Pax6 Controls Progenitor Cell Identity and Neuronal Fate in Response to Graded Shh Signaling

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Summary

Distinct classes of motor neurons and ventral interneurons are generated by the graded signaling activity of Sonic hedgehog (Shh). Shh controls neuronal fate by establishing different progenitor cell populations in the ventral neural tube that are defined by the expression of Pax6 and Nkx2.2. Pax6 establishes distinct ventral progenitor cell populations and controls the identity of motor neurons and ventral interneurons, mediating graded Shh signaling in the ventral spinal cord and hindbrain.

Introduction

The control of motor behavior in vertebrates depends on the coordinated activity of motor neurons (MNs) and interneurons located within the spinal cord and brain stem. Motor neurons differentiate from progenitor cells in the ventral half of the neural tube in response to the inductive actions of Sonic hedgehog (Shh), a protein secreted by the notochord (Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995; Chiang et al., 1996; Ericson et al., 1996). The first step in this Shh-dependent program appears to be the repression of expression of Pax3 and Pax7, forming a generic population of ventral progenitors (Goulding et al., 1993; Liem et al., 1995; Ericson et al., 1996; Tremblay et al., 1996). The eventual generation of MNs from this early progenitor cell population requires a second phase of Shh signaling (Ericson et al., 1996). How the further diversification of MN subtypes and the generation of ventral interneurons from early ventral progenitor cells are controlled remains unclear.

To determine how ventral interneuron fates are established we identified markers that define two classes of interneurons, termed V1 and V2, which are generated at different positions in the ventral neural tube, dorsal to MNs. Shh signaling is necessary for the generation of both classes of ventral interneurons. Moreover, different concentrations of Shh are required for the induction of V1 and V2 interneurons and for MNs, with the requisite concentration related to the dorsoventral position at which each neuronal class is generated. Thus, graded Shh signaling within the ventral neural tube appears to define the identity and position of generation of distinct ventral neuronal subtypes.

These results pose the problem of how graded Shh signals are interpreted by ventral progenitor cells and lead to the generation of distinct neuronal subtypes. As one approach to this problem, we have focused on the regulation and function of two homeobox genes, Pax6 and Nkx2.2, that are expressed by undifferentiated cells in the ventral region of the neural tube (Walther and Gruss, 1991; Ekker et al., 1995; MacDonald et al., 1995). At forebrain levels, the expression of Pax6 and Nkx2.2 is altered by misexpression of Shh (Barth and Wilson, 1995; Ekker et al., 1995; MacDonald et al., 1995), and in the spinal cord, the expression of Pax6 is regulated by notochord-derived signals (Goulding et al., 1993). Moreover, mutations in the Pax6 gene underlie the pronounced defects in eye and forebrain development in Small Eye (Sey) mutant mice (Hill et al., 1991; Stoykova et al., 1996; Warren and Price, 1997) These findings raise the possibility that Pax6 and Nkx2.2 are involved in the Shh-mediated control of neuronal identity and pattern at caudal levels of the CNS.

To test this possibility, we analyzed the relationship between Shh signaling, Pax6 and Nkx2.2 expression, and neuronal fate in the spinal cord and hindbrain. We show that Pax6 expression in the ventral neural tube exhibits a ventral^{low}–dorsal^{high} gradient and that Nkx2.2 expression is restricted to progenitors that do not express Pax6. In vitro assays reveal that these two progenitor cell populations are established by graded Shh signaling. In addition, an analysis of ventral patterning in the spinal cord and hindbrain of Sey mutant mice shows that Pax6 regulates the expression of Nkx2.2 and thus the establishment of distinct ventral progenitor cell populations. Pax6 expression controls the identity of MNs and ventral interneurons. These findings identify Pax6 as an essential intermediary in graded Shh signaling in the caudal neural tube, acting to control ventral progenitor cell identity and the fate of MNs and ventral interneurons.

Results

Shh Induces Different Ventral Neuronal Classes at Distinct Concentration Thresholds

To examine how the identity and pattern of neuronal subtypes in the ventral spinal cord is generated, we focused on three classes of ventral neurons. One class of interneurons, V1, is defined by coexpression of En1, Lim1/2, and Pax2 (Figure 1a) and is generated in the dorsal-most region of the ventral spinal cord (Figures 1b, 1c, and 1f–1h). A second class of interneurons, V2, is defined by coexpression of Chx10, Lim3, and Gsh4
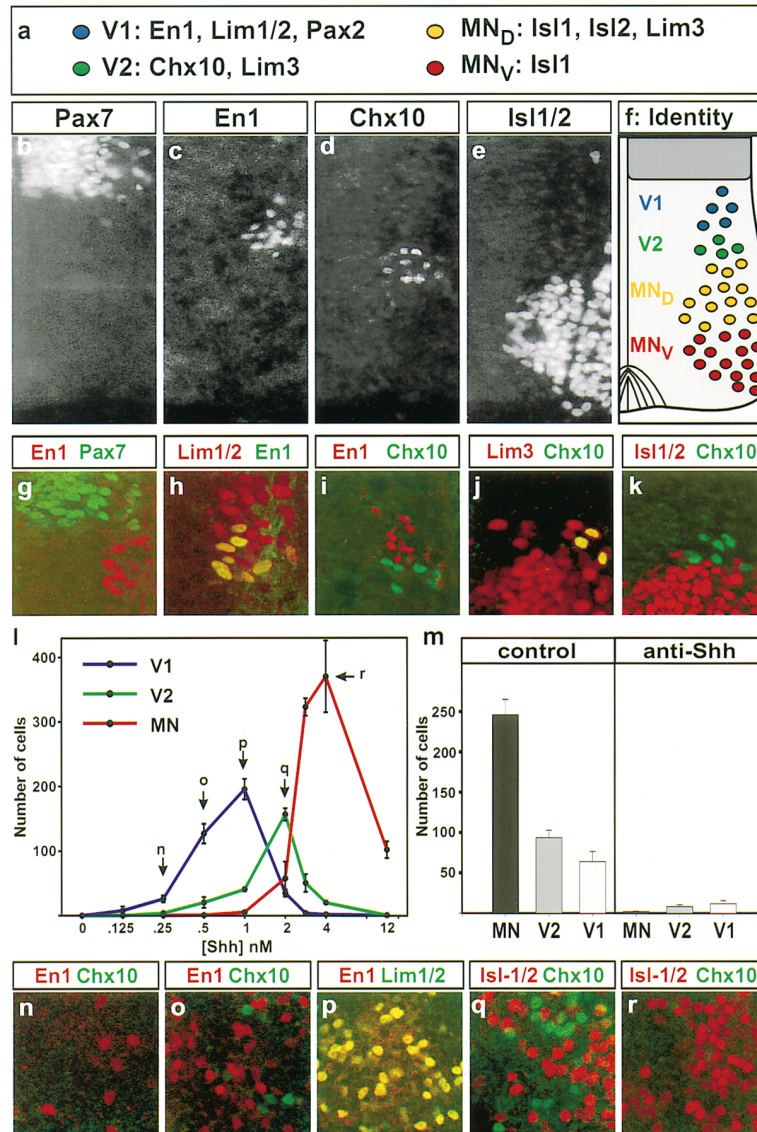
Figure 1. Induction of Distinct Classes of Ventral Neurons at Different Shh-N Concentrations

(a) Neuronal subtype identity defined by homeodomain protein expression.

(b–f) Localization of neuronal subtypes in the ventral cervical spinal cord of stage 22 chick embryos. (b) Pax7 expression in dorsal progenitor cells. (c) En1 expression defines V1 neurons generated ventral to Pax7 cells. (d) Chx10 expression defines V2 neurons. (e), Isl1/2 expression defines MNs. (f) Summary of position of neuronal generation in the ventral spinal cord.

(g–k) Characterization of V1 and V2 neurons. (g) En1 cells are generated ventral to Pax7 cells. (h) En1 cells express Lim1/2. Dorsal Lim1/2 cells that do not express En1 derive from Pax7 progenitors and are D3 dorsal interneurons. (i) En1 and Chx10 cells represent distinct neuronal populations. (j) Chx10 cells coexpress Lim3. These cells also express Gsh4 (not shown). (k) Chx10 cells are distinct from Isl1/2 MNs.

(l) Quantitative analysis of the induction of En1 (V1), Chx10 (V2), and Isl1/2 (MN) neurons in stage 10 [i] explants at Shh-N concentrations indicated in (l). [i] explants contain ∼1000 cells.

(m) Requirement for Shh signaling in ventral neuronal differentiation. Cells in stage 10 ventral neural tube/floor plate [vf] explants grown in the absence of anti-Shh IgG (control) generate many MNs, V1, and V2 interneurons. In the presence of anti-Shh IgG, progenitor cells generate few, if any, MNs, V1, or V2 interneurons. Pax7 expression is reinitiated in the presence of anti-Shh IgG (not shown).

Each point in (l) and (m) represents the mean ± SE for at least 6 explants from two or more separate experiments.

(n–r) Representative examples of En1, Chx10, Lim1/2, and Isl1/2 expression in cells in stage 10 [i] explants exposed to different concentrations of Shh-N, as indicated in (l).

(Figure 1a) and is generated in the intermediate region of the ventral spinal cord, ventral to V1 interneurons (Figures 1d, 1f, and 1i–1k). The third class, MNs, is defined by expression of Isl1 (Figure 1a) and is generated ventral to V2 interneurons (Figures 1e, 1f, and 1k).

We first addressed whether the differentiation of V1 and V2 neurons can, like MNs, be induced by Shh and if so, at what concentrations. To test this, stage 10 chick intermediate neural plate ([i]) explants were grown in vitro for 44 hr, alone or in the presence of recombinant Shh-N. Neither V1 nor V2 neurons, nor MNs were generated when [i] explants were grown in the absence of Shh-N (Figure 1l). The generation of all three neuronal classes was induced by Shh-N (Figures 1l and 1n–1r), but at different concentration thresholds. The generation of V1 neurons required the lowest concentration, V2 neurons an ∼2-fold higher concentration and MNs a further ∼2-fold elevation in concentration (Figure 1l).

These results suggest that the identity and position of generation of neuronal subtypes in the spinal cord is achieved by the exposure of ventral progenitor cells to different Shh-N concentrations.

We next examined whether the generation of V1 and V2 neurons, as for MNs, requires Shh signaling. To test this, ventral neuronal differentiation was assayed in stage 10 ventral neural tube/floor plate [vf] explants that contain an endogenous source of Shh (Ericson et al., 1996). Explants were grown for 44 hr alone, or in the presence of anti-Shh IgG. In the absence of anti-Shh IgG, [vf] explants generated ∼250 MNs, ∼100 V2 neurons, and ∼60 V1 neurons (Figure 1m). In the presence of anti-Shh IgG, [vf] explants generated <3 MNs and ∼10 V1 and ∼10 V2 neurons (Figure 1m). In [vf] explants grown in the presence of anti-Shh IgG, cells reinitiated expression of Pax7, a marker of dorsal progenitor cells (data not shown; see Ericson et al., 1996). Consistent
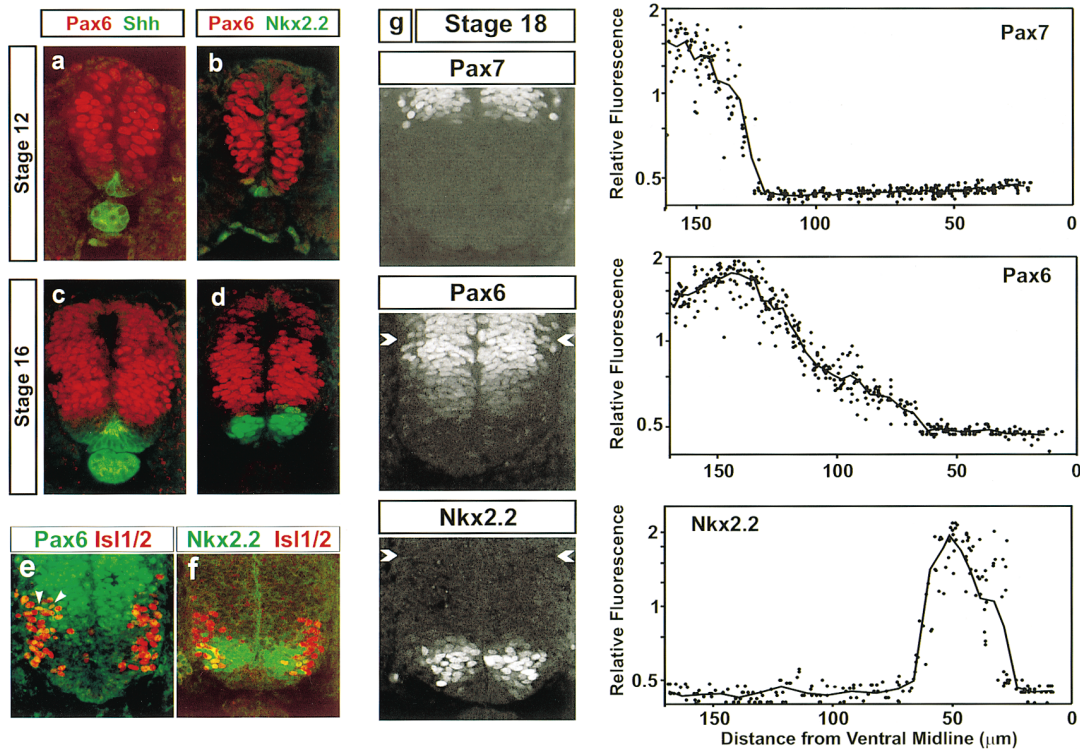
Figure 2. Pax7, Pax6, Nkx2.2, and Shh Expression in the Ventral Neural Tube

(a–d) Expression of Shh, Pax6, and Nkx2.2 in chick cervical (12 somite level) neural tube (stage 12) and spinal cord (stage 16).

(e) Dorsally located MNs derive from Pax6 progenitors. This is demonstrated by the coexpression of Pax6 and Isl1/2 in medially positioned MNs (arrowheads) in stage 18 cervical spinal cord. Pax6 expression is extinguished soon after the differentiation of postmitotic MNs.

(f) Ventrally located MNs derive from Nkx2.2 progenitors, as assessed by the coexpression of Nkx2.2 and Isl1/2 in medially positioned MNs (orange cells) in stage 18 cervical spinal cord.

(g) Comparison of the domains of Pax7, Pax6, and Nkx2.2 in the ventral spinal cord (stage 18). Arrowheads indicate ventral boundary of Pax7. Right-hand plots show quantitative analysis of levels of Pax7, Pax6, and Nkx2.2 in the nuclei of individual cells in stage 18 ventral spinal cord, plotted as a function of cell distance from the ventral midline. Each point represents a single nucleus.

with this, ~200 Lim1/2⁺ D3 dorsal interneurons were generated in such explants, whereas few, if any, D3 interneurons were generated in the absence of anti-Shh IgG (data not shown). Thus, the generation of V1 and V2 neurons as well as MNs requires Shh signaling.

Graded Shh Signaling Generates Distinct Progenitor Cell Populations in the Ventral Neural Tube
We next examined whether graded Shh signaling generates different classes of ventral neurons in the spinal cord through the establishment of distinct ventral progenitor cell populations. This analysis has focused on two homeobox genes, *Pax6* and *Nkx2.2*. Both genes are expressed in dividing progenitor cells within the ventral neural tube, and their expression is rapidly extinguished from most postmitotic neurons (Walther and Gruss, 1991; Price et al., 1992; Shimamura et al., 1995).

From stages 10 to 12, Pax6 was expressed by cells at all dorsoventral positions of the neural tube, with the exception of the ventral midline (Figures 2a and 2b, and data not shown). Nkx2.2 was also detected at low levels but was restricted to ventral midline cells (Figure 2b).

From stages 12 to 16, the level of Pax6 in cells adjacent to the floor plate decreased below the limit of detection, and Nkx2.2 expression was initiated within these cells (Figures 2c and 2d). To define the spatial profile of Pax6 and Nkx2.2 expression in more detail, we quantified the level of both proteins in the nuclei of individual progenitor cells at stage 18, the peak of neurogenesis. Within the ventral neural tube, the domain defined by the absence of Pax7 expression, there was an extended ventral[low]–dorsal[high] gradient of Pax6 (Figure 2g). In contrast, the boundaries of Nkx2.2 and Pax7 expression were more abrupt (Figure 2g).

We next determined the relationship between Pax6 and Nkx2.2 cells and their ventral neuronal progeny at cervical levels of the spinal cord. Both V1 and V2 neurons derive from Pax6 progenitor cells, as assessed by their position and by the detection of Pax6 in medial, and thus newly generated, neurons (data not shown). In contrast, MNs appear to have a dual origin. Dorsally positioned MNs derive from Pax6 progenitors (Figure 2e), whereas ventrally positioned MNs derive from Nkx2.2 progenitor cells (Figure 2f). These two populations of MNs could be distinguished by their expression of LIM homeodomain
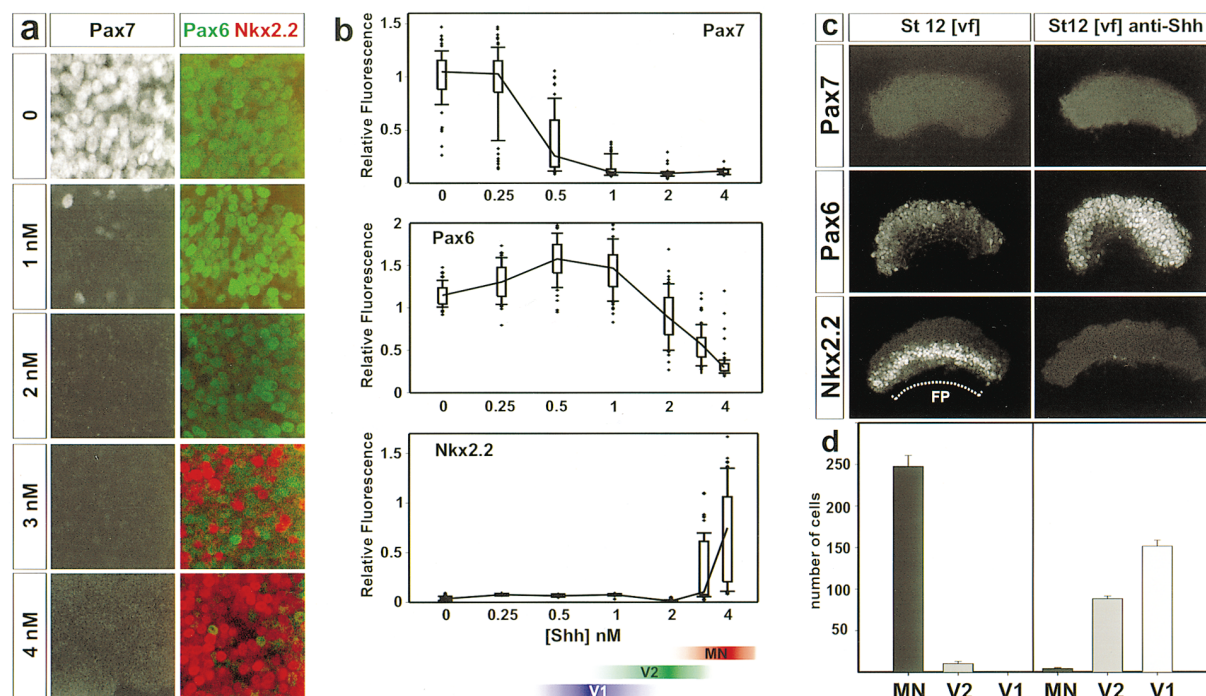
Figure 3. Regulation of Pax7, Pax6, and Nkx2.2 Expression and Neural Fate by Shh In Vitro

(a) Stage 10 [i] explants were grown for 22 hr in different concentrations of Shh-N, and the expression of Pax7, Pax6, and Nkx2.2 was determined. In explants exposed to 3 nM Shh-N, 32 + 3% of cells expressed Nkx2.2, and at 4 nM Shh-N, 70 + 5% of cells expressed Nkx2.2 (n = 6 explants).

(b) Quantitative analysis of the level of Pax7, Pax6, and Nkx2.2 in the nuclei of individual cells in [i] explants exposed to different Shh concentrations. Box indicates 50th and bar indicates 95th percentile values. Results are representative of three experiments. Colored bars beneath plots indicate approximate concentration range at which V1 and V2 neurons and MNs are generated (see Figure 1l).

(c and d) Cells in stage 12 [vf] explants grown in the absence of anti-Shh IgG do not express Pax7, express low levels of Pax6 in cells distant from the floor plate (FP), and express Nkx2.2 in cells close to the floor plate (c). At 38 hr, these explants generate many MNs, few V2 neurons, and no V1 neurons (d). In the presence of anti-Shh IgG, cells do not reinitiate Pax7 expression, express high levels of Pax6, and do not express Nkx2.2 (c). Cells in these explants generate few MNs but many V2 and V1 interneurons (d). Histograms in (d) represent mean ± SE for 5–6 explants from at least three experiments.

proteins. Dorsal MNs coexpressed Isl1, Isl2, and Lim3, whereas ventral MNs expressed Isl1 but not Isl2 or Lim3 (data not shown). The functional relevance of the distinct progenitor cell origins of dorsally and ventrally generated MNs is addressed below.

To examine whether the Pax6 gradient and the complementarity in Pax6 and Nkx2.2 expression is established by Shh signaling, we assayed [i] explants grown for 22 hr, alone or with Shh-N. In the absence of Shh-N, over 95% of cells coexpressed Pax7 and Pax6, and no cells expressed Nkx2.2 (Figures 3a and 3b). Exposure of [i] explants to concentrations of Shh-N that generated predominantly V1 neurons resulted in the repression of Pax7, whereas the level of Pax6 was elevated (Figures 3a and 3b). At a Shh-N concentration optimal for the generation of V2 neurons, Pax6 expression was maintained by all cells, but at a lower level, and Nkx2.2 cells were not detected (Figures 3a and 3b). At 1.5- to 2-fold higher Shh-N concentrations that generated predominately MNs, Pax6 expression was further decreased, and Nkx2.2 was now expressed at a high level by many cells, although, as in vivo, cells did not coexpress Pax6

and Nkx2.2 (Figures 3a and 3b). Thus, graded Shh signaling is sufficient to establish graded Pax6 expression and distinct Pax6 and Nkx2.2 cell populations.

We next examined if Shh signaling is required for the generation of different ventral progenitor cell populations and whether Pax6 and Nkx2.2 expression is predictive of neuronal identity. To test this, we blocked Shh signaling in [vf] explants at stage 12, by which time Pax7 expression has been stably repressed (Figure 3c; Ericson et al., 1996). In [vf] explants grown in the absence of anti-Shh IgG, Pax6 was expressed at a low level by cells located at a distance from the floor plate, and Nkx2.2 at a high level by cells adjacent to the floor plate (Figure 3c). These explants generated ∼250 MNs, ∼10 V2 neurons, and 0 V1 neurons (Figure 3d). In contrast, in [vf] explants grown in the presence of anti-Shh IgG, Pax6 was expressed at high levels by over 95% of progenitor cells, and no cells expressed Nkx2.2 (Figure 3c). These explants generated <5 MNs, ∼90 V2 neurons, and ∼150 V1 neurons (Figure 3d). Thus, blockade of Shh signaling shortly before the onset of neurogenesis results in the maintenance of a high level of Pax6 and

the absence of Nkx2.2 in ventral progenitors and in the generation of V1 and V2 neurons rather than MNs. Shh signaling is therefore required for the establishment of the distinct Pax6 and Nkx2.2 ventral progenitor cell populations, and the state of Pax6 and Nkx2.2 expression by ventral progenitors is predictive of the fate of their neuronal progeny.

Control of Ventral Progenitor Cell Identity by Pax6

To address the role of *Pax6* in the control of ventral cell fates at caudal levels of the neuraxis, we analyzed the *Sey* mouse that carries a point mutation in the *Pax6* gene that is predicted to generate a truncated protein that retains only the paired domain (Hill et al., 1991).

We first examined the role of Pax6 in establishing progenitor cell domains at spinal cord and hindbrain levels. In wild-type embryos examined at e10–e12, the expression of Pax6 in the ventral neural tube was graded in a manner similar to that observed in chick, and the complementarity in expression of Pax6 and Nkx2.2 was also evident (Figure 4a). In the neural tube of *Sey/Sey* embryos, no expression of Pax6 was detected using antibodies directed against the paired domain (Figure 4b), providing evidence that the *Sey* mutation eliminates Pax6 activity. At all levels of the spinal cord and hindbrain of *Sey/Sey* embryos, there was a marked increase in the number (∼3-fold) of Nkx2.2 cells and a dorsal expansion in the Nkx2.2 domain (Figure 4b). The expanded Nkx2.2 domain never occupied the entire ventral neural tube, although scattered Nkx2.2 cells were located dorsally (Figure 4b). The location and level of Shh expression (Figures 4c and 4d) and the position of the ventral boundaries of *Pax3* and Pax7 expression (Figures 4e–4h) was not altered in *Sey/Sey* embryos, suggesting that the expanded Nkx2.2 domain is not the result of enhanced Shh signaling. These results suggest that the domain occupied normally by Nkx2.2 progenitor cells is defined indirectly through Shh-mediated repression of Pax6 and not by a direct requirement for high level Shh signaling.

Since eye development is sensitive to Pax6 dosage (Hill et al., 1991; Schedl et al., 1996), we examined whether ventral progenitor cell pattern is altered in *Sey* heterozygotes. In *Sey/+*, embryos, the pattern of Nkx2.2 expression was not different from that of wild-type littermates (data not shown), and no MN or interneuron patterning defects (see below) were detected at spinal cord and hindbrain levels. The lack of a heterozygote phenotype may be explained by the finding that Pax6 levels in cells in the ventral neural tube of *Sey/+* embryos were 80%–85% of the level detected in wild-type littermates (data not shown).

Impaired Generation of Ventral Interneurons in the Absence of Pax6 Function

We next examined whether the loss of Pax6 function influences the generation of specific ventral neuronal types at caudal levels of the neural tube. We discuss first the fate of ventral interneurons.

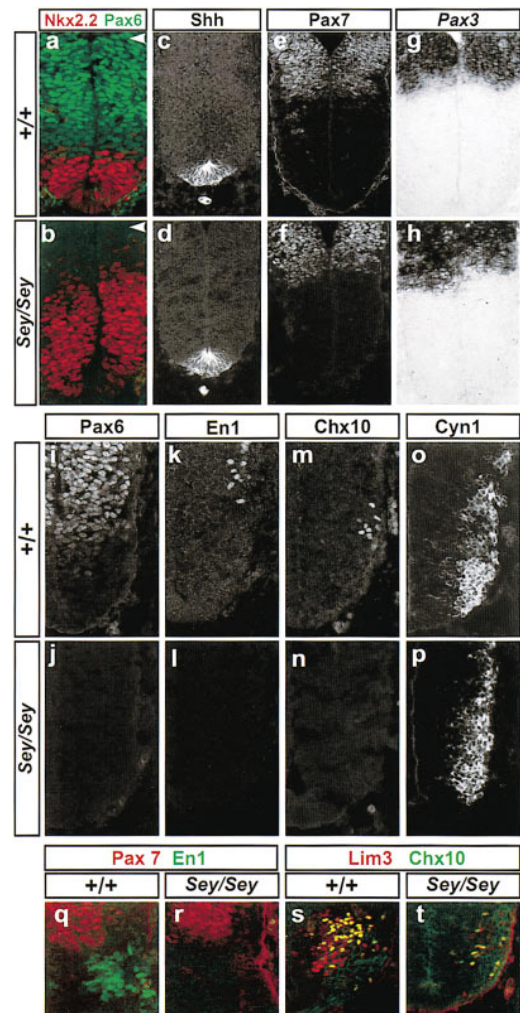In wild-type mouse embryos, as in the chick, V1 and

Figure 4. Alteration in Progenitor Cell Domains and Ventral Interneuron Fate in *Sey/Sey* Embryos

(a and b) Pattern of Pax6 and Nkx2.2 at the C1-R7 level of e10 wild-type (a) and *Sey/Sey* (b) embryos. In wild-type embryos, Pax6 and Nkx2.2 are expressed in complementary domains in the ventral neural tube (a). In *Sey/Sey* embryos, Pax6 is not detectable and the Nkx2.2 domain is expanded dorsally (b). Arrowheads indicate ventral boundary of Pax7 expression.

(c–h) The domains of Shh (c and d), Pax7 (e and f), and Pax3 (g and h) expression are similar in the ventral neural tube of wild-type and *Sey/Sey* embryos.

(i–p) Sections through the C1-R7 level of e10 (28 somite) wild-type (i, k, m, and o) and *Sey/Sey* (j, l, n, and p) embryos. In wild-type embryos, Pax6 is expressed in ventral progenitors (i), and En1 (V1) (k) and Chx10 (V2) (m) cells are detected. Cyn-1 expression (o) defines postmitotic neurons. In *Sey/Sey* embryos, Pax6 is not detected (j), and no En1 (l) or Chx10 (n) cells are detected. No Lim1/2 or Pax2 cells were detected in the normal domain of V1 neuron generation (not shown). Cyn-1 neurons are generated within the domain normally occupied by En1 and Chx10 cells (p).

(q–t) At e11, no En1 (V1) cells are detected in *Sey/Sey* embryos (r), whereas by this stage many En1 cells are detected in wild-type embryos (q) ventral to Pax7 expressing cells. By e11, some Chx10, Lim3 (V2) interneurons have been generated (t), although the number of cells is greatly reduced (s). Cells labeled with Lim3 but not Chx10 in (s) are MNs.

V2 neurons are generated from Pax6 progenitors from e10 to e12 (Figures 4i, 4k, 4m, 4q, and 4s). In Sey/Sey embryos examined over the period e10–e12, V1 neurons were not generated at any rostrocaudal level (Figures 4l and 4r, and data not shown). However, neurons were generated within this dorsal domain, as assessed by expression of Cyn1 (Figures 4o and 4p). Similarly, V2 neurons were not detected in Sey/Sey embryos examined at e10 (Figure 4n). From e11 to e12, however, V2 neurons were detected, although at reduced (∼50%) numbers (Figures 4s and 4t). These results show that Pax6 is required for the generation of V1 neurons and has a profound influence on the generation of V2 neurons.

Control of Hindbrain Motor Neuron Identity by Pax6

We next addressed the fate of MNs. In Sey/Sey embryos, we detected marked changes in the number and/or subtype identity of MNs, with the precise phenotype varying at different rostrocaudal levels. This variation appears to reflect distinctions in the progenitor cell origin of different MN classes (see below). We have analyzed MN development at the C1-R7 level of the rostral spinal cord/caudal hindbrain and at the C4-C3 level of the cervical spinal cord.

At the C1-R7 level in wild-type embryos, the neural tube generates somatic MNs of the hypoglossal motor nucleus (hMN) and visceral MNs of the vagal motor nucleus (vMN). The axons of hMNs project via the hypoglossal (XIIth cranial) nerve, whereas the axons of vMNs project along the vagus (Xth cranial) nerve (Kuratani et al., 1988; Kuratani and Tanaka, 1990). To identify these two MN subclasses in wild-type embryos, we monitored the profile of LIM homeodomain protein expression, the migration of MN cell bodies, and motor axon projections (Simon et al., 1994; Varela-Echavarría et al., 1996). We also examined the dorsoventral position of MN generation and the status of Pax6 and Nkx2.2 expression in their progenitor cells.

Two classes of MNs could be distinguished in wild-type embryos at e10–e11. One class was generated in a position just ventral to that of V2 neurons and coexpressed Isl1, Isl2, and transiently, Lim3 and Gsh4 (Figures 5a–5e, 5k, and 6i, and data not shown). Based on their dorsal position and the coexpression of Pax6 in medial Isl1/2 cells (Figure 5a), these MNs derive from Pax6 progenitor cells. The cell bodies of these MNs extended axons that projected through ventral roots to form the hypoglossal nerve (Figures 6a and 6d, and data not shown) and thus are hypoglossal (h) MNs. By e12, hMNs retained expression of Isl1 and Isl2, but most cells no longer expressed Lim3 or Gsh4 (Figure 6l, and data not shown). The second class of MNs was generated in a position ventral to that of hMNs, from Nkx2.2 progenitor cells (Figure 5b), and coexpressed Isl1 and Gsh4 but not Isl2 or Lim3 (Figures 5d, 5e, and 5k, and data not shown). These MNs migrated dorsally (Figures 6g and 6h), and by e12, MNs with this LIM homeodomain code were located in a dorsolateral position (Figures 6j and 6k). These MNs extended axons into the vagus nerve (Figures 6b and 6c) and thus are vagal (v) MNs. hMNs and vMNs are therefore generated in approximately equal numbers but derive from different progenitor cell populations, defined by expression of Pax6 and Nkx2.2.
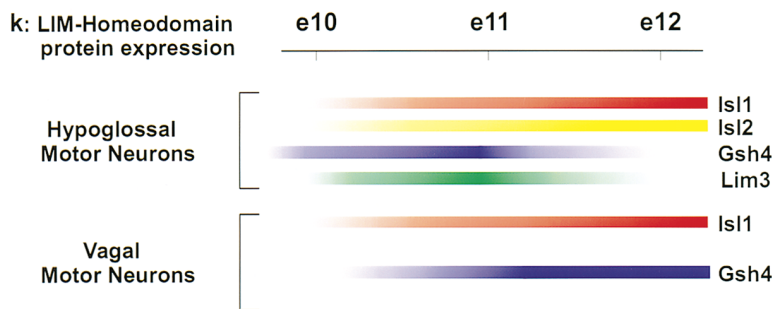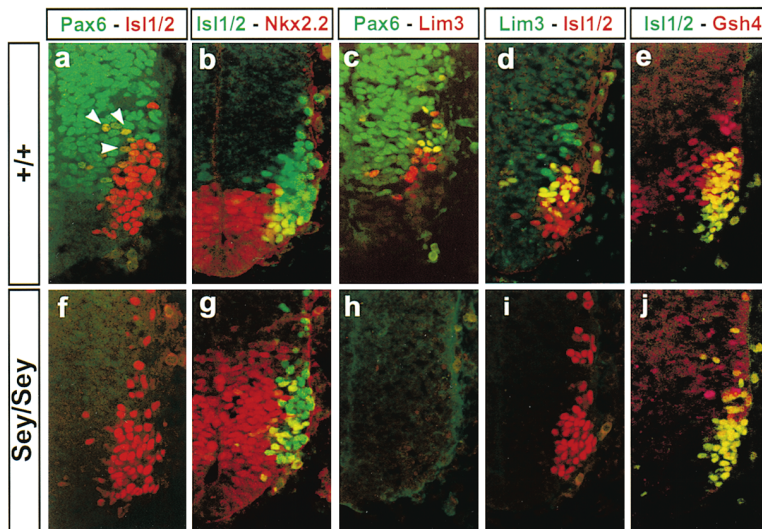
We next examined the differentiation of hMNs and vMNs in e10–e12 Sey/Sey embryos. Nkx2.2 cells occupied the entire domain from which MNs were generated, and Nkx2.2 was detected in medially positioned MNs at all dorsoventral levels (Figures 5f and 5g), suggesting that all MNs now derive from Nkx2.2 progenitor cells. The total number of MNs, defined by Isl1 expression, was not changed in Sey/Sey embryos (Figures 5a and 5f); however, LIM homeodomain protein expression, MN migration, and axonal projections were profoundly altered. From e10, all MNs coexpressed Isl1 and Gsh4 (Figure 5j; Figure 6r and data not shown), but none expressed Isl2 or Lim3 (Figures 5d and 5i; Figures 6o and 6p, and data not shown). At e11, there was a ∼2-fold increase in the number of MNs that migrated dorsally (Figure 6n and data not shown), and these MNs settled in a dorsal position characteristic of vMNs (Figures 6q and 6r). Moreover, by e12, no MNs were detected in a ventral position characteristic of hMNs, (Figures 6q and 6s). Analysis of the organization of cranial nerves at e10 and e11 revealed that the ventral roots that form the hypoglossal nerve were absent in Sey/Sey embryos, whereas the vagus nerve was present (Figures 6u–6w and data not shown). A very low number of apoptotic cells was detected in the neural tube of both wild-type and Sey/Sey embryos between e10 and e12 (data not shown), suggesting that the absence of hMNs does not result from their early death. Thus, at C1-R7 levels, the loss of Pax6 results in an expansion in the domain Nkx2.2 and in an apparent transformation of hMNs into vMNs.

Control of Spinal Motor Neuron Fate by Pax6

In wild-type embryos at the C4-C3 level, the neural tube generates three classes of MNs: somatic MNs of the median motor column (MMC) (Callister et al., 1987), phrenic MNs (Goshgarian and Rafols, 1981), and special visceral MNs of the spinal accessory nucleus (SAN) (Krammer et al., 1987; Liinamaa et al., 1997). At e12, the cell bodies of MMC neurons are located in a ventromedial position and project axons ventrally through segmental motor nerves (Figure 6f), whereas SAN neurons are located dorsolaterally and project axons via the spinal accessory (XIth cranial) nerve (Figure 6e). MMC neurons expressed Isl1, Isl2, Lim3, and Gsh4 (Figure 7d and data not shown), whereas SAN neurons expressed Isl1 alone (Figure 7d and data not shown). An additional medial group of MNs that projected axons through the ventral roots expressed Isl1 but not Isl2, and Lim 3 and Gsh4 only transiently (Figure 7d and data not shown). These cells are likely to be phrenic (P) MNs.

The majority of MNs appeared to derive from Pax6 progenitors, although a few MNs did coexpress Nkx2.2 transiently (Figures 7b and 7c). This raised the question of whether Nkx2.2 progenitor cells give rise to another ventral cell type. We found that at this level, cells adjacent to the floor plate that express Sim1 were contained within the Nkx2.2 progenitor cell domain (Figure 7e; data not shown, Fan et al., 1996).

C1 - R7 Level

Figure 5. Alteration in LIM Homeodomain Protein Expression by Motor Neurons in the Caudal Hindbrain of *Sey/Sey* Embryos

Sections show images of the C1-R7 level of the rostral spinal cord/caudal hindbrain in e10 wild-type and *Sey/Sey* embryos.

(a and b) Dorsal Isl1/2 MNs are generated from Pax6 progenitor cells, as assessed by the position of MNs and by the coexpression of Pax6 and Isl1/2 in medially located MNs (orange cells, arrowheads) (a). Ventrally located Isl1/2 MNs appear to derive from Nkx2.2 progenitors, as assessed by the detection of cells that coexpress Nkx2.2 and Isl1/2 (b).

(c) Lim3 cells derive from the Pax6 progenitor cell domain.

(d) Lim3 is coexpressed only by dorsal Isl1/2 MNs. The dorsal group of Lim3 interneurons does not express Isl1/2 but coexpresses Chx10 (not shown).

(e) Gsh4 is expressed by both dorsally and ventrally positioned MNs. Expression in ventrally located MNs occurs slightly after that in dorsal MNs. Medially located Gsh4 cells in a dorsal position have not begun to express Isl1/2.

(f and g) In *Sey/Sey* embryos, Isl1/2 MNs are generated (f) and appear to derive from Nkx2.2 progenitors (g). These cells do not express Isl2 (data not shown). Scattered dorsal Isl1 cells are evident (f). The total number of MNs was not changed (wild type: 93 ± 4 Isl1 MNs/section; *Sey/Sey*: 92 ± 7 Isl1 MNs/section mean ± SE; n = 12 sections).

(h) In *Sey/Sey* embryos, no Lim3 cells are present.

(i) In *Sey/Sey* embryos, Isl1+ MNs do not coexpress Lim3.

(j) In e10 *Sey/Sey* embryos, most MNs have begun to express Gsh4. Note the absence of dorsomedial Gsh4 cells that do not express Isl1/2.
(k) Summary of the developmental change in LIM homeodomain protein expression by hypoglossal and vagal MNs over the period e10–e12.

In *Sey/Sey* embryos, the dorsal expansion in Nkx2.2 cells occupied much of the domain in which MNs were normally generated but was less extensive than that detected at the C1-R7 level (Figures 7f and 7h). The total number of MNs was decreased by ~45% (Figures 7g–7i). This reduction appeared to encompass MMC and P MNs, but MNs that expressed Isl1 alone in a dorsolateral position characteristic of SAN neurons were still detected (Figure 7i). Consistent with this, the spinal accessory nerve appeared unaffected (Figures 6u–6w and data not shown). The reduction in MN number was accompanied by a marked increase in the number of *Sim1* cells and in a dorsal expansion in the domain of *Sim1* expression (Figure 7j).

This analysis provides evidence that at all rostrocaudal levels, the elimination of Pax6 results in a dorsal-to-ventral transformation in the identity of progenitor cells. At the C1-R7 level, this transformation diverts cells from hMN to vMN fate, whereas at the C4-C3 level the equivalent transformation diverts cells, albeit incompletely, from MN to *Sim1* fate.

Discussion

This study provides evidence that the identity and position of differentiation of neuronal subtypes in the spinal cord is initiated by the exposure of ventral progenitor cells to small differences in Shh concentration and that one critical output of graded Shh signaling is the repression of Pax6. Repression of Pax6 permits the generation of a second population of ventral progenitor cells, defined by the expression of Nkx2.2. Elimination of Pax6 results in a dorsal-to-ventral transformation in the identity of ventrally located progenitor cells and a consequent change in MN fate. In addition, the lack of Pax6 function in dorsally-located ventral progenitor cells results in the loss of certain ventral interneurons. Pax6 is therefore a key intermediary in the Shh-dependent control of neuronal subtype identity in the ventral spinal cord and hindbrain. We discuss the implications of these findings for the signaling role of Shh, for the control of ventral progenitor cell identity and for the specification of ventral neuronal subtypes.

Graded Shh Signaling in the Neural Tube
Several subclasses of neurons are generated at different dorsoventral positions in the ventral spinal cord and hindbrain. The present studies provide evidence that the specification of neuronal subtype identity is initiated by the exposure of ventral progenitor cells to different concentrations of Shh. The Shh concentration threshold required to generate specific neuronal subtypes in vitro
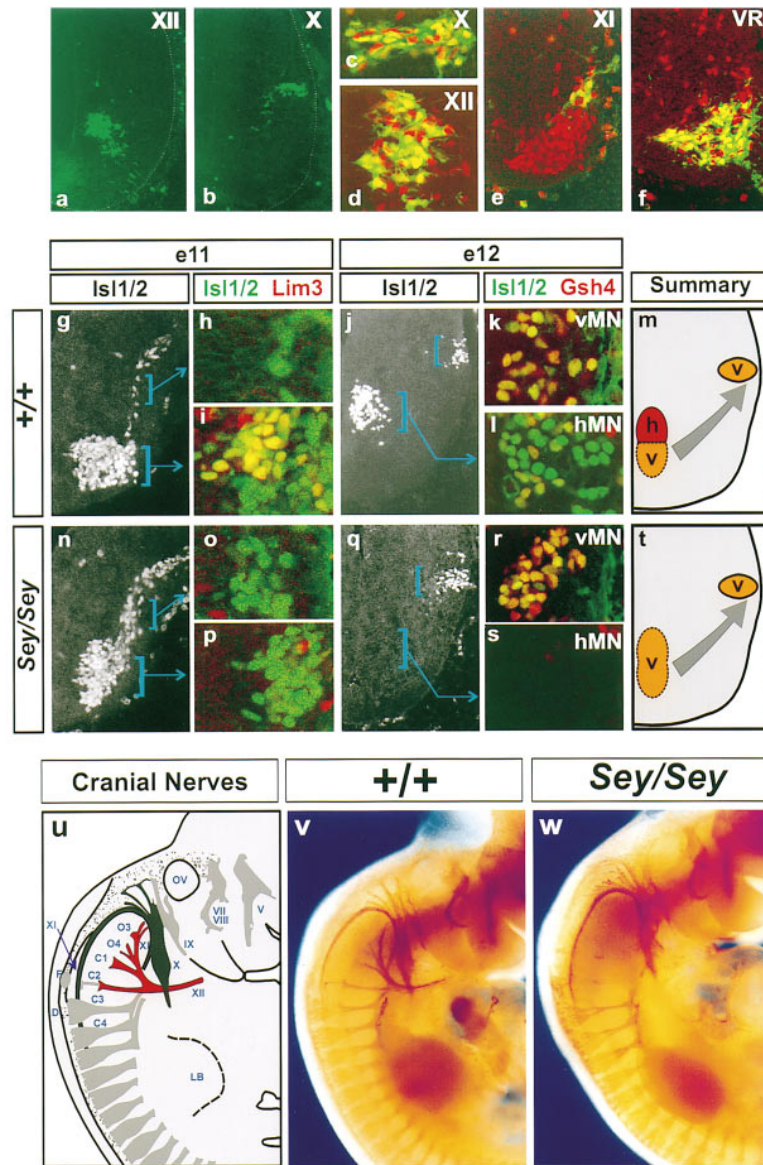
Figure 6. Alteration in Motor Neuron Fate in the Caudal Hindbrain of *Sey/Sey* Embryos

(a and b) At the R8 level of e12 wild-type embryos, ventrally located motor neurons (hMNs) are retrogradely labeled from the hypoglossal (XII) nerve (a), whereas dorsally located motor neurons (vMNs) are retrogradely labeled from the vagus (X) nerve (b).

(c and d) Retrogradely labeled vMNs (c) and hMNs (d) coexpress Isl1.

(e) At the C4 level, dorsally located motor neurons are retrogradely labeled from the spinal accessory (XI) nerve.

(f) At the C4 level, ventrally located MNs are retrogradely labeled from the C4 ventral root (VR).

(g–i) In e11 wild-type embryos, some Isl1^{+} MNs appear to migrate dorsally. These cells do not express Lim3 (h), whereas many ventromedial MNs express Lim3 (i). In contrast, ventrolateral cells do not express Lim3 (i).

(j–k) In e12 wild-type embryos, Isl1^{+} MNs (vMNs) have settled in a dorsolateral position, whereas (hMNs) do not migrate dorsally. Most hMNs have ceased expression of Lim3 and Gsh4 (i), while vMNs maintain expression of Gsh4 (k).

(m) Summary of migration of vMNs.

(n) In *Sey/Sey* embryos analyzed at e11, there is a 2-fold increase (wild type: 17 ± 3 MNs/section; *Sey/Sey*: 37 ± 5 MNs/section mean ± SE; n = 8 sections) in dorsally migrating MNs.

(o and p) MNs in *Sey/Sey* embryos express Isl1 but not Lim3 or Isl2 (not shown).

(q–t) At e12, all MNs occupy a dorsolateral position and ventral MNs are missing. (q), Dorsally positioned MNs coexpress Isl1 and Gsh4.

(u–w) Whole-mount labeling of e11 wild-type and *Sey/Sey* embryos using anti-neurofilament antibody reveals that the hypoglossal motor nerve (XII) is absent in *Sey/Sey* embryos, whereas the vagus (X) and spinal accessory (XI) nerves are present.

is inversely related to the distance from the midline at which each neuronal type is generated in vivo. Together with the requirement for high Shh concentrations in floor plate differentiation (Roelink et al., 1995), these findings suggest that the identity of all ventral cell types is controlled by graded Shh signaling. Since the progenitors of MNs and ventral interneurons remain sensitive to the ambient Shh concentration at the time they leave the cell cycle (Ericson et al., 1996), graded Shh signaling is likely to define distinct neuronal fates through a direct action on ventral progenitor cells and not through a cascade of inductive interactions.

The pattern of expression of Pax6 also provides evidence that ventral progenitor cells themselves respond to Shh signaling in a graded manner. The ventral[low]–dorsal[high] gradient in expression of Pax6 detected in vivo can be reconstructed in vitro by exposure of neural cells to an ~10-fold range in Shh concentration, suggesting that a similar concentration range may be sufficient in

vivo to specify distinct ventral progenitor cell populations. The sensitivity of ventral progenitor cells to small differences in Shh concentration may provide an explanation for the holoprosencephalic phenotype in humans lacking one copy of the *Shh* gene (Roessler et al., 1996). It remains unclear how individual cells are able to perceive small differences in ambient Shh concentration and respond with the generation of distinct cell types. Nevertheless, the onset of Nkx2.2 expression in response to a 1.5-fold change in Shh concentration indicates that abrupt changes in gene expression and progenitor cell identity can occur in response to graded Shh signaling. Studies on mesodermal patterning in *Xenopus* have revealed sharp transitions in gene expression in response to 2-fold increments in concentration of Activin (Green et al., 1992; Gurdon et al., 1994). Thus, the ability of cells to discriminate small differences in the concentration of inductive signals may be widespread in vertebrate embryos.
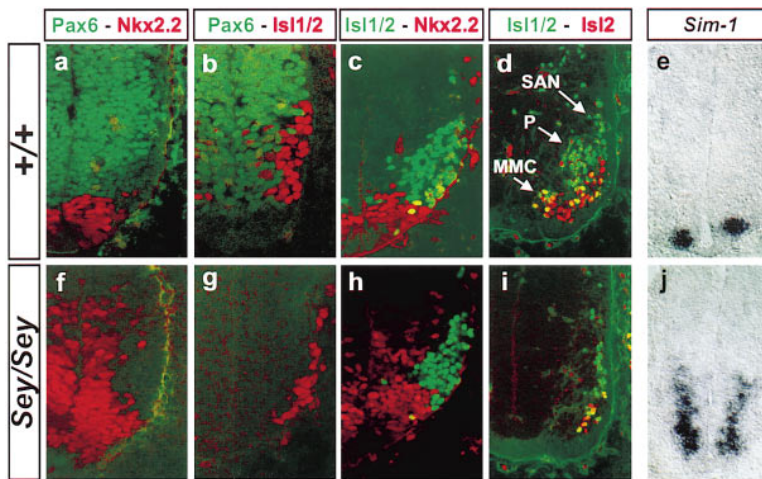
Figure 7. Alteration in Ventral Cell Pattern in the Cervical Spinal Cord in *Sey/Sey* Embryos

Images show an analysis of ventral patterning at the C4-C3 level of the cervical spinal cord.

(A) Pax6 and Nkx2.2 define distinct progenitor cell domains in the ventral spinal cord of e10 wild-type embryos.

(b and c) At e10, most Isl1/2 MNs derive from Pax6 progenitors (b), although a few ventral MNs derive from Nkx2.2 progenitors (c).

(d) By e12, ventrally positioned MMC MNs (MMC) coexpress Isl1 and Isl2, whereas dorsolaterally positioned SAN neurons do not express Isl2. A third group of MNs located medial and ventral to SAN neurons that transiently express Lim3 and Gsh4 (not shown) and do not express Isl2 are likely to be phrenic (P) MNs.

(e) *Sim1* expression in e11 embryos defines cells adjacent to the floor plate within the Nkx2.2 progenitor domain (not shown, see panels [a] and [c]).

(f) In e10 *Sey/Sey* embryos, there is a dorsal expansion in the Nkx2.2 domain within the ventral spinal cord.

(g–i) In *Sey/Sey* embryos, there is an ~45% reduction (wild-type embryos: 85 ± 3 MNs/section, *Sey/Sey* embryos: 50 ± 4 MNs/section, n = 10 sections) in the number of MNs. The reduction in MN is prominent ventrally (h and i) and appears to deplete MMC MNs and P MNs (i). Cells in a dorsolateral position characteristic of SAN MNs are present in *Sey/Sey* embryos (i).

(j) In e11 *Sey/Sey* embryos, there is an increase in number of *Sim1* cells and a dorsal expansion in the domain of *Sim1* expression.

Control of Progenitor Cell Identity

The differential sensitivity of Pax7, Pax6, and Nkx2.2 expression to Shh signaling in vitro may explain the progression of progenitor cell specification within the ventral neural tube. The initial establishment of a general ventral progenitor cell population appears to involve the repression of Pax7 and *Pax3* expression (Liem et al., 1995; Ericson et al., 1996). The expression of Pax7 in vitro is repressed by Shh at a concentration ~8-fold lower than that required to repress Pax6. Thus, the early exposure of neural cells to low concentrations of Shh provided by the notochord may be sufficient to extinguish expression of Pax7 from ventral cells without repressing Pax6. The stable inheritance of the early state of Pax7 expression (Ericson et al., 1996), may contribute to the establishment of a sharp boundary of Pax7 expression in the neural tube. In contrast, the repression of Pax6 requires higher concentrations of Shh, and, in addition, Pax6 expression remains sensitive to the ambient Shh concentration during the period of neurogenesis. These two features of the control of Pax6 may underlie the formation of the ventral^{low}–dorsal^{high} gradient of Pax6 in the ventral neural tube in response to graded Shh signaling.

The extinction of Pax6 expression at high Shh concentrations appears to control the generation of a distinct Nkx2.2 progenitor cell domain. The marked dorsal expansion of Nkx2.2 expression in *Sey/Sey* mice indicates additionally that the dorsal extent of the Nkx2.2 progenitor cell domain is defined indirectly by the Shh concentration required for extinction of Pax6. The dorsal limit of the expanded Nkx2.2 domain in *Sey/Sey* embryos could reflect the position at which Shh activity falls below a threshold necessary to activate Nkx2.2 expression. Alternatively, Shh signaling could, in a Pax6-independent manner, define additional domains of gene expression, and Nkx2.2 expression may be inhibited dorsally by such genes. The patterns of expression of the *Dbx1* and *Dbx2* genes (Lu et al., 1994; Shoji et al., 1996) makes them candidates as inhibitors of more dorsal Nkx2.2 expression.

Although our results show that the expression of Pax6 is graded and that Pax6 controls cell fate within the ventral neural tube, it remains unclear whether the graded expression of Pax6 is important for the control of neuronal fate or is an incidental consequence of the requirement for graded Shh signaling. The level of Pax6 expression is critical for appropriate eye development (Schedl et al., 1996), but in the spinal cord and hindbrain it is possible that Pax6 functions at a single threshold level, defining solely Pax6^{off} and Pax6^{on} progenitor cells states. If this is the case, Shh signaling could, independently of Pax6, establish additional distinctions in progenitor cell identity that contribute to the specification of ventral interneuron fate.

Pax6 Function in the Control of Progenitor Cell Identity and Neuronal Fate

Our analysis suggests that Pax6 functions in the ventral spinal cord and hindbrain in a cell-autonomous manner, as in the control of eye development (Quinn et al., 1996). However, Pax6 appears to have somewhat different functions in the control of progenitor cell fate at different dorsoventral positions. In the dorsal region of the ventral neural tube, the loss of Pax6 function does not lead to a detectable ventral transformation in progenitor cell identity but nevertheless eliminates V1 interneurons. In *Sey/Sey* embryos, neurons are generated within the dorsal domain that normally gives rise to V1 neurons, and thus it remains possible that the loss of Pax6 results in a switch in V1 interneuron fate.

The loss of Pax6 function more ventrally does result in a dorsal-to-ventral transformation in progenitor cell identity. At the C1-R7 level, this transformation does not change the total number of MNs, indicating that Pax6 is not required directly for MN generation, but instead

appears to alter MN subtype identity from somatic (hMN) to visceral (vMN). The loss of hMNs has also been detected in rat embryos that carry a mutation in the *Pax6* gene (Osumi et al., 1997). At the C4-C3 level, however, an equivalent transformation converts progenitor cells from a MN fate toward a more ventral cell fate, characterized by expression of *Sim1*. Ventrally, therefore, the loss of Pax6 function leads to a conserved transformation in the identity of ventral progenitor cells, but the consequences for MN fate differ according to position along the rostrocaudal axis of the neural tube. The marked reduction in V2 neuron generation may also result from the dorsal encroachment of Nkx2.2 progenitor cells into the domain that normally generates V2 neurons.

The transformation of somatic hMNs to visceral vMNs detected in *Sey/Sey* embryos indicates that an important determinant of the subtype identity of MNs is the status of Pax6, and indirectly of Nkx2.2, expression by their progenitor cells. Since the state of Pax6 expression by ventral progenitors is controlled by Shh, these results provide evidence that graded Shh signaling controls not only the selection of MN and ventral interneuron identity, but also MN subtype identity. Shh may therefore control MN subtype diversity at other levels of the neuraxis through the establishment of distinct Nkx2.2 and Pax6 progenitor cell populations.

V1 neurons comprise the population of En1 interneurons that is missing from the spinal cord of *Isl1* mutant mice in which MN generation is inhibited (Pfaff et al., 1996). One potential explanation for this finding was that MNs provide an inductive signal required for the differentiation of V1 neurons. However, the present findings show that the generation of V1 neurons can be dissociated from that of MNs. The requirement for *Isl1* in the differentiation of V1 neurons may therefore be the consequence of a depletion in the number of ventral progenitors (Pfaff et al., 1996).

The emergence of a link between Shh signaling, progenitor cell identity and neuronal fate in the spinal cord and hindbrain may provide insight into the mechanisms of ventral patterning at rostral levels of the neuraxis. Pax6, Nkx2.2, and the related gene *Nkx2.1* are expressed in ventral domains at forebrain levels (Walther and Gruss, 1991; Price et al., 1992; Shimamura et al., 1995). Moreover, Shh has been shown to regulate neuronal fate and the pattern of expression of these genes in the developing forebrain (Barth and Wilson, 1995; Ekker et al., 1995; Ericson et al., 1995; MacDonald et al., 1995). In addition, there are profound defects in eye development and other aspects of forebrain patterning in mice lacking *Pax6* (Hill et al., 1991; Stoykova and Gruss, 1994; Stoykova et al., 1996; Warren and Price, 1997) and *Nkx2.1* function (Kimura et al., 1996). The relationship between Shh signaling, Nkx2.2 and Pax6 progenitor cell identity, and neuronal fate defined at spinal cord and hindbrain levels may thus be conserved at rostral levels of the vertebrate CNS.

Experimental Procedures

Neural Plate Explant Culture
Neural plate tissue was isolated from Hamburger and Hamilton (1951) (HH) stage 10–12 chick embryos from the 12 somite level

(Ericson et al., 1991, 1996; Yamada, et al., 1991, 1993). Shh-N protein was generated as described (Ericson et al., 1996).

Immunohistochemistry
Immunohistochemical localization of proteins was carried out using antibodies as described (Ericson et al., 1992, 1996; Tsuchida et al., 1994). Pax6 was detected with a MAb raised against aa 1–223 of chick Pax6 (A. K. et al., unpublished data) and with rabbit anti-Pax6 (MacDonald et al., 1995). En1 was detected with a rabbit Ab (Davis et al., 1991) and a MAb raised against aa 66–85 of the chick En1. Chx10 was detected using a rabbit anti-Chx10 (Liu et al., 1994). Isl2 was detected with a rabbit anti-Isl2 antisera. MAb anti-Nkx2.2 was raised against chick Nkx2.2. Rabbit anti-Gsh4 (Li et al., 1994) and rabbit and MAb anti-Lim3 (Zhadanov et al., 1995) were raised against GST fusion proteins. Pax2 was detected with rabbit anti-Pax2 antisera (Ryan et al., 1995). MAb Cyn-1 recognizes a neuron-specific intracellular epitope. Immunostaining of whole mount embryos was performed using MAb 2H3 (Dodd et al., 1988). Apoptotic cells were identified using TUNEL (Pfaff et al., 1996).

In Situ Hybridization Histochemistry
Sectioned embryos were hybridized with digoxigenin-labeled antisense riboprobes (Schaeren-Wiemers and Gerfin-Moser, 1993). Probes used were *Sim1* (Fan et al., 1996) and *Pax3* (Goulding et al., 1994).

Retrograde Labeling of Motor Neurons
The motor nuclei of cranial nerves X, XI, and XII and MNs projecting through the ventral root at C4 were identified by retrograde labeling as described (Varela-Echavarría et al., 1996).

Quantification of Nuclear Homeodomain Protein Levels
The relative fluorescence intensity of Pax6, Pax7, or Nkx2.2 was quantified by photon counting of nuclei using a MRC600 confocal microscope (Bio-Rad) and Optimas 5.2 image analysis software (Optimas Inc.).

Genotyping of *Sey* Mice
The *Small Eye* allele (Hill et al., 1991) was maintained on a CBA background. Genotype was determined by Southern blotting (Schedl et al., 1996). *Sey/Sey* (25), *Sey/+* (9), and +/+ (29) embryos were analyzed.

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