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

Sonja Kriks1, Guillermo M. Lanuza1,*, Rumiko Mizuguchi1,*, Masato Nakafuku2 and Martyn Goulding1,†

1Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
2Department of Cell and Developmental Biology, Children’s Medical Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
*These authors contributed equally to this work
†Author for correspondence (e-mail: goulding@salk.edu)

Accepted 25 April 2005
Development 132, 2991-3002
Published by The Company of Biologists 2005
doi:10.1242/dev.01878

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 Dlx2 (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 dl3 identity, and that Gsh1 activates the Mash1-dependent differentiation of dl3 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 dl3 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.

Mash1 WT, GCACCGCAAAGGCTCAAGGTGCTCTT and ATAC-CATGTGAGACTTCTCTGCTAGG; Mash1 KO, AGCGTGAGCTTACAGGTAACCCAC and TCCAGTCTCCATATGACAAACGGT; Gsh2 WT, CAAGGTTGTCGAATGAGTGAGGG and CTTCAGCGC-GACGTTCGAAAC; Neo, CAAGATGGATGCACCGCAG and CGATTTTCG-CTTGGTGTC; and Mash1 WT, CTCCGGGAGCATGTCGCCCAA and CCAGGACTCAATACCGAGG.

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-Isil1/2 (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, ImmunologicaDirect), 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 pIRE-EGFP expression vector (Invitrogen, modified by M. Dottori) that contains the chick beta-actin promoter 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
Spinal interneuron specification by Gsh2 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 Ngln1-expressing progenitors dorsally, and by Ngln1+/Dbx2+ progenitors ventrally (Fig. 1I,J,M and Fig. 3E,I). The dorsal population of Ngln1+ cells differentiates as dI2 neurons (Gowan et al., 2001), whereas the Ngln1+/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). Ngln1 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 Ngln1 (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.

**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 Ngln1 is expressed in adjacent domains dorsal and ventral to Gsh2+ and Mash1+ progenitors. (K) Gsh2 is homogeneously expressed in all Pax3+ dI3 progenitors. (L) Ngln1 marks 50% of Pax3+ cells in the dI2 ventricular domain. (M) Ngln1 and Gsh2 are expressed in adjacent domains in the dorsal neural tube; this image is an overlay of staining for Gsh1/Gsh2 and Ngln1 of the adjacent sections shown in K and L. (N) Ventricular dividing cells, marked by BrdU, express Gsh2 in dI3 progenitors. (O) Ngln1 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.

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
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 dl4 and dl5 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 dl3 progenitor domain. This slight dorsal shift in Gsh1 expression may account for the small increase in Pax2+ dl4 neurons in the Gsh2 mutants.

The observed expansion of dl2 neurons in the Gsh2 mutant spinal cord (Fig. 2F,L) suggested that the progenitor program that specifies dl2 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 dl3 progenitors was also observed (Fig. 3G,H), further suggesting a switch from dl3 to dl2 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. 3J), suggesting that Gsh1 alone may maintain the integrity of the dl5/dl6 boundary. In summary, there is an expansion of the Ngn1+ dl2 progenitor domain in the Gsh2 mutant spinal cord, such that it directly abuts Gsh1/Mash1-expressing dl4 progenitors.

Gsh1 single mutants show no phenotype in the dorsal spinal cord

The relocation of the Ngn1 boundary to the dl3/dl4 boundary in the Gsh2 mutant spinal cord, coupled with the lack of any change in the dl5/dl6 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 dl2, dl3, dl4 and dl5 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 dl3, dl4 and dl5 progenitors.

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

At E10.5, Msx1 and Olig3 expression extend to the dl3/dl4 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...
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).

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
Spinal interneuron specification by 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, Gsh1 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). 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.
Mash1 and Gsh1/Gsh2, it does not suppress dl4 development, as Pax2 expression is retained in the dl4 domain (Fig. 8H). This finding is also consistent with the continued generation of Pax2+ dl4 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 dl3 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 dl5/dl6 boundary (Fig. 8I-L). Although the dorsal limit of the dl3 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 dl2 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 dl3 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 dl3 generation zone (Fig. 8N). These cells are likely to be the descendants of the ectopic Mash1 cells that are located dorsal to dl3 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 dl3 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 dl3 neurons, Gsh1 and Gsh2 regulate the development of dl5 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 dl1 neurons are produced.
Spinal interneuron specification by Gsh2

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 modulator 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.

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 dll3-dl5 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 (Toresson 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 dl1-dl3 identity and is expressed in dl1-3 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 dl5 and dl6 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 dl5/dl6 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 dl3 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 dl4 neuronal progenitors, as dl4 neurons are still generated in Gsh1/Gsh2 mutants (data not shown)? It has been noted that dl4 neurons also develop in the absence of dorsal Wnt/BMP signaling (Muller et al., 2002) and Shh signaling (M.G., unpublished), suggesting that dl4 progenitors may represent a developmental ground state for the caudal neural tube. Consistent with this hypothesis, we have observed an expansion of the dl4 progenitor domain in older embryos that parallels a reduction in TGFβ3 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.

The authors are very grateful to Steve Potter and Kenny Campbell for providing the Gsh1 and Gsh2 heterozygous mice used in this study. The Mash1 heterozygous mice were very kindly provided by Francois Guillemot. We would also like to thank Leping Cheng and Quifu Ma for the Ngn1/Ngn2 mutant embryos. An antibody to Gsh2 was a kind gift from Kenny Campbell. Antibodies to Lmx1b, Tlx3 and Ngn1 were kindly provided by Tom Jessell, Thomas Muller and Jane Johnson. We are also grateful to Mirella Dottori for helping with the initiation of this project. We thank Jane Johnson and Thomas Muller for graciously sharing data prior to publication. This research was supported by grants from the National Institutes of Health to M.G. G.M.L. was supported by a fellowship from the HFSP.

**References**


