 1998; Ma et al., 1998), mouse *Dbx2* (Shoji et al., 1996), mouse *Otp* (Simeone et al., 1994), mouse *Msx1* (Robert et al., 1989), mouse *Olig3* and mouse *Msx3* (G.M.L., this study).

Imaging

Fluorescence labeling in spinal cord sections was visualized using a Zeiss LSM 510 confocal microscope. Brightfield in situ images were captured by digital photography on a Zeiss Axioplan2 microscope with an Axiocam digital camera. All figures were assembled for publication as Photoshop/Canvas images.

Results

Gsh1 and Gsh2 expression in the dorsal spinal cord

Gsh2 and its closely related homolog *Gsh1* are expressed in progenitors in the dorsal ventricular zone (Szucsik et al., 1997; Weiss et al., 1998). Using antibodies that recognize either the Gsh2 protein alone, or Gsh1 and Gsh2 together, we carefully mapped the expression domains of both proteins in the developing dorsal spinal cord. Gsh1 and Gsh2 are first expressed from E9.5-E10.5 in a subset of Pax7$^+$ progenitors at dorsal hindbrain and cervical spinal cord levels (Fig. 1A,B, data not shown). Whereas Gsh2 is initially expressed in dI3 progenitors located just ventral to the Ngn1$^+$ dI2 progenitor domain (Fig. 1C,K,L), by E11.5 Gsh2 expression extends ventrally into the dI4 and dI5 progenitors (Fig. 1D). Gsh1

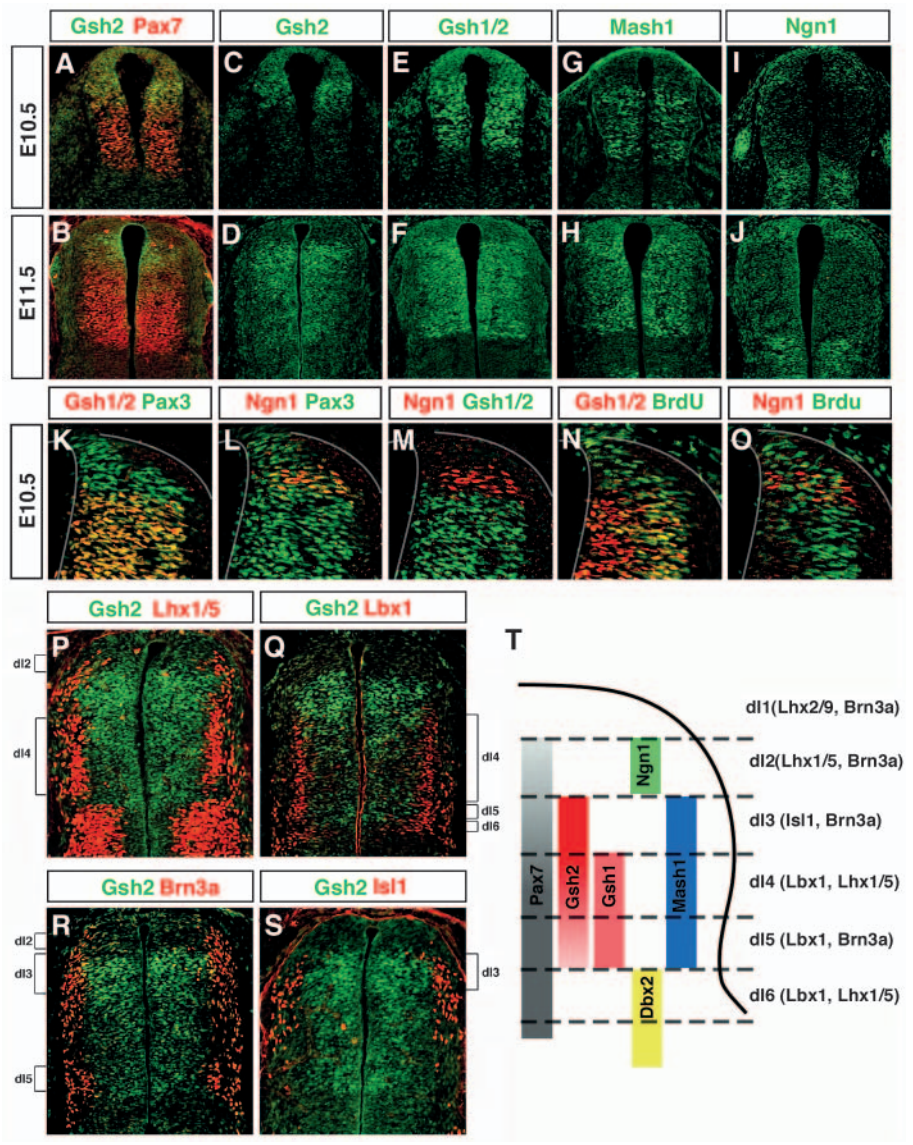

Fig. 1. Gsh2 is expressed in the progenitors of the dI3, dI4 and dI5 neurons in the embryonic spinal cord. (A-D) A subset of Pax7$^+$ cells express Gsh2. (E,F) At E10.5 and E11.5, Gsh1/Gsh2 expression encompasses dI3, dI4 and dI5 progenitor domains. (G,H) Mash1 is expressed in the same dorsal progenitor domain as Gsh1/Gsh2. (I,J) The bHLH protein Ngn1 is expressed in adjacent domains dorsal and ventral to Gsh2$^+$ and Mash1$^+$ progenitors. (K) Gsh2 is homogeneously expressed in all Pax3$^+$ dI3 progenitors. (L) Ngn1 marks 50% of Pax3$^+$ cells in the dI2 ventricular domain. (M) Ngn1 and Gsh2 are expressed in adjacent domains in the dorsal neural tube; this image is an overlay of staining for Gsh1/Gsh2 and Ngn1 of the adjacent sections shown in K and L. (N) Ventricular dividing cells, marked by BrdU, express Gsh2 in dI3 progenitors. (O) Ngn1 is expressed by some of the cells marked after a 1.5-hour pulse of BrdU. (P-S) The Gsh1/Gsh2-positive domain gives rise to dI3, dI4 and dI5 neurons. (P,Q) dI4 neurons expressing both Lhx1/Lhx5 and Lbx1 arise ventrolateral to the most dorsal Gsh2$^+$ domain, whereas the most ventral Gsh2$^+$ domain gives rise to Lbx1$^+$ dI5 neurons that do not express Lhx1/Lhx5. (R,S) Isl1$^+$ and Brn3a$^+$ dI3 neurons arise from the most dorsal Gsh2$^+$ progenitor domain. (T) Schematic summary of the expression of several transcription factors in alar plate progenitors.

expression at E10.5 is more restricted and encompasses only the dI4 and dI5 progenitor domains (Fig. 1E,F and Fig. 3C). In comparing the expression domains of Gsh1/Gsh2 and the proneural bHLH protein, Mash1, we noted that Gsh1/Gsh2 expression delineates a dorsal territory in the ventricular zone that gives rise to Mash1$^+$ progenitors (Fig. 1E-H). This Gsh1$^+$/Gsh2$^+$/Mash1$^+$ domain is bordered by the Ngn1-expressing progenitors dorsally, and by Ngn1$^+$/Dbx2$^+$ progenitors ventrally (Fig. 1I,J,M and Fig. 3E,I). The dorsal population of Ngn1$^+$ cells differentiates as dI2 neurons (Gowan et al., 2001), whereas the Ngn1$^+$/Dbx2$^+$ cells give rise to dI6 interneurons (Muller et al., 2002).

Progenitor cells in the dorsal ventricular zone express Pax3. By comparing Gsh1/Gsh2 expression with Pax3 expression, we found that Gsh1/Gsh2 are expressed in >98% of dI3-dI5 progenitors (Fig. 1K), including many cells that are in S phase (Fig. 1N). Ngn1 is expressed in dI2 progenitors that are adjacent to Gsh1$^+$/Gsh2$^+$ dI3 cells (Fig. 1M). Interestingly, approximately 50% of the cells in the presumptive dI2 progenitor domain express Ngn1 (Fig. 1L), including a proportion of BrdU-labeled S-phase cells (Fig. 1O).

To confirm that Gsh2 is expressed in dI3, dI4 and dI5 progenitors, the spatial relationship of the Gsh2$^+$ progenitor domain was mapped with respect to postmitotic dorsal interneuron subtypes. A comparison of Gsh2 with Lhx1/Lhx5 (dI2, dI4 and dI6 neurons), Lbx1 (dI4, dI5, dI6 neurons), Brn3a, which is expressed in dI1-3/dI5 neurons and Isl1, a marker for differentiating dI3 neurons, demonstrated that Gsh2 is selectively expressed in the progenitors for dI3, dI4 and dI5 interneurons (Fig. 1P-S). Gsh1, however, is expressed only in dI4/dI5 progenitors (data not shown). Interestingly, Gsh2 expression levels are higher in cells that lie medial to the generation zone for Isl1$^+$ dI3 neurons (Fig. 1S), indicating an elevated expression in dI3 progenitors.

Altered dorsal interneuron development in *Gsh2* mutant mice

Previous studies in *Drosophila* have suggested that *Ind*, the fly homolog of *Gsh1* and *Gsh2*, functions as an essential determinant of intermediate neuroblast identity in the ventral neuroectoderm (Weiss et al., 1998). This finding, together with our observation that *Gsh2* expression demarcates a domain that gives rise to three dorsal interneuron cell types, led us to wonder whether *Gsh2* might also play an essential role in controlling the identity of dorsal interneuron subtypes. To investigate this, we assessed the development of the early interneuron subtypes that emerge from the dorsal neural tube of *Gsh2* mutant mice (Szucsik et al., 1997). No changes were

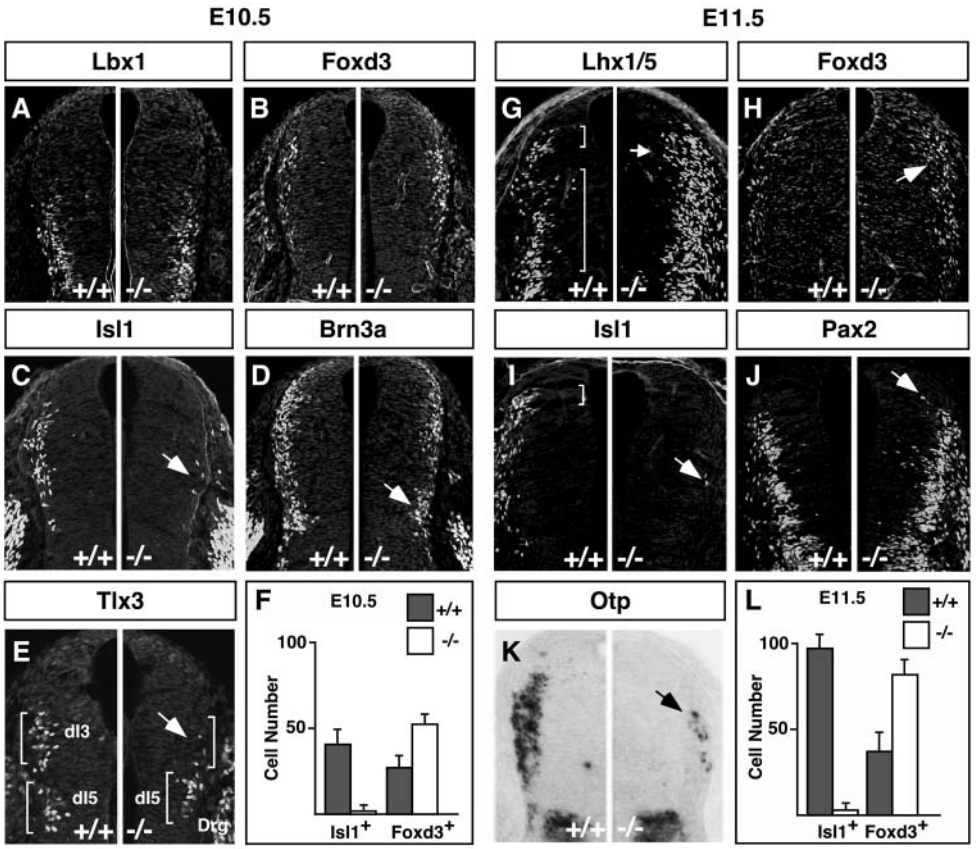

Fig. 2. *Gsh2* mutant embryos do not generate dI3 neurons. (A-F) Changes in dorsal neuron differentiation in E10.5 *Gsh2* knockout (KO) embryos. (A) Lbx1$^+$ dI4-dI6 neurons are still generated in the *Gsh2*$^{-/-}$ mutant spinal cord. (B) Foxd3$^+$ dI2 neurons are increased in *Gsh2*$^{-/-}$ mutant embryos, whereas Isl1$^+$ dI3 neurons are almost completely absent (C). The arrow in C indicates the few remaining dI3 cells. (D) Ventral Brn3a expression (arrow) is unchanged in the mutant, indicating a normal development of dI5 neurons. (E) The dorsal population of Tlx3$^+$ neurons (arrow) is missing in the *Gsh2* mutant cord, confirming the loss of dI3 neurons, while Tlx3 expression in dI5 neurons is not affected. (F) The loss of Isl1$^+$ dI3 neurons is partially offset by an increase in Foxd3$^+$ dI2 neurons. (G-L) Changes in transcription factor expression at E11.5 in *Gsh2* mutant embryos. In *Gsh2*$^{-/-}$ mutants, Lhx1/5$^+$ (G) and Foxd3$^+$ neurons (H) are generated from the dI3 progenitor domain (arrows in G and H) at the expense of Isl1$^+$ (I) and *Otp*$^+$ (K) dI3 neurons (arrows). (J) There is a slight dorsal expansion of dI4 neurons expressing Pax2 (arrow). (L) Cell counts of Foxd3- and Isl1-positive cells in *Gsh2* KO mice (–/–) show a loss of >95% of dI3 neurons and a concomitant increase in dI2 neurons, when compared with age matched wild-type embryos (+/+). Drg, dorsal root ganglion.

seen in the expression of NeuN (Neuna60 – Mouse Genome Informatics) or other pan-neural postmitotic markers (data not shown), indicating that *Gsh2* is not required for progenitors to exit the cell cycle and initiate a generic program of neuronal differentiation.

Using a battery of dorsal interneuron markers, the specification of neurons that arise from the dorsal Pax3/Pax7 territory at E10.5 and E11.5 was then examined. Little or no change was noted in the expression of Lbx1 at E10.5 (Fig. 2A), or of Pax2 at E11.5 (Fig. 2J), indicating that dI4-dI6 neurons differentiate normally. Lbx1, however, marks only two of the three neuronal cell types that arise from Gsh2$^+$ progenitors, prompting us to use additional markers to assess dorsal interneuron development. The expression of Brn3a (Fig. 2D, arrow) and Tlx3 in dI5 neurons (Fig. 2E) was unchanged in the *Gsh2* mutant spinal cord, demonstrating that *Gsh2* is not required for the generation of dI5 neurons.

In contrast to the normal generation of dI4 and dI5 neurons, Isl1-expressing dI3 neurons, which also arise from Gsh2$^+$ progenitors, were all but absent from the E10.5 *Gsh2* mutant spinal cord (Fig. 2C,I). In particular, we observed a >90% reduction in the number of Isl1$^+$ dI3 neurons at both E10.5 and E11.5 (Fig. 2F,L). Examination of Tlx3 expression in the E10.5 mutant spinal cord also revealed a selective reduction in the most dorsal population of Tlx3$^+$ neurons (Fig. 2E, arrow) that are dI3 neurons (Qian et al., 2002). Further evidence of the specific loss of postmitotic dI3 neurons comes from the near absence of the dorsal *Otp* expression domain in the *Gsh2* mutant spinal cord (Fig. 2K, arrow).

In adjacent sections, a concomitant increase in the number of Foxd3$^+$ dI2 neurons (Fig. 2B,H) was observed, demonstrating that putative dI3 neurons differentiate as dI2 neurons in the *Gsh2* mutant cord. Interestingly, the increase in Foxd3$^+$ cell numbers did not completely offset the loss of Isl1$^+$ dI3 neurons (Fig. 2F,L), suggesting that some dI3 neurons may adopt a dI4 cell fate. Further evidence for the respecification of dI3 neurons comes from the observation that the gap that normally separates the Lhx1$^+$/Lhx5$^+$ dI2 neuronal domain from the Lhx1$^+$/Lhx5$^+$ dI4 neuronal domain was no longer present in *Gsh2* mutants (Fig. 2G, see arrow). This expansion in the dorsal Lhx1/Lhx5 expression domain is consistent with the ectopic generation of dI2 neurons and, to a lesser extent, dI4 neurons, from putative dI3 progenitors.

Gsh2 is required for the proper formation of the dI3 progenitor domain

The loss of dI3 neurons in the *Gsh2* mutant spinal cord led us to question whether the patterning of neuronal precursors in the dorsal spinal cord is altered in these mice. In particular, we were interested in ascertaining why dI3 neurons are selectively lost, whereas dI4 and dI5 neurons that also arise from Gsh2$^+$ progenitors persist. Using an antibody that recognizes both

Gsh1 and Gsh2, we observed that Gsh1 continues to be expressed in the dorsal ventricular zone of *Gsh2* mutant mice (Fig. 3B). However, the expression domain of Gsh1 was more restricted than that of Gsh2 (Fig. 3A), encompassing only the dI4 and dI5 progenitor populations. This more restricted pattern of *Gsh1* expression was confirmed by in situ analyses using a probe that was specific for *Gsh1* (Fig. 3C,D). Although we did observe a slight shift in the dorsal boundary of *Gsh1* expression in E10.5 *Gsh2* mutants (Fig. 3D, arrow), this shift encompassed only part of the presumptive dI3 progenitor domain. This slight dorsal shift in *Gsh1* expression may account for the small increase in $Pax2^+$ dI4 neurons in the *Gsh2* mutants.

The observed expansion of dI2 neurons in the *Gsh2* mutant spinal cord (Fig. 2F,L) suggested that the progenitor program that specifies dI2 progenitor identity might expand ventrally as far as the *Gsh1* expression domain. Consistent with this hypothesis, we observed a pronounced ventral expansion of the dorsal *Ngn1* expression domain (Fig. 3E,F, brackets), together with a less marked expansion of the dorsal *Ngn2* expression domain (Fig. 3K,L, brackets). An associated reduction of Mash1 expression in putative dI3 progenitors was also observed (Fig. 3G,H), further suggesting a switch from dI3 to dI2 progenitor identity. No changes, however, were observed in the expression domains of *Msx1* and *Olig3* (data not shown), or in the *Dbx2* expression domain (Fig. 3I,J), suggesting that *Gsh1* alone may maintain the integrity of the dI5/dI6 boundary. In summary, there is an expansion of the $Ngn1^+$ dI2 progenitor domain in the *Gsh2* mutant spinal cord, such that it directly abuts Gsh1/Mash1-expressing dI4 progenitors.

Gsh1 single mutants show no phenotype in the dorsal spinal cord

The relocation of the *Ngn1* boundary to the dI3/dI4 boundary in the *Gsh2* mutant spinal cord, coupled with the lack of any change in the dI5/dI6 border, indicated that *Gsh1* and *Gsh2* could have overlapping and partially redundant functions in the dorsal neural tube. To test this, we assessed the differentiation of dorsal interneuron cell types in *Gsh1* and *Gsh1/Gsh2* mutants. In *Gsh1* single mutants, we observed a normal compliment of dI2, dI3, dI4 and dI5 neurons, as evidenced by the unchanged expression of Isl1, Pax2, Lhx1/Lhx5 and Lmx1b (Fig. 4A-D). Not surprisingly, Gsh2 expression was maintained in the *Gsh1* mutant spinal cord, which probably accounts for the lack of change in Mash1 expression (Fig. 4E,F). Our findings support the idea that *Gsh1* and *Gsh2* are regulated independently of each other in dorsal progenitors, and that *Gsh2* function alone is sufficient for the correct specification of dI3, dI4 and dI5 progenitors.

Gsh1/2 double mutants show limited changes in dorsal progenitor patterning

At E10.5, *Msx1* and *Olig3* expression extend to the dI3/dI4 boundary, and expression of both genes is unaltered in the *Gsh2* mutant spinal cord (data not shown). We therefore questioned whether *Gsh1* and *Gsh2* together might refine the expression of the Class A progenitor

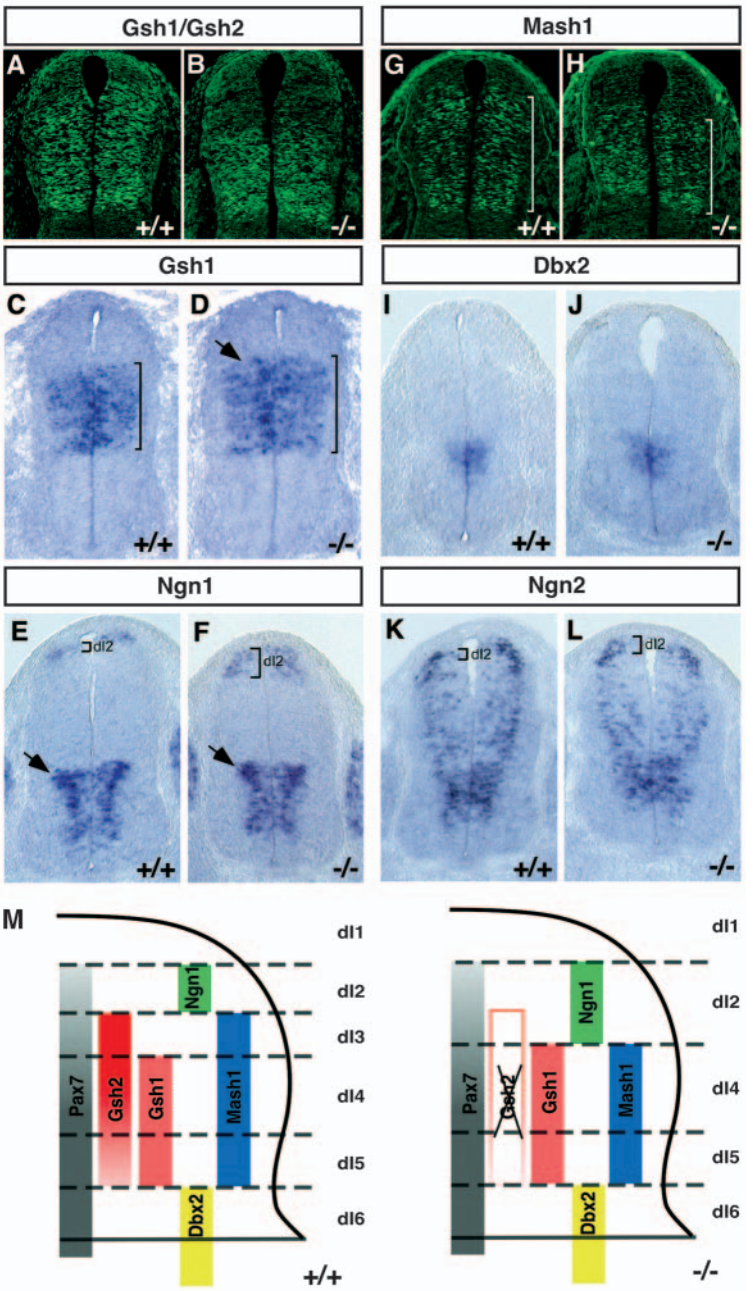

Fig. 3. *Gsh2* is required to establish the boundary between the dI2 and dI3 progenitor domains. (A-D) *Gsh1* expression in dI4 and dI5 progenitors (brackets) is largely unchanged in $Gsh2^{-/-}$ embryos, although the *Gsh1* domain shows a slight dorsal expansion when compared with wild-type (WT) embryos (arrow in D). (E,F) *Ngn1* expression expands ventrally into the presumptive dI3 progenitor domain, as does *Ngn2* expression (K,L), although to a lesser extent than *Ngn1*. (G,H) Mash1 expression is reduced in presumptive dI3 progenitors but is maintained in the adjacent dI4 and dI5 progenitor domains (brackets). (I,J) The expression of *Dbx2* in dI6 progenitors is unchanged demonstrating that the boundary between the dI5 and dI6 progenitors is not altered in the *Gsh2* mutant cord. The ventral boundary of *Ngn1* at the dI5/dI6 border is also unchanged (E,F, see arrows). (M) Schematic summary of the changes in dorsal progenitor specification in the alar plate, showing a concomitant reduction of Mash1 expression and expansion of *Ngn1* expression in the dI3 progenitor domain.

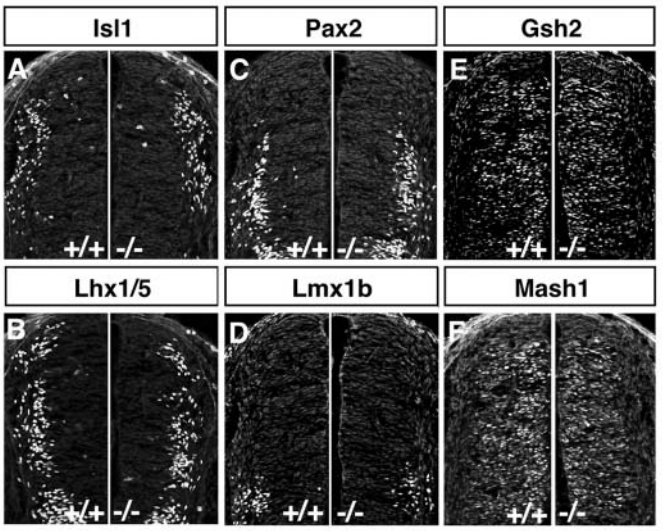

Fig. 4. *Gsh1* mutant embryos do not present any obvious defects in early dorsal interneuron development. (A-D) Expression of postmitotic marker proteins for the six different interneuron populations in the dorsal half of the spinal cord is unchanged in E11.5 *Gsh1* mutant embryos. (E,F) Gsh2 and Mash1 also show normal expression patterns, confirming the normal development of dI3-dI5 progenitor populations.

genes *Msx1* and *Olig3*, as well as *Dbx2*, which is expressed in dI6 progenitors. We reasoned that *Gsh1* might normally prevent *Msx1* and *Olig3* from being expressed in dI4 and dI5 progenitors, and, as a consequence, that *Msx1* and *Olig3*-expressing Class A progenitors would expand into the dI4/dI5 progenitor territory of the *Gsh1/Gsh2* mutant spinal cord. Although, we occasionally detected low-level expression of *Msx1* in the dI4 and dI5 progenitor domains, there was no major change in *Msx1* expression in *Gsh1/Gsh2* mutants (Fig. 5A,B). *Olig3* expression was also largely unaltered in E10.5 double mutants (Fig. 5C,D). These findings suggest that strong cross-repressive interactions between *Gsh1/Gsh2* and the Class A genes *Msx1/Olig3* are unlikely to position the border between Class A TGFβ-dependent progenitors and Class B TGFβ-independent progenitors. Furthermore, the observation that *Dbx2* expression is unchanged in the *Gsh1/Gsh2* double mutant spinal cord (Fig. 5E,F) demonstrates that the positioning of the dI5/dI6 boundary is not mediated by *Gsh1/Gsh2* repression of *Dbx2*. Nevertheless, in comparison to *Gsh2* single mutants, *Ngn1* expression expands more ventrally to encompasses dI4 and dI5 progenitors in addition to dI3 progenitors (Fig. 5G,H).

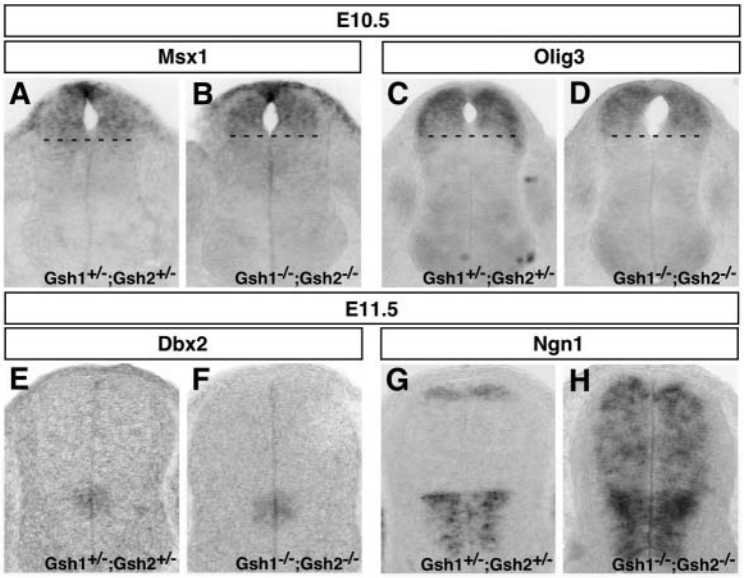

Fig. 5. Expression of *Msx1*, *Olig3*, *Dbx2* and *Ngn1* in the *Gsh1/Gsh2* mutant spinal cord. (A-D) *Msx1* and *Olig3* expression in Class A progenitors is unchanged in E10.5 *Gsh1/Gsh2* double mutants. The dashed line indicates the boundary between dI3 and dI4 progenitors. (E,F) Expression of *Dbx2* in dI6 progenitors is unchanged in the double mutant spinal cord, indicating the Gsh proteins do not repress *Dbx2*. (G,H) *Ngn1* expression expands into the dI3-dI5 progenitor domain in the *Gsh1/Gsh2* mutant cord.

Mash1 functions as a proneural determinant of dI3 identity

The changes in *Mash1* and *Ngn1* expression in the *Gsh2* mutant spinal cord led us to investigate whether the regulation of *Mash1* might be a nodal point for integrating the patterning signals that activate the dI3 differentiation program. In the *Mash1* mutant spinal cord, there is a marked reduction in the number of Isl1^{+} dI3 neurons (Fig. 6A,C). This reduction in dI3 neurons is accompanied by a concomitant increase in Foxd3^{+} dI2 neurons, together with an expansion of the dorsal Lhx1/Lhx5 expression domain (Fig. 6G,H). At E10.5, we also observed an expansion in *Ngn1* expression, similar to that observed in the *Gsh2* mutant spinal cord (Fig. 6E,F). However, at later developmental times, *Ngn1* expression was once again primarily restricted to dI2 progenitors (S.K., unpublished data). Gsh2 expression at E10.5 seems to be reduced dorsally in *Mash1* mutant spinal cords (Fig. 6B, arrowhead). This reduction is not visible at E11.5, probably because Ngn1 is again restricted to dI2 progenitors at this time (Fig. 6D). Taken together, these findings provide evidence that Mash1 promotes the differentiation of dI3 neurons, and suggest that Mash1 participates in the early formation of the dI2/dI3 boundary, either by directly blocking *Ngn1* expression, or by activating Gsh2 expression, which may in turn repress *Ngn1*.

Mash1 and *Gsh2* are sufficient for the induction of dI3 neurons

When *Mash1* was ectopically expressed in the chick neural tube, we observed a dramatic upregulation of Isl1 (Fig. 6I,J, arrow) and Tlx3 (data not shown) expression on the electroporated side, indicating that Mash1 alone is sufficient to activate the dI3 differentiation program. A concomitant reduction in Lhx1/Lhx5 expression in presumptive dI2 and dI4 neurons was also noted (Fig. 6K,L), which is consistent with newborn neurons adopting a dI3 identity. Although we saw no evidence of Gsh2 upregulation 36-48 hours after electroporation, at 20 hours after electroporation there was a clear induction of Gsh2 in *Mash1*-overexpressing cells (Fig. 6M,N), demonstrating that Mash1 induces the transient

expression of Gsh2. Because Mash1 functions as a proneural differentiation factor, the loss of Gsh2 induction 36 hours after electroporating Mash1 may reflect the downregulation of Gsh2 that normally occurs in postmitotic neurons. Nonetheless, the induction of Gsh2 by Mash1 reveals the presence of a positive-feedback mechanism, which may enable Mash1 to either upregulate or maintain Gsh2 expression in dI3 progenitors.

The induction of Gsh2 in dorsal progenitors, including in some cells that are dorsal to the normal dI3 progenitor domain (Fig. 6M,N, arrowhead), suggested that *Mash1* and *Gsh2* might function by repressing the proneural program of dI2 neurons. We reasoned that *Ngn1* might also be repressed by *Mash1*. Consistent with this hypothesis, we observed a marked reduction in *Ngn1* expression following *Mash1* overexpression (Fig. 6O,P). These effects were typically seen at early times after electroporation, when Mash1^{+} cells were still in the ventricular zone. Thus, it appears that *Mash1* can repress *Ngn1* in dI2 progenitors as they begin to differentiate.

It was unclear, however, whether the repression of *Ngn1* was directly due to Mash1 activity or whether it represented an indirect pathway that is mediated by *Gsh2*. To help distinguish between these possibilities, we investigated whether *Gsh2* represses *Ngn1* and promotes the differentiation of dI3 neurons in the chick neural tube. *Gsh2* did strongly repress *Ngn1* and *Ngn2* in the dorsal dI2 progenitor domain (Fig. 7G-I, arrowheads), as well as ventrally. Interestingly, *Gsh2* expression also produced sporadic induction of Isl1 on the electroporated side of the neural tube (Fig. 7A-C). However, the induction of Isl1 by *Gsh2* was qualitatively different than that by *Mash1*, in that it was far less robust (cf. Fig. 6I,J). Whereas *Mash1* induced Isl1 in a cell autonomous fashion (Fig. 6I), in the *Gsh2*-electroporated neural tubes, many of the ectopic Isl1^{+} cells did not express GFP, which marks cells carrying the *Gsh2* expression vector. This finding is consistent with the lack of induction of Mash1 by *Gsh2* (Fig. 7D-F), and it suggests that *Gsh2* is unlikely to be a direct activator of Mash1 expression.

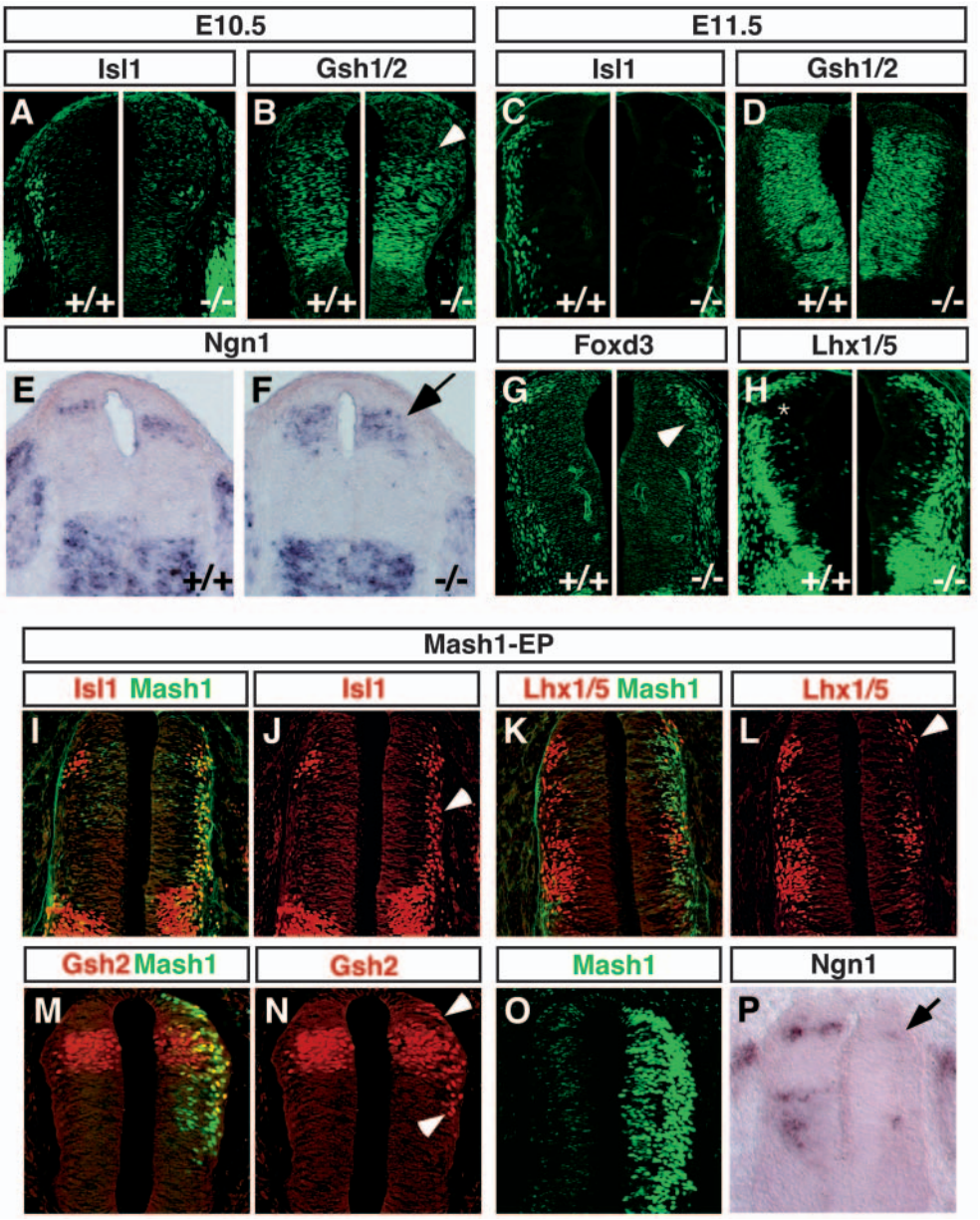

Fig. 6. Reduced generation of dI3 neurons in *Mash1*$^{-/-}$ embryos. (A,C) Immunohistochemical detection of Isl1 protein shows that dI3 neurons are significantly reduced in *Mash1* mutants at E10.5 and E11.5. (B,D) Gsh1/Gsh2 expression is slightly reduced in E10.5 *Mash1* knockouts (B, arrowhead) but is unchanged in E11.5 *Mash1*$^{-/-}$ embryos (D). (G,H) Concomitant with the decrease in Isl1^{+} cells, there is an expansion of Foxd3^{+} (arrowhead) and Lhx1/Lhx5^{+} (asterisk) dI2 neurons into the prospective dI3 population. (E,F) At E10.5, *Ngn1* expression expands ventrally into the prospective dI3 progenitors in the *Mash1* mutant (arrow). (I-P) Electroporation (EP) of *Mash1* in the chick neural tube; the electroporated side is shown on the right, control side on the left. (I,J) Isl1 is strongly induced after *Mash1* overexpression, suggesting an induction of dI3 neurons (arrowhead). (K,L) Moreover, *Mash1* misexpression leads to a reduction of Lhx1/Lhx5, indicating that *Mash1* initiates the dI3 differentiation program at the expense of dI2 and dI4 neurons. (M,N) Ectopic Gsh2^{+} cells are found 24 hours after *Mash1* overexpression (arrowheads in N), suggesting a positive-feedback mechanism onto *Gsh2* to maintain dI3 progenitor identity. (O,P) *Mash1* overexpression represses ventral and dorsal *Ngn1* expression (arrow) 20 hours after electroporation.

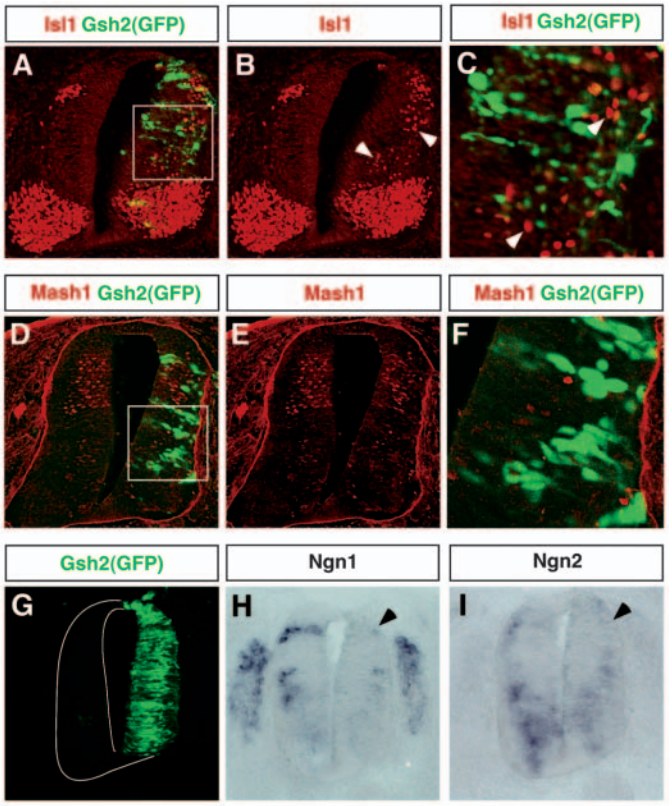

Fig. 7. *Gsh2* can induce Isl1$^+$ dI3 neurons. Framed areas in A and D are shown enlarged in C and F, respectively. (A-C) Misexpression of *Gsh2* leads to an induction of ectopic Isl1$^+$ dI3 neurons that is less dramatic than the induction seen following *Mash1* overexpression. Arrowheads in B indicate ectopic Isl1$^+$ cells. Additionally, this induction occurs in a non-cell autonomous manner (C, arrowheads). (D-F) Isl1 induction by Gsh2 is independent of Mash, as only very few ectopic Mash1$^+$ cells are seen following *Gsh2* overexpression. (G-I) Both *Ngn1* and *Ngn2* are strongly repressed (arrowheads) by *Gsh2* misexpression, and this repression is apparent in both their dorsal and ventral expression domains.

Ngn1 antagonizes dI3 differentiation by repressing dI3 progenitor development

The observed expansion of *Ngn1* expression in the *Gsh2* and *Mash1* mutant spinal cords led us to wonder whether *Ngn1* suppresses dI3 development. In chick embryos electroporated with a *Ngn1* expression vector, a marked reduction in the number of dorsal, but not ventral, Isl1$^+$ cells was noted (Fig. 8A,B, arrowhead). This indicates that *Ngn1* selectively suppresses dI3 interneuron development, but not motoneuron development. This reduction in dI3 neurons was accompanied by a suppression of *Mash1* expression in dorsal progenitors (Fig. 8E,F), suggesting a switch in dI3 progenitor cell fate. Consistent with this hypothesis, we observed the near complete loss of Gsh1/Gsh2 expression on the electroporated side of the neural tube (Fig. 8C,D). This suggests that *Ngn1* overexpression blocks the development of dI3 progenitors. The effects of *Ngn1* overexpression on dI4, V0 and V1 progenitors were less clear, as Pax2 expression was not markedly changed on the *Ngn1*-electroporated side of the neural tube (Fig. 8G,H). Nevertheless, it appears that although *Ngn1* does repress *Mash1* and *Gsh1/Gsh2*, it does not suppress dI4 development, as Pax2 expression is retained in the dI4 domain (Fig. 8H). This finding is also consistent with the continued generation of Pax2$^+$ dI4 neurons in the *Gsh1/Gsh2* and *Mash1* mutant spinal cord (S.K. and M.G., unpublished).

In view of the expansion of *Ngn1* expression in both the *Gsh2* and *Mash1* mutant spinal cord, we asked whether the loss of *Ngn1* leads to an expansion of the dI3 progenitor domain. An expansion of the Gsh1/Gsh2 and Mash1 progenitor domain was noted in the *Ngn1/Ngn2* double mutants; however, this primarily involved the expression of Mash1 and the Gsh proteins in cells located ventral to the dI5/dI6 boundary (Fig. 8I-L). Although the dorsal limit of the dI3 domain appeared to be largely unchanged, we occasionally observed ectopic Mash1-expressing cells dorsal to this boundary (Fig. 8L, arrowheads). The limited dorsal-ward expansion of Mash1 expression is probably due to the expansion of Math1 expression into the dI2 progenitor domain in the *Ngn1/Ngn2* mutant neural tube (Gowan et al., 2001). Math1 has a demonstrated role in repressing Mash1 expression (Nakada et al., 2004). Similarly, the generation domain of dI3 neurons as indicated by Tlx3 expression was largely normal in the *Ngn1/Ngn2* mutant neural tube, although in some instances, isolated Tlx3$^+$ cells appeared to be located dorsal to the normal dI3 generation zone (Fig. 8N). These cells are likely to be the descendents of the ectopic Mash1 cells that are located dorsal to dI3 progenitor domain (Fig. 8L).

Discussion

The role of *Gsh2* in patterning Class A progenitors

This study addresses the important issue of how neural progenitors in the dorsal spinal cord are patterned and specified by focusing on the transcriptional pathways that specify dI3 neurons. The Gsh1 and Gsh2 homeodomain transcription factors are key components of a transcriptional regulatory network that specifies early dorsal cell fates. While Gsh2 alone is essential for the generation of dI3 neurons, Gsh1 and Gsh2 regulate the development of dI5 neurons (S.K., unpublished). Interestingly, our studies find no evidence that cross-repressive interactions between homeodomain transcription factors play a primary role in segregating different classes of dorsal progenitors, raising the possibility that the spatial regulation of proneural bHLH determination genes may be the key step in generating dorsal progenitors with different developmental fates.

Our results provide evidence that early patterning genes, such as *Gsh2*, function by restricting the expression domains of neuronal determination genes, such as *Mash1*, *Math1* and *Ngn1/Ngn2*, to distinct subsets of dorsal progenitors. This finding raises the interesting possibility that the proneural determination genes function as the primary determinants of cell identity in the dorsal neural tube, and that they do so by initiating specific differentiation programs in subsets of progenitors as they emerge from the ventricular zone. Further support for this model comes from previous analyses of *Math1* and *Ngn1/Ngn2* mutant mice (Bermingham et al., 2001; Gowan et al., 2001), and from our analysis of the *Mash1* mutant phenotype (this study). In all three instances, the loss of proneural gene activity results in clear alterations to cell fate. In *Ngn1/Ngn2* mutant embryos, extra dI1 neurons are produced

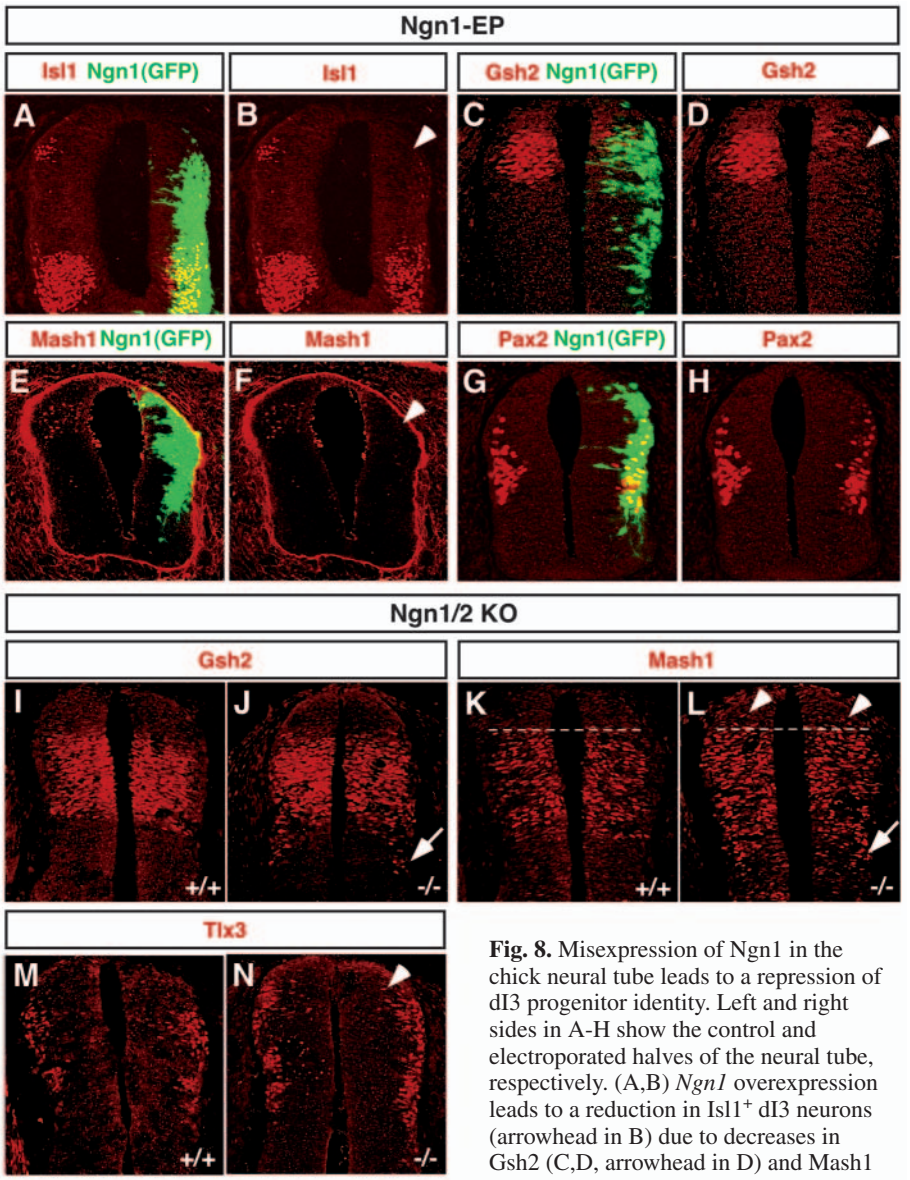

Fig. 8. Misexpression of Ngn1 in the chick neural tube leads to a repression of dI3 progenitor identity. Left and right sides in A-H show the control and electroporated halves of the neural tube, respectively. (A,B) *Ngn1* overexpression leads to a reduction in Isl1^{+} dI3 neurons (arrowhead in B) due to decreases in Gsh2 (C,D, arrowhead in D) and Mash1 (E,F, arrowhead in F) protein expression. (G,H) Pax2^{+} dI4 and dI6 interneurons are unaffected by *Ngn1* overexpression. (I-L) Expression patterns of Gsh2 and Mash1 in *Ngn1/Ngn2* double knockouts. (I,J) Gsh2 expression expands beyond the dI5/dI6 boundary in *Ngn1*$^{-/-}$/*Ngn2*$^{-/-}$ embryos (arrow), whereas the dorsal boundary is unchanged. (K,L) Mash1 is not markedly expanded dorsally in *Ngn1/Ngn2* deficient mice, although a few ectopic Mash1^{+} cells are found dorsal to the dI2/dI3 boundary (arrowheads). Mash1 extends ventrally in the *Ngn1/Ngn2* double-mutant spinal cord, when compared with wild type (arrow). (M,N) Tlx3 is hardly changed in *Ngn1/Ngn2* double knockouts, although a few Tlx3^{+} cells are found dorsal to the dI2/dI3 boundary (arrowhead).

at the expense of dI2 neurons, and the ectopic generation of dI1 neurons is accompanied by a ventral expansion of Math1 (Gowan et al., 2001). Conversely, in *Math1* mutant embryos, there is switch from dI1 to dI2 identity, along with a dorsal expansion of *Ngn1* and *Ngn2* expression. In this study, we show that there are fewer dI3 neurons and an increased number of dI2 neurons in *Mash1* mutant embryos (Fig. 6). This increase in the generation of dI2 neurons is due to the ectopic expression of *Ngn1/Ngn2* in presumptive dI3 progenitors. Thus, alterations to proneural bHLH expression in progenitors cause a switch in cell fate in all three Class A neuronal cell types. Further support for the above model comes from misexpression analyses in the chick, where overexpression of *Math1*, *Ngn1/Ngn2* or *Mash1* redirects the differentiation program of dorsal progenitors (Gowan et al., 2001; Nakada et al., 2004) (this study). Taken together, these findings provide support for a model in which the Math1, Ngn1/Ngn2 and Mash1 bHLH factors function as primary determinants of Class A identity.

The observation that *Ngn1* expression expands into the dI3 progenitor domain in the *Gsh2* mutant spinal cord (Fig. 3), suggests that a primary role of *Gsh2* is to repress expression of 'dI2' determination genes in prospective dI3 progenitors. This repression is, however, independent of *Mash1*, as Mash1 was not induced following *Gsh2* overexpression (Fig. 7). Our findings are also consistent with the reduction of *Mash1* expression in the *Gsh2* mutant spinal cord being due to a ventral expansion of *Ngn1* expression, as *Ngn1* was able to strongly repress *Mash1* transcription when overexpressed in the chick spinal cord (Fig. 8). *Ngn1* also promotes the differentiation of dI2 neurons (Gowan et al., 2001), possibly in combination with *Olig3* (Muller et al., 2005), and thus, the induction/repression of *Ngn1* appears to be a crucial component of a binary genetic switch that specifies dI2 versus dI3 cell fates. The primary function of *Gsh2* may therefore be to repress *Ngn1* (and to a lesser extent *Ngn2*), thereby permitting *Mash1* expression in dI3 progenitors. Interestingly, we found that *Mash1* transiently induces Gsh2 expression in the neural tube in a cell autonomous manner, suggesting that *Mash1* functions in a positive-feedback loop that consolidates *Gsh2* expression in prospective dI3 progenitors.

In the chick and mouse spinal cord, the three early born Class A-type neurons that arise from the dorsal alar plate depend on roof plate-derived signals for their development (Liem et al., 1997; Muller et al., 2002). Genetic ablation of the roof plate or abrogation of BMP-signaling leads to the loss of all three cell types (Lee et al., 2000; Lee and Jessell, 1999; Wine-Lee et al., 2004). The progenitors for Class A neurons express *Msx1*, probably in response to BMP/TGFβ signaling from the dorsal midline. Interestingly, at E10.5, the *Gsh2* expression domain overlaps with *Msx1* in dI3 progenitors. This indicates that *Gsh2*, rather than acting to repress *Msx1*, acts as a modifier of the BMP-dependent Class A progenitor program. Olig3 expression is unchanged in both

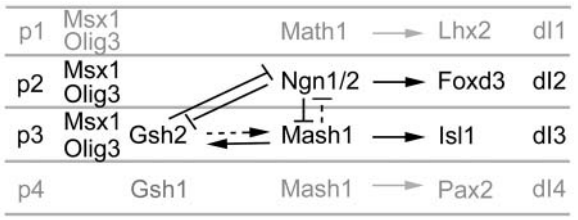

Fig. 9. Schematic of the proposed genetic interactions between *Gsh2*, *Ngn1* and *Mash1*. Arrows symbolize an induction; bars signify a repression. Solid lines indicate probable direct genetic effects, whereas dashed lines indicate likely indirect interactions.

the *Gsh2* and *Gsh1/Gsh2* mutant spinal cord, which is again consistent with Gsh2 acting as a transcriptional modifier to suppress the dI2 program in *Msx1*$^{+}$/*Olig3*$^{+}$ Class A progenitors (Fig. 9).

Gsh2 function in the spinal cord and telencephalon

Gsh2 also has a demonstrated role in the dorsoventral patterning of the telencephalon, where it is required for the proper specification of striatal precursors that are generated from the ganglionic eminence (GE) (Toresson et al., 2000; Yun et al., 2001). The striatum and cerebral cortex each develop from two dorsoventrally distinct telencephalic domains that abut each other. The more ventral progenitors for the lateral ganglionic eminence (LGE) express *Gsh2*, whereas cortical progenitors in the adjacent dorsal domain express *Pax6*. There are, however, differences between the telencephalon and the spinal cord in the regulatory interactions involving *Gsh2* and *Pax6*. Whereas cross-repressive interactions between *Gsh2* and *Pax6* determine the boundary between the cortex and LGE, *Gsh2* and *Pax6* are not expressed in a strictly complementary manner in the spinal cord. Instead, *Pax6* is broadly expressed in the ventricular zone, where it largely overlaps with Gsh2 in dI3-dI5 progenitors (S.K., unpublished). Moreover, in the *Gsh2* mutant spinal cord *Pax6* expression is largely unchanged, indicating that if *Gsh2* represses *Pax6*, it does so rather weakly.

The *Mash1* and *Ngn1/Ngn2* proneural genes show parallel patterns of expression in the developing telencephalon and spinal cord. Differentiating neurons in the *Gsh2*$^{+}$ LGE express *Mash1*, whereas those in the dorsal telencephalon express *Ngn1* and *Ngn2*. Moreover, the loss of *Gsh2* leads to a reduction of *Mash1* expression in both the spinal cord and LGE, and this is accompanied by a ventral expansion of the *Ngn1/Ngn2* expression domain. Similarities in the expression profiles for *Gsh1* and *Gsh2* are also seen between the developing spinal cord and the telencephalon, with *Gsh1* showing a more restricted domain of expression in both structures (Toresson and Campbell, 2001) (this study). In the developing telencephalon, *Gsh2* is highly expressed in both the medial ganglionic eminence (MGE) and LGE, whereas *Gsh1* is present at high levels in the MGE and at diminished levels in the LGE. Consequently, the loss of striatal cell types and the associated expansion of cortical progenitors is more pronounced in the *Gsh1/Gsh2* double mutants (Torreson and Campbell, 2001; Yun et al., 2003). *Gsh1* and *Gsh2* therefore have overlapping and parallel functions in both the telencephalon and the spinal cord where they specify different dorsoventral progenitor domains.

DV patterning in vertebrates and invertebrates

In the embryonic *Drosophila* central nervous system, CNS neurons and glia arise from three dorsoventral columns of progenitors in the neuroectoderm that express the *Msh* (dorsal column), *Ind* (intermediate column) and *Vnd* (ventral column) homeodomain transcription factors. Transcriptional cross-repressive interactions between these three transcription factors play a primary role in establishing the columnar identity of these neural progenitors (McDonald et al., 1998; Weiss et al., 1998; von Ohlen and Doe, 2000). Although the spatial expression of the *Vnd*, *Ind* and *Msh* transcription factors in the *Drosophila* embryonic nervous system mirrors the expression of their vertebrate homologs in the embryonic spinal cord, there appear to be key differences in the mechanisms used to establish these expression domains. Whereas *Msh* and *Ind* transcriptionally repress each other, thereby establishing two non-overlapping domains of *Msh* and *Ind* expression in the neuroectoderm, the expression domains of *Msx1* and *Msx3* in the neural tube clearly overlap with those of *Gsh2* and *Gsh1* at E10.5, respectively (Fig. 5; S.K., unpublished). Moreover, *Msx1* and *Msx3* (data not shown) expression is largely unchanged in the *Gsh2* and in the *Gsh1/Gsh2* mutant spinal cord (Fig. 5, data not shown), suggesting that Gsh1 and Gsh2 do not regulate the transcription of either gene. *Olig3*, which functions as a determinant of dI1-dI3 identity and is expressed in dI1-3 progenitors at E10.5 like *Msx1* (Muller et al., 2005), also exhibits an unchanged expression pattern in the *Gsh1/Gsh2* mutant spinal cord (Fig. 5). Indeed, we have been unable to identify any dorsal determinant, with the exception of the proneural determination genes *Ngn1*, *Ngn2* and *Mash1*, whose expression changes in embryos lacking either *Gsh2*, *Gsh1*, or *Gsh1* and *Gsh2* together.

Parallels have been drawn between the dorsoventral specification of neural progenitors in the *Drosophila* ventral neuroectoderm and in the ventricular zone of the vertebrate spinal cord. While the invertebrate and vertebrate homologs of Vnd/Nkx, Ind/Gsh and Msh/Msx are expressed in a similar array of dorsoventral stripes, an additional fourth progenitor domain that expresses the Dbx class of homeodomain transcription factors is present in vertebrates (Fjose et al., 1994; Pierani et al., 2001). These progenitors occupy an intermediate position between the *Gsh1*$^{+}$/*Gsh2*$^{+}$ domain and ventral progenitors that express *Nkx2.2*, *Nkx2.9* and *Nkx6.1* genes. Thus it appears that the early vertebrate neural tube broadly comprises four DV progenitor territories, which are subsequently subdivided into 11 distinct progenitor domains. Although a Dbx gene homolog is present in *Drosophila* (J. Skeath and H. Broihier, personal communication), its expression in the developing ventral cord appears to be restricted to distinct subsets of neuroblasts and postmitotic neurons. In the vertebrate neural tube, *Dbx2* functions as a Class 1 gene and its ventral border of expression is regulated by Nkx6.1-dependent repressor activity (Vallstedt et al., 2001). *Gsh1/Gsh2* and *Dbx2* form a boundary between dI5 and dI6 progenitors. However, this boundary of expression remains unaltered in the *Gsh1/Gsh2* double mutants (Fig. 5), indicating that there is no cross-repression between *Gsh1/Gsh2* and *Dbx2* that plays a role in establishing the dI5/dI6 progenitor border. Thus, although some of the DV patterning activities of these homeodomain transcription factors have been conserved in invertebrates and vertebrates, their expression patterns have

diverged, as have the regulatory interactions that determine their expression in CNS progenitors.

Conclusions

In this study, we provide evidence that the Gsh class of homeodomain transcription factors are key components of the genetic program that specifies dI3 interneuron identity in the dorsal spinal cord (Fig. 9). Our findings also raise the intriguing possibility that the genetic interactions governing Class A neuron cell fate differ from those previously described in the ventral neuroectoderm of *Drosophila* and the ventral neural tube. A number of outstanding issues remain. Do the Gsh proteins function as transcriptional repressors and, if so, what are their transcriptional targets? Do the genetic interactions between *Gsh2* and *Ngn1* represent direct interactions or is there an intermediate factor that mediates repression of Ngn1 by Gsh2? What is the developmental status of dI4 neuronal progenitors, as dI4 neurons are still generated in *Gsh1/Gsh2* mutants (data not shown)? It has been noted that dI4 neurons also develop in the absence of dorsal Wnt/BMP signaling (Muller et al., 2002) and Shh signaling (M.G., unpublished), suggesting that dI4 progenitors may represent a developmental ground state for the caudal neural tube. Consistent with this hypothesis, we have observed an expansion of the dI4 progenitor domain in older embryos that parallels a reduction in TGFβ signals in the dorsal neural tube (Gross et al., 2002). Finally, Gsh1/Gsh2 and Mash1 are expressed in late born dorsal progenitors, and it would therefore be interesting to know whether genetic interactions involving *Gsh1/Gsh2* and *Mash1* regulate the development of late-born neurons that populate the substantia gelatinosa.

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