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Two Critical Periods of Sonic Hedgehog Signaling Required for the Specification of Motor Neuron Identity

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Summary

Antibodies that block Sonic Hedgehog (SHH) signaling have been used to show that SHH activity is required for the induction of floor plate differentiation by the notochord and independently for the induction of motor neurons by both the notochord and midline neural cells. Motor neuron generation depends on two critical periods of SHH signaling: an early period during which naive neural plate cells are converted into ventralized progenitors and a late period that extends well into S phase of the final progenitor cell division, during which SHH drives the differentiation of ventralized progenitors into motor neurons. The ambient SHH concentration during the late period determines whether ventralized progenitors differentiate into motor neurons or interneurons, thus defining the pattern of neuronal cell types generated in the neural tube.

Introduction

The patterning of cell types in vertebrate embryos depends on the function of organizing centers, specialized cell groups that direct the fate of nearby cells through the secretion of inductive factors (Gurdon, 1987). The identity and pattern of cell types generated in the ventral neural tube is controlled initially by an axial mesodermal organizing center, the notochord (Jessell and Dodd, 1992). The notochord secretes a locally acting factor that induces the differentiation of floor plate cells at the ventral midline of the neural tube and a diffusible factor that can initiate motor neuron differentiation (reviewed by Placzek, 1995).

The secreted glycoprotein Sonic Hedgehog (SHH) is expressed by the notochord and later by the floor plate and is a strong candidate as a local inducer of floor plate differentiation (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995; Hynes et al., 1995b; Ericson et al., 1995; Marti et al., 1995a; Tanabe et al., 1995). Exposure of neural plate cells to the biologically active amino-terminal fragment of SHH (SHH-N) (Lee et al., 1994; Porter et al., 1995) is sufficient to induce expression of floor plate markers, notably the transcription factor HNF3β (for hepatocyte nuclear factor 3β; Roelink et al., 1995; Hynes et al., 1995b). The induction of HNF3β in neural plate cells is an early response to notochord-derived signals (Ruíz i Altaba et al., 1995a), and ectopic expression of HNFβ induces floor plate differentiation (Ruíz i Altaba et al., 1993, 1995b; Sasaki and Hogan, 1994; Hynes et al., 1995a). These findings suggest that in vivo, the high level of SHH associated with the surface of notochord cells (Marti et al., 1995b; Roelink et al., 1995) exposes midline neural plate cells to a concentration of SHH-N above the threshold for induction of HNF3β, leading subsequently to the expression of other floor plate genes. Nevertheless, it remains to be shown that SHH is required for the notochord-mediated induction of floor plate differentiation.

The role of SHH in the specification of motor neuron identity is less clear. In part, this uncertainty reflects the fact that the generation of motor neurons is a protracted process. Neural plate cells are exposed to signals from the notochord soon after neural plate formation (Liem et al., 1995), but postmitotic motor neurons are first generated ~18 hr later (Ericson et al., 1992; Pfaff et al., 1996), after neural plate cells have undergone one or more rounds of cell division (Langman et al., 1966). Thus, even though SHH is able to induce motor neurons in vitro at concentrations below the threshold for floor plate differentiation (Marti et al., 1995a; Roelink et al., 1995; Tanabe et al., 1995), it remains possible that SHH induces an intermediary factor in neural plate cells and that this factor rather than SHH itself is responsible for motor neuron differentiation. SHH secreted from the notochord might therefore control cell pattern in the ventral neural tube entirely through local signaling, with long-range cell patterning mediated by a secondary diffusible factor. Indeed, many of the long-range patterning activities of Hedgehog in Drosophila depend on the induction of diffusible intermediary factors, notably the transforming growth factor β (TGFβ)-like protein Decapentaplegic (DPP) (Zecca et al., 1995; Nellen et al., 1996; Lecuit et al., 1996). Antibodies directed against SHH have been shown to inhibit the induction of motor neuron differentiation by the notochord in neural plate explants (Marti et al., 1995a). However, the notochord normally induces floor plate cells under these conditions, and thus it remains unclear whether the requirement for SHH signaling is in floor plate differentiation, motor neuron differentiation, or both.

In this study, we have addressed whether SHH is required for the generation of floor plate cells and motor neurons by blocking SHH signaling in vitro with anti-SHH antibodies. We show that SHH function is required for the short-range induction of floor plate cells by the notochord and independently for the induction of motor neurons both by the notochord and midline neural cells.

These results raise the additional question of when and over what range SHH signaling is required for the generation of motor neurons. We provide evidence that motor neuron generation depends on two critical periods of SHH signaling, with long range actions at different concentration thresholds. During the early period, SHH derived from the notochord converts naive neural plate

cells into ventralized progenitors. During the late period, which extends well into S phase of the final progenitor cell division, SHH derived from the floor plate acts at a distance to direct the differentiation of ventralized progenitors into postmitotic motor neurons. These results provide strong evidence against a cascade model in which a SHH-inducible intermediary factor is sufficient for motor neuron differentiation. The ambient SHH concentration during the late period determines whether ventralized progenitors differentiate into motor neurons or interneurons, thus defining the pattern of differentiation of specific neuronal cell types in the ventral neural tube.

Results

SHH Is Required for the Induction of Floor Plate and Motor Neuron Differentiation

To determine whether SHH is required for the induction of ventral cell types in the caudal neural tube, we generated polyclonal (H4) and monoclonal (MAb 5E1) antibodies against the biologically active amino-terminal fragment of SHH (SHH-N). The H4 and 5E1 antibodies recognize the ~20 kDa SHH-N protein by Western blotting and enzyme-linked immunosorbent assay (data not shown). Both antibodies also react with Indian Hedgehog (IHH) (Vortkamp et al., 1996), but since neither IHH nor Desert Hedgehog (DHH) is expressed by notochord or neural tube cells (Bitgood et al., 1996; data not shown), in the present studies the H4 and 5E1 antibodies recognize SHH selectively.

Affinity-purified H4 immunoglobulin G (IgG) was tested first for its ability to block the SHH-N-mediated induction of floor plate and motor neuron differentiation in intermediate neural plate ([i]) explants (Yamada et al., 1993). Explants exposed to recombinant SHH-N (4 nM) for 48 hr gave rise to HNF3β^+ floor plate cells and Isl1/Isl2$^+$ motor neurons (Figures 1A and 1E). Addition of H4 IgG blocked the SHH-N-mediated induction of floor plate cells and motor neurons (Figures 1B and 1F; data not shown). Three results demonstrate the specificity of action of the H4 antibody. First, increasing the concentration of SHH-N to 13 nM in the presence of H4 IgG restored motor neuron differentiation (205 $\pm$ 27 Isl1/Isl2$^+$ cells, n = 5). Second, the blocking activity of H4 was mimicked by MAb 5E1 IgG (0 $\pm$ 0 Isl1/Isl2$^+$ cells, n = 5), but not by rabbit-anti SHH (H2) IgG (data not shown), a reagent that reacts only with denatured SHH protein (Roelink et al., 1995). Third, H4 IgG had no effect on the bone morphogenetic protein 4 (BMP4)-mediated induction of neural crest cell differentiation in [i] explants (data not shown; see Liem et al., 1995).

To examine whether induction of floor plate differentiation by the notochord is mediated by SHH, we grew conjugates of notochord and [i] explants in the presence or absence of H4 IgG. Floor plate differentiation, detected by HNF3β expression, was blocked in the presence of H4 IgG (Figures 1C and 1D). These results indicate that SHH is required for the notochord-mediated induction of floor plate differentiation.

To determine whether motor neuron generation requires SHH signaling, previously described midline sources of motor neuron–inducing factors (Placzek et al., 1991; Yamada et al., 1993) were tested for their sensitivity to anti-SHH antibodies. The notochord-mediated induction of motor neurons was blocked by H4 IgG (Figures 1G and 1H), consistent with previous results (Marti et al., 1995a). Motor neuron induction by the notochord under transfilter conditions that reveal the activity of a diffusible factor (Tanabe et al., 1995) was also blocked by H4 IgG (Figures 1I and 1J). In addition, motor neuron induction by the floor plate or by stage 10 ventral midline tissue was blocked by H4 IgG (Figures 1K and 1L; see also Figure 5A). In contrast, H4 IgG did not affect the ability of the floor plate to induce commissural axon extension from rat E11 dorsal spinal cord explants in vitro (Tessier-Lavigne et al., 1988; Placzek et al., 1990; data not shown), an activity mediated by netrins (Serafini et al., 1994). These results provide evidence that SHH signaling is required for the induction of motor neuron differentiation by both midline mesodermal and neural cells.

Early Action of Notochord-Derived SHH on Neural Plate Cells

To begin to determine when SHH signaling is required for motor neuron generation, we analyzed the time at which neural plate cells are first exposed to a SHH-mediated signal. Previous studies have indicated that notochord-derived signals repress expression of the homeobox genes *pax3* and *msx1* from medial regions of the neural plate (Liem et al., 1995). PAX7 is also initially expressed in some cells at the midline of the newly formed neural plate (data not shown), but its expression is lost rapidly from medial neural plate cells (Figure 2B) and subsequently is restricted to the dorsal neural tube (Figure 2D). To determine whether signals from the notochord are responsible for the absence of PAX7 expression, in medial neural plate cells, stage 10 [i] explants (Figure 2E) were grown alone or with the notochord for 22 hr. Over 95% of cells in explants grown alone expressed PAX7 (Figure 2F), whereas expression was eliminated from neural plate cells grown in contact with the notochord (Figure 2G). The notochord-mediated repression of PAX7 expression was mimicked by SHH (Figure 2H) and blocked by H4 IgG (Figure 2I). Thus, SHH is necessary and sufficient for the notochord-mediated repression of PAX7 expression in neural plate cells in vitro. The absence of PAX7 expression by medial neural plate cells in vivo therefore shows that these cells have been exposed to notochord-derived SHH at the time of neural plate formation.

We next examined whether persistent SHH signaling is required to maintain the repression of PAX7 expression. To test this, stage 10 ventral neural plate explants were isolated at a stage at which expression of PAX7 had already been extinguished (Figure 2J) and were grown in vitro for 22 hr. Reexpression of PAX7 was detected in >95% of cells (Figures 2K and 2L), suggesting that cells in the medial neural plate require continued SHH signaling from the notochord to maintain the repression of PAX7 expression. In contrast, cells in explants derived from stage 12 ventral neural tube devoid of floor plate (Figure 2E) did not reexpress PAX7

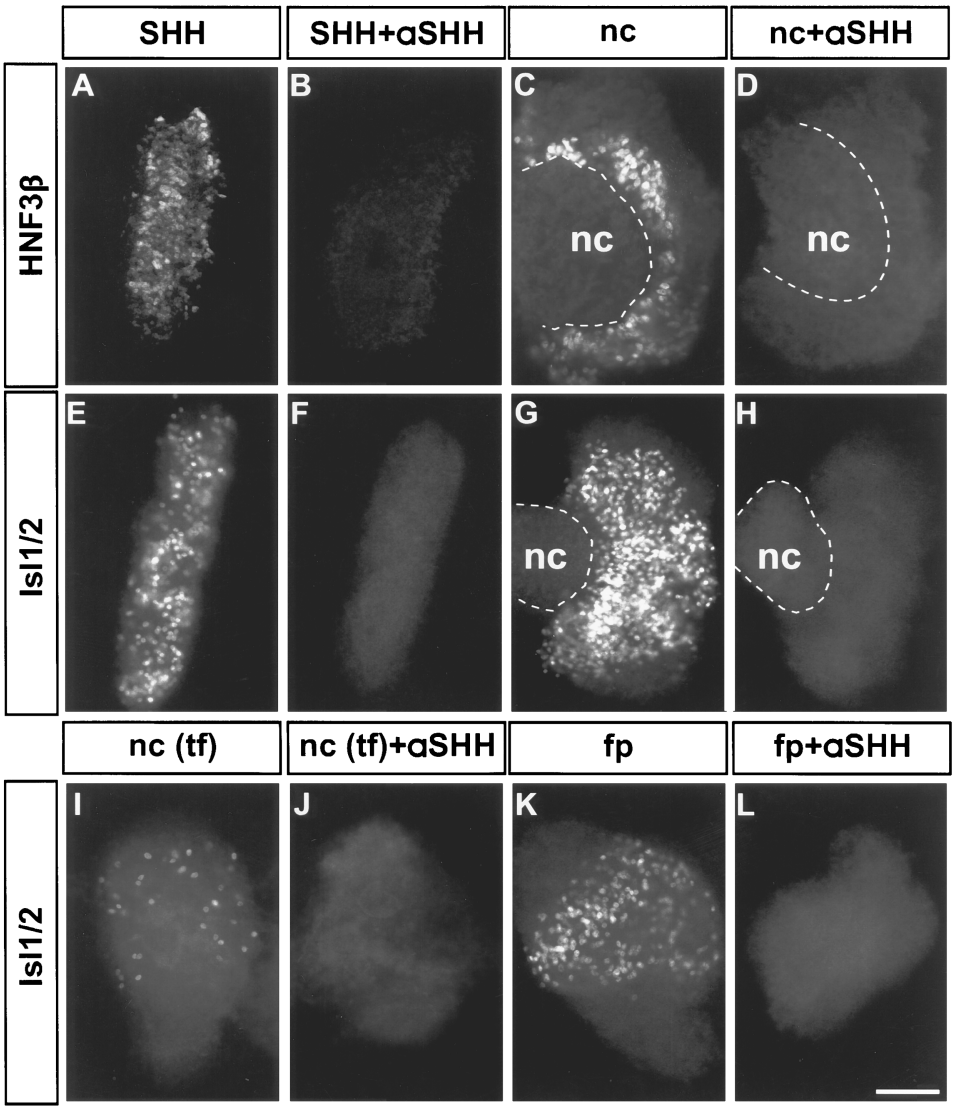

Figure 1. Blockade of Floor Plate and Motor Neuron Differentiation by Anti-SHH Antibodies

Stage 10 [i] explants were grown for 48 hr.

(A and B) Induction of HNF3β$^+$ cells by SHH-N (4 nM) (149 ± 20 cells, n = 6; [A]) is blocked by H4 IgG (0 cells, n = 6; [B]).

(C and D) Notochord-mediated induction of HNF3β$^+$ cells (168 ± 19, n = 8; [C]) is blocked by H4 IgG (0 cells, n = 15; [D]).

(E and F) Induction of Isl1/Isl2$^+$ cells by SHH-N (4 nM) (383 ± 20 cells, n = 8; [E]) is blocked by H4 IgG (0 cells, n = 9; [F]).

(G and H) Notochord-mediated induction of Isl1/Isl2 neurons (282 ± 41, n = 10; [G]) is blocked by H4 IgG (8 ± 4 cells, n = 10; [H]).

(I and J) Notochord transfilter induction of Isl1/Isl2 neurons (27 ± 10, n = 6; [I]) is blocked by H4 IgG (0 cells, n = 10 explants; [J]).

(K and L) Floor plate induction of Isl1/Isl2 neurons (144 ± 13, n = 6; [K]) is blocked by H4 IgG (1 ± 1 cells, n = 10; [L]).

Values indicate mean ± SEM. Scale bar, 120 μm.

when grown alone (Figures 2M and 2N), or in the presence of H4 IgG (Figure 2O). Thus, by stage 12 the extinction of PAX7 expression by ventral neural tube cells is independent of SHH signaling.

This analysis of PAX7 expression suggests that the development of neural plate cells into motor neurons is divisible into two distinct periods, an early (stage 10 to stage 12) period during which naive (PAX7^{on}) neural plate cells are converted to stable ventralized (PAX7^{off}) progenitors and a late (post–stage 12) period during which ventralized progenitor cells give rise to postmitotic motor neurons. The appreciation of this subdivision has permitted us to examine more precisely the temporal requirement for SHH signaling in motor neuron generation.

Early Exposure of Neural Plate Cells to SHH Is Required for Floor Plate and Motor Neuron Differentiation

To determine whether SHH signaling during the early period is required for the eventual differentiation of ventral cell types, stage 10 [i] explants were exposed to SHH-N for different periods of time, and floor plate and motor neuron generation was assessed. Exposure of

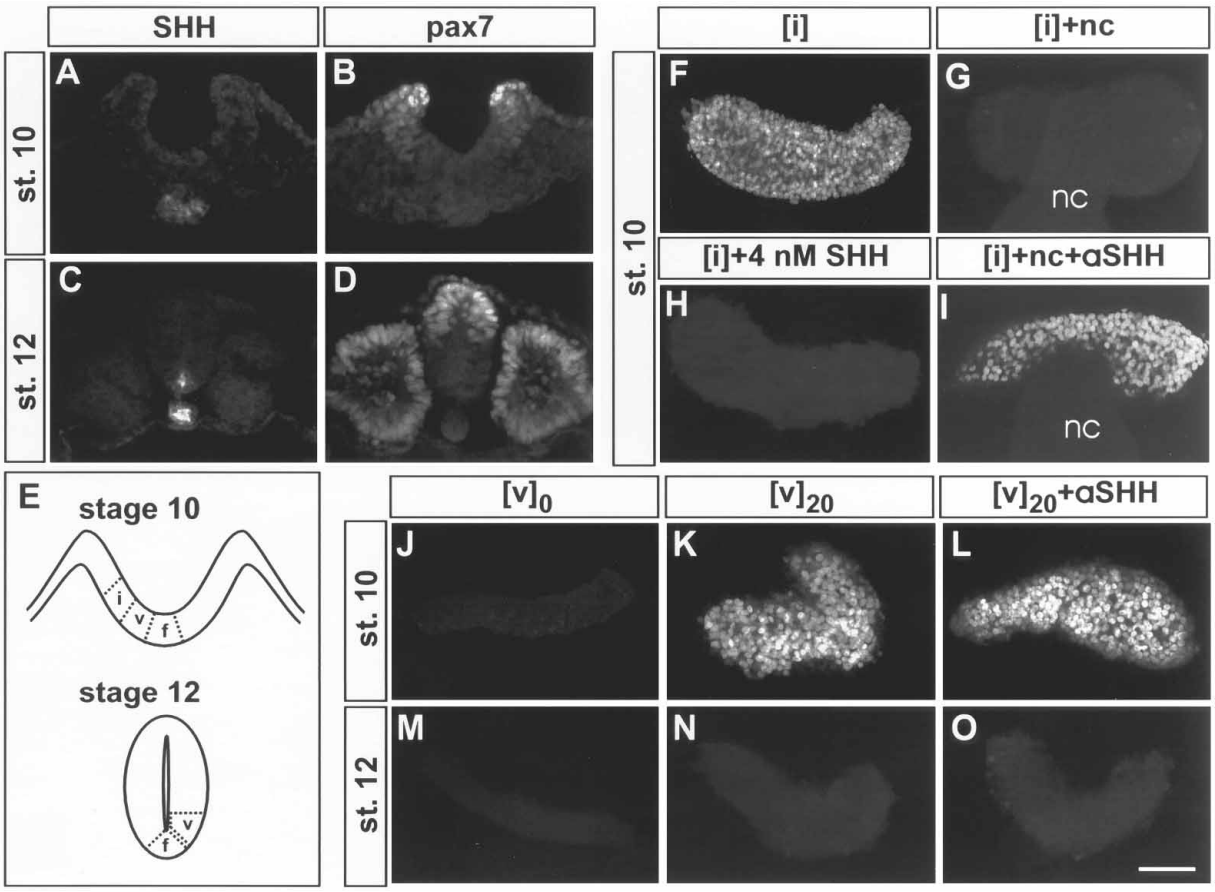

Figure 2. Notochord-Derived SHH Represses PAX7 Expression from Ventral Neural Cells

(A) SHH is expressed in the notochord but not by midline neural plate cells at stage 10.
(B) PAX7 expression is extinguished in the medial neural plate at stage 10.
(C) SHH is expressed by floor plate cells at stage 12.
(D) PAX7 expression is restricted to the dorsal neural tube at stage 12.
(E) Regions isolated for use in explant assays.
(F) PAX7 is expressed by >95% of cells in [i] explants grown alone for 22 hr.
(G) SHH-N (4 nM) represses PAX7 expression.
(H) Notochord represses PAX7 expression.
(I) H4 IgG blocks the notochord repression of PAX7 expression. PAX7 is detected in >90% of cells in neural plate explants after 22 hr.
(J) Cells in stage 10 ventral neural plate explants do not express PAX7 at the time of isolation.
(K) Over 90% of cells in stage 10 [v] explants reexpress PAX7 after 22 hr.
(L) H4 IgG does not influence reexpression of PAX7 in stage 10 [v] explants.
(M) Cells in stage 12 [v] explants do not express PAX7 at the time of isolation.
(N) Cells in stage 12 [v] explants do not reexpress PAX7 after 22 hr in vitro.
(O) Addition of H4 IgG does not result in reexpression of PAX7 in stage 12 [v] explants after 22 hr.
Results derived from five to ten explants. Scale bar in (A)–(D), 150 μm; in (F)–(O), 100 μm.

explants to 4 nM SHH-N resulted in the detection of many floor plate cells and motor neurons by 25 hr (Figures 3A and 3E). In contrast, explants grown in the absence of SHH-N for the first 12 hr followed by addition of 4 nM SHH-N for an additional 25 hr did not contain floor plate cells or motor neurons at 37 hr (Figures 3B and 3F). Thus, neural plate cells rapidly lose the competence to generate floor plate cells and motor neurons unless exposed to SHH during this critical early period. Repression of PAX7 expression was detected in all cells in explants exposed to 4 nM SHH for 12 hr, whereas in the absence of SHH, maintained PAX7 expression was detected in over 95% of cells (data not shown).

These observations raised the issue of whether the concentration of SHH necessary to maintain the competence of neural plate cells for floor plate and motor neuron generation during this early period is the same as that required for the eventual differentiation of these ventral cell types. To test this, neural plate explants were exposed to 0.4 nM SHH-N for 37 hr. At this concentration no floor plate cells or motor neurons were generated (Figures 3C and 3G). In contrast, exposure of neural plate explants to 0.4 nM SHH-N for 12 hr followed by addition of 4 nM SHH-N for an additional 25 hr resulted in the generation of both floor plate cells and motor neurons at 37 hr (Figures 3D and 3H). Exposure of explants to 0.4 nM SHH-N for 12 hr repressed PAX7 expression in ~50% of cells (data not shown).

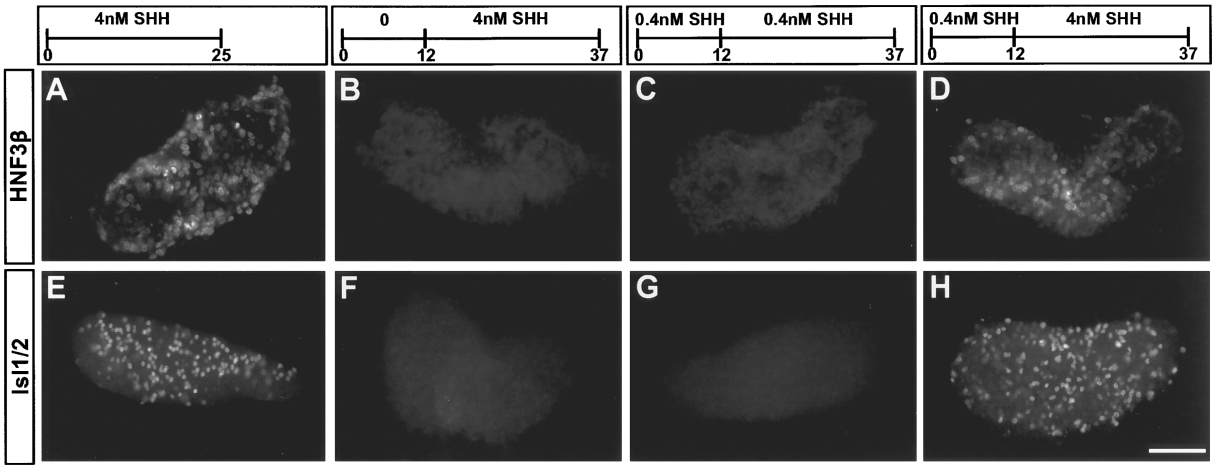

Figure 3. Early Requirement for SHH in Floor Plate and Motor Neuron Generation

(A and E) Floor plate cells (A) and motor neurons (E) are generated in stage 10 [i] explants grown in 4 nM SHH-N for 25 hr (150 ± 17 HNF3β$^+$ cells, n = 5; 118 ± 14 Isl1/Isl2$^+$ cells, n = 6) or 37 hr (168 ± 15 HNF3β$^+$, n = 7; 375 ± 40 Isl1/Isl2$^+$ cells, n = 4).

(B and F) Neither floor plate cells (B) (0 HNF3β$^+$ cells, n = 6) nor motor neurons (F) (1 ± 0 cells, n = 7) are generated when [i] explants are grown for 12 hr in the absence of SHH-N followed by 25 hr in 4 nM SHH-N. Similar results were obtained when the period in 4 nM SHH-N was extended to 33 hr (2 ± 1 Isl1/Isl2$^+$ cells, n = 6) or with 12 nM SHH-N (1 ± 1 Isl1/Isl2$^+$ cells; n = 5).

(C and G) Neither floor plate cells (C) (0 HNF3β$^+$ cells, n = 6) nor motor neurons (G) (0 cells, n = 6) are generated when [i] explants are grown for 37 hr in 0.4 nM SHH-N.

(D and H) Floor plate cells (D) (83 ± 10 HNF3β$^+$ cells, n = 7) and motor neurons (H) (211 ± 32 Isl1/Isl2$^+$, n = 7) are generated when [i] explants are grown for 12 hr in 0.4 nM SHH-N followed by 25 hr in 4 nM SHH-N.

These observations provide evidence that naive neural plate cells require exposure to SHH, acting at a low concentration threshold, during a critical period soon after neural plate formation to maintain the capacity to generate floor plate cells and motor neurons. They also show that the early repression of PAX7 expression by neural plate cells predicts their competence for later floor plate and motor neuron generation.

Ventralized Progenitors Require Additional SHH Signaling for Motor Neuron Generation

We next examined whether neural cells that have been converted to a ventralized state in vivo are able to differentiate into floor plate cells or motor neurons in the absence of further SHH signaling. Ventral midline neural plate tissue (f region, see Figure 2E) isolated from stage 10 embryos generated numerous HNF3β$^+$ and SHH$^+$ cells when grown alone or in the presence of H4 IgG for 24 hr (Figures 4A–4D). This result indicates that midline cells have been exposed to SHH for a period of time sufficient to specify floor plate identity shortly after neural plate formation.

To determine whether the early exposure of adjacent ventral neural cells to SHH is also sufficient to specify motor neuron identity, ventral neural tube explants devoid of midline tissue (v region, Figure 2E) were isolated at stage 12 and grown in vitro for 24 hr. Cells in such explants did not express HNF3β nor *Shh* mRNA (Figures 4G and 4H) and did not contain detectable SHH immunoreactivity (Figures 2A and 2C; data not shown). Nevertheless, these explants generated ~50 motor neurons (Figure 4E), raising the possibility that some ventralized progenitors are able to differentiate into motor neurons independent of further SHH signaling. This is not the case, however, since motor neuron generation in such explants was blocked by addition of H4 IgG (Figure 4F). Thus, the differentiation of ventralized progenitor cells into motor neurons requires additional SHH signaling. In addition, since SHH synthesis is restricted to notochord and floor plate cells, the blockade of motor neuron generation by anti-SHH antibodies provides strong evidence that the SHH protein required locally for the conversion of ventralized progenitor cells into motor neurons is obtained by diffusion from midline cells.

Prolonged Requirement for Floor Plate–Derived SHH in the Conversion of Ventralized Progenitors into Motor Neurons

To test whether floor plate cells can serve as a source of SHH required for the conversion of adjacent ventralized progenitors into motor neurons, we isolated explants comprising the prospective or definitive floor plate together with flanking ventral neural tissue from stage 10–17 embryos and analyzed floor plate and motor neuron differentiation after 24–48 hr in vitro. HNF3β$^+$ floor plate cells and SHH expression were detected in these explants, and many motor neurons were generated (Figure 5). Addition of H4 IgG completely blocked motor neuron generation in explants derived from stage 10–12 embryos (Figures 5A and 5B). In contrast, floor plate differentiation, assessed by HNF3β and SHH expression, was not inhibited (Figures 4A–4D; data not shown). Thus, the blockade of motor neuron differentiation in the presence of anti-SHH antibodies does not result from the inhibition of floor plate differentiation or of SHH expression.

Motor neuron generation was markedly reduced but not completely abolished by addition of H4 IgG to explants derived from stage 13 and 14 embryos (Figures 5A and 5B). This observation raised the question of why some ventral progenitor cells in explants isolated at stage 13 (about 2 hr before the appearance of Isl1/Isl2$^+$

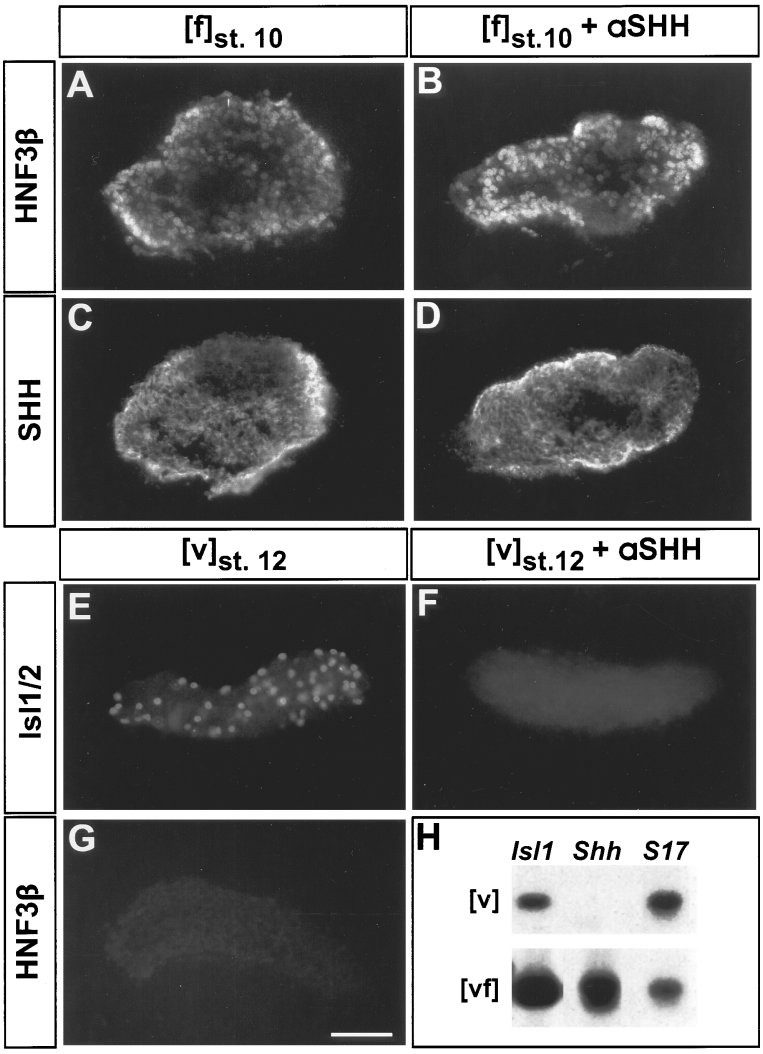

Figure 4. Ventralized Progenitor Cells Require Additional SHH Signaling for Motor Neuron Generation

(A and B) HNF3β$^+$ cells are generated in stage 10 [f] explants grown for 36 hr in the absence (A) (211 ± 19 cells; n = 6) or presence (B) (171 ± 16 cells, n = 6) of H4 IgG.

(C and D) SHH-immunoreactive cells are detected in [f] explants grown for 24 hr in the absence (C) or presence (D) of H4 IgG.

(E) Stage 12 [v] explants isolated in the absence of floor plate cells generate motor neurons (54 ± 10 cells, n = 7 explants) after 36 hr.

(F) Stage 12 [v] explants grown in the presence of anti-SHH H4 IgG do not generate motor neurons (0 cells, n = 7 explants).

(G) Stage 12 [v] explants do not contain HNF3β$^+$ cells.

(H) RT–PCR analysis of *Isl1*, *Shh*, and *S17* expression in stage 12 [v] or [vf] explants grown for 24 hr in vitro. This RT–PCR assay is sensitive at *Shh* transcript levels 200 fold below those present in floor plate cells. Similar results were observed in two assays.

Scale bar in (A)–(D), 70 μm; in (E), (G), and (H), 60 μm.

motor neurons; Figure 5B; data not shown) were able to generate motor neurons in a SHH-independent manner. We reasoned that the SHH-independent progenitors might be most advanced in their developmental program and destined to give rise to the first motor neurons detected in vivo. A prediction of this reasoning is that the first motor neurons to be generated in vitro in stage 13 floor plate/ventral explants should be insensitive to blockade by anti-SHH antibodies. Consistent with this premise, the generation of motor neurons over the first 10 hr period was not inhibited by the addition of H4 IgG, whereas motor neuron generation over the subsequent 26-hr period was completely blocked (Figure 6A). The lack of an early inhibitory effect of H4 IgG does not result from a delay in the blockade of SHH signaling, since motor neuron generation over the first 10 hr in stage 12 floor plate/ventral explants was inhibited (Figure 5B and data not shown). These results suggest that the inability of H4 IgG to block motor neuron generation completely at stages 13 and 14 can be explained by the presence of increasing numbers of advanced progenitors. Motor neuron generation was also inhibited, albeit less markedly, in explants isolated at stages 15–17 (Figure 5B), a time at which the first postmitotic motor neurons have already been generated. These results indicate that floor plate–derived SHH is required over a prolonged period for the conversion of ventralized progenitors into motor neurons.

Motor Neuron Progenitors Maintain a Dependence on SHH Late into S Phase of Their Final Cell Division

Ventralized progenitor cells lose their dependence on SHH signaling only a few hours before the appearance of the first postmitotic motor neurons (see Figure 5B), and thus probably within their final cell division cycle (Langman et al., 1966). To determine more precisely the stage of the cell cycle at which the SHH dependence of ventralized progenitors is lost, we maintained stage 13 floor plate/ventral explants in vitro in the continued presence of bromodeoxyuridine (BrdU) and analyzed the proportion of motor neurons that had incorporated BrdU after 36 hr. In the presence of H4 IgG, ~40 motor neurons were generated (see Figure 5B). Of these, 83% had not incorporated BrdU (Figures 6B–6E), indicating that the vast majority of SHH-independent motor neuron progenitors had completed their final round of DNA synthesis. The remaining 17% showed low levels of BrdU

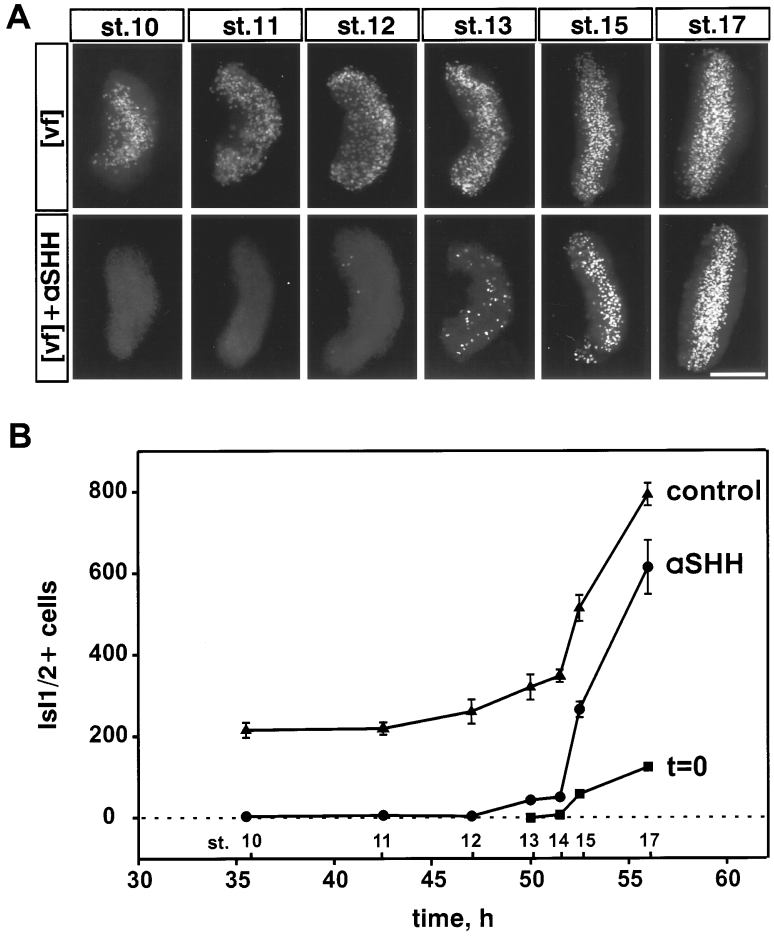

Figure 5. Prolonged Requirement for SHH in the Conversion of Ventral Progenitors into Motor Neurons

(A and B) Isl1/Isl2+ cells in [vf] explants comprising midline and adjacent ventral neural cells grown in vitro for 24–48 hr, in the absence (control) or presence (aSHH) of H4 IgG. (B) shows also the number of Isl1/Isl2$^+$ present in the explant at the time of isolation (t = 0). Values in (B) represent mean + SEM for five explants.

Scale bar in (A), 180 μm.

labeling (Figures 6B–6E), suggesting that these cells were late in their final S phase at the time that they attained independence of SHH signaling. In the absence of H4 IgG, ~300 motor neurons were generated (Figure 5B), of which 86% incorporated BrdU, mostly at high levels (Figures 6B and 6F–6H). The 14% of motor neurons that had failed to incorporate BrdU in explants grown without H4 IgG (Figure 6B) presumably derived from the SHH-independent ventral progenitors revealed in the presence of H4 IgG. Taken together, these results indicate that motor neuron progenitors depend on local SHH signaling until late into their final division cycle.

Ventralized Progenitors Differentiate into Interneurons When SHH Signaling Is Blocked

Blocking the late period of SHH signaling prevents motor neuron generation, raising the issue of the fate of progenitors in this condition. We have considered three possible fates: reversion to a naive state, death, and the adoption of alternative neuronal fates.

Two lines of evidence indicate that ventralized progenitors do not revert to a naive state under conditions in which the late phase of SHH signaling is blocked. First, as described above, PAX7 was not reexpressed in stage 12 ventral explants grown in the presence of anti-SHH antibodies (see Figure 2O). Second, stage 12 ventral or floor plate/ventral explants grown in the presence of both H4 IgG and BMP4 did not give rise to neural crest cells (data not shown), a predicted outcome (Liem et al., 1995) if these cells had reverted to a naive state. Moreover, ventralized progenitor cells appear not to die after blockade of SHH signaling. Incubation of stage 12 floor plate/ventral explants with acridine orange, a marker of pyknotic cells, revealed no difference in the number of labeled cells in the presence and absence of H4 IgG (data not shown).

In view of these results, we determined whether ventralized progenitors might generate interneurons rather than motor neurons under conditions in which the late period of SHH signaling is blocked. To assess interneuron differentiation, we monitored a class of Lim1/Lim2$^+$ interneurons that is generated over the same period as motor neurons in the intermediate region of the neural tube, adjacent to the motor neuron domain (Figure 7K; Tsuchida et al., 1994). Stage 12 floor plate/ventral explants gave rise to many motor neurons and virtually no Lim1/Lim2$^+$ interneurons when grown for 36 hr in vitro (Figures 7A and 7D). In contrast, in the presence of H4 IgG, the blockade of motor neuron differentiation (Figure 7B) was accompanied by the generation of ~150 Lim1/Lim2$^+$ interneurons (Figure 7E). Motor neuron generation in these explants was also blocked by forskolin (Figure 7C), a compound that appears to inhibit the transduction of SHH signals by activating protein kinase A (Fan et al., 1995; Perrimon, 1995), and in this condition also, ~150 Lim1/Lim2 interneurons were generated (Figure 7F). These findings show that ventralized progenitor

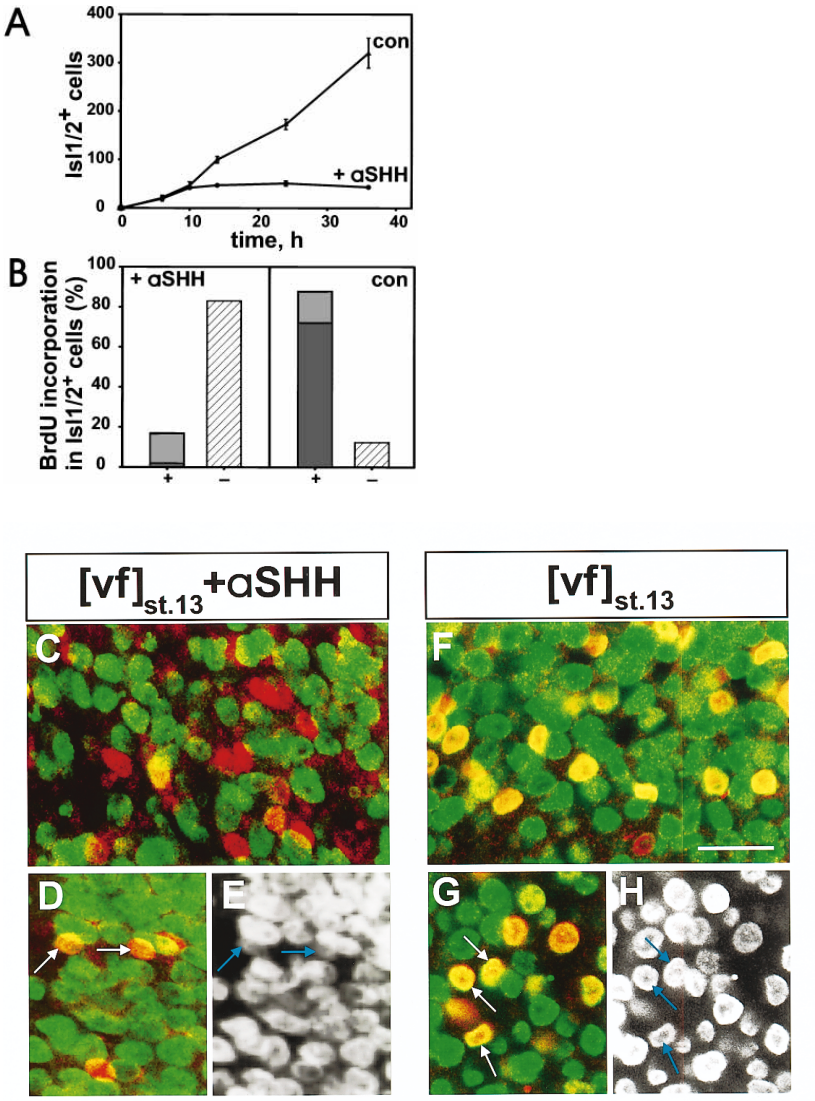

Figure 6. Dependence of Ventralized Progenitors on SHH Signaling Persists Until Late in their Final Cell Cycle

(A) Analysis of Isl1/Isl2 motor neuron generation in stage 13 [vf] explants grown in vitro in the presence (anti-SHH) or absence (control) of H4 IgG.

(B) BrdU incorporation into Isl1/Isl2$^+$ neurons in stage 13 [vf] explants grown in the presence or absence of H4 IgG. Shaded column indicates Isl1/Isl2$^+$ neurons that have incorporated BrdU. Light shading indicates cells that incorporated low levels of BrdU (low is defined subjectively as a labeling intensity below one third that of the most intensely labeled cells). Dark shading indicates cells that incorporated high levels of BrdU. Hatched column indicates Isl1/Isl2$^+$ neurons that did not incorporate BrdU. Histograms are derived from analysis of 155 Isl1/Isl2$^+$ neurons in eight explants grown in H4 IgG and from 318 Isl1/Isl2$^+$ neurons in six control explants.

(C–E) Stage 13 [vf] explants grown in vitro in the presence of BrdU for 36 hr in H4 IgG. BrdU$^+$ cells are green and Isl1/Isl2$^+$ motor neurons red. Of Isl1/Isl2$^+$ neurons, 83% had not incorporated BrdU (C, D). The 17% of Isl1/Isl2$^+$ neurons that were double-labeled incorporated low levels of BrdU (D, E).

(E) BrdU labeling of the field shown in (D) colabeled with anti Isl1/Isl2. Labeling is very weak in motor neurons that have incorporated BrdU (blue arrows).

(F–H) In the absence of H4 IgG, most Isl1/Isl2$^+$ motor neurons had incorporated BrdU (yellow cells) (F, G).

(H) BrdU labeling of the field shown in (G) colabeled with anti-Isl1/Isl2. Virtually all Isl1/Isl2$^+$ motor neurons express high levels of BrdU (blue arrows).

Scale bar, 18 μm.

cells generate Lim1/Lim2 interneurons rather than motor neurons when the late period of SHH signaling is blocked. However, in contrast with motor neurons, the conversion of naive neural plate cells into ventralized progenitors appears not to be a prerequisite for the differentiation of Lim1/Lim2 interneurons, since such neurons were generated (166 ± 27, Lim1/Lim2$^+$ cells, n = 8) in [i] explants grown alone for 36 hr.

These results raise the possibility that a common ventralized progenitor generates motor neurons or interneurons as a function of the ambient SHH concentration. We have not tested this possibility directly. However, one prediction of this idea is that when the SHH concentration is close to the threshold for motor neuron generation, the selection of distinct interneuron or motor neuron fates might be perturbed, with the possible consequence that markers that normally define motor neurons and interneurons might be coexpressed in the same neuron. To test this idea, [i] explants were exposed for 36 hr to 1.2 nM SHH-N, a concentration just above the threshold for induction of motor neurons (Roelink et al., 1995) that resulted in the generation of ~30 Isl1/Isl2$^+$ neurons and ~170 Lim1/Lim2$^+$ interneurons (Figure 7H). Remarkably, 85% of the Isl1/Isl2+ neurons coexpressed Lim1/Lim2 (Figure 7H). Such double-labeled cells were not detected when the SHH-N concentration was increased or decreased ~3-fold (Figures 7G and 7I). Indeed, raising the SHH concentration ~3-fold resulted in the generation of ~300 Isl1/Isl2$^+$ neurons and almost completely suppressed the generation of Lim1/Lim2 interneurons (Figure 7I). Moreover, in [i] explants grown in contact with the notochord, ~5% of Isl1/Isl2 neurons coexpressed Lim1/Lim2, and these cells were located close to the junction of the motor neuron and Lim1/Lim2 interneuron populations (Figure 7J).

These observations prompted us to examine whether such double-labeled neurons could be detected in vivo. Analysis of the neural tube from stages 15 to 19 revealed that ~4% of Isl1/Isl2 neurons coexpressed Lim1/Lim2, but cells of this phenotype were not detected at later stages (Figures 7K and 7L). Strikingly, the cells that

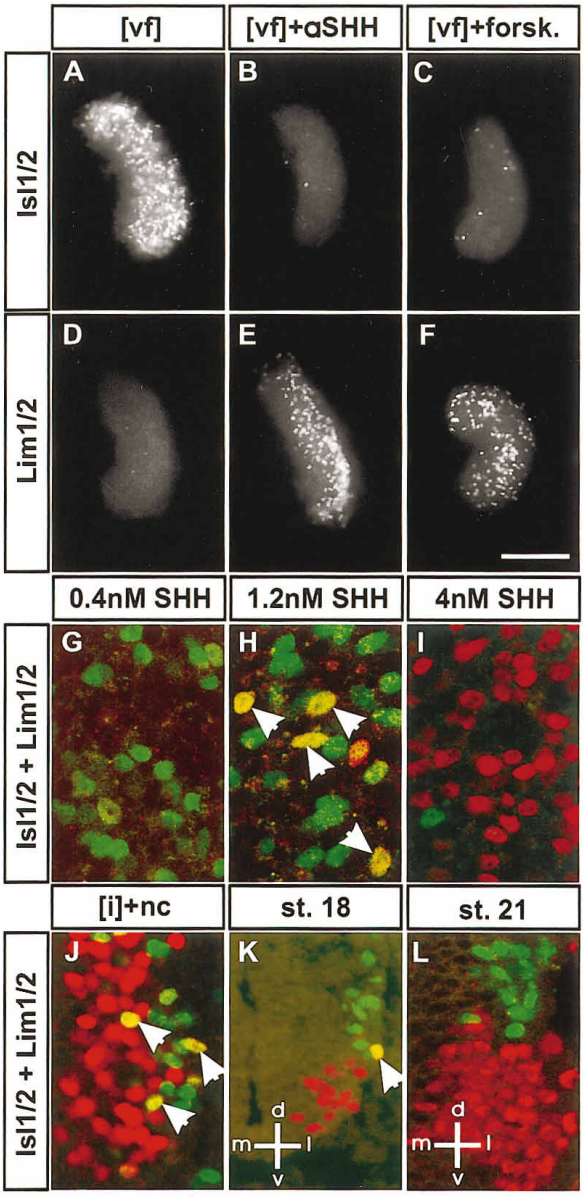

Figure 7. Selection of Motor Neuron and Interneuron Fates by Ventralized Progenitors Is Dependent on Late SHH Signaling

(A and D) Stage 12 [vf] explants grown in vitro for 36 hr generate Isl1/Isl2$^+$ motor neurons (260 ± 30, n = 5; [A]) but few Lim1/Lim2 interneurons (2 ± 1 cells, n = 5; [D]).

(B and E) Stage 12 [vf] explants grown in vitro for 36 hr generate few if any Isl1/Isl2 motor neurons (4 ± 1, n = 5; [C]) but many (158 ± 21, n = 5) Lim1/Lim2 interneurons (E).

(C and F) Stage 12 [vf] explants grown in vitro for 36 hr in forskolin (25 μM) generate few Isl1/Isl2$^+$ motor neurons (7 ± 2, n = 5; [C]) but many (155 ± 16, n = 5) Lim 1/Lim2 interneurons (F).

(G) In the presence of SHH-N (0.4 nM), Lim1/Lim2 neurons (191 ± 30, n = 6) but not Isl1/Isl2 neurons are generated.

(H) In the presence of SHH-N (1.2 nM), both Isl1/Isl2 neurons (28 ± 7, n = 5) and Lim1/Lim2 neurons (171 ± 19; n = 5) are generated, and 85% of the Isl1/Isl2$^+$ neurons (n = 70 cells) coexpress Lim1/Lim2 (arrows).

(I) In the presence of SHH-N (4 nM), many (280 ± 30, n = 5) Isl1/Isl2 neurons and few Lim1/Lim2 neurons (1 ± 1, n = 7) are generated. No double labeled cells are detected.

(J) Coexpression of Lim1/Lim2 is detected in ~5% of Isl1/Isl2$^+$ neurons in [i] explants grown in contact with the notochord for 36 hr. Double-labeled cells are located at the border of the Isl1/Isl2 and Lim1/Lim2 neuronal populations.

(K) Coexpression of Isl1/Isl2 and Lim1/Lim2 (yellow cell, arrow) in stage 18 neural tube. Double labeled cells are located at the border between the motor neuron and interneuron populations and are detected only between stages 15 and 19.

(L) Absence of double-labeled cells at stage 21.

Scale bar in (A)–(F), 150 μm; in (G)–(I), 30 μm; in (J), 40 μm; in (K) and (L), 50 μm.

coexpressed Isl1/Isl2 and Lim1/Lim2 were invariably found at the border of the motor neuron and Lim1/Lim2 interneuron populations (Figure 7K).

These results suggest that the selection of motor neuron and interneuron fates in the ventral neural tube is normally controlled by the ambient SHH concentration at the time of the final division of ventralized progenitor cells. They are also consistent with, but do not prove, the idea that motor neurons and Lim1/Lim2 interneurons can derive from a common ventralized progenitor cell.

Discussion

The present studies establish that SHH-mediated signaling is required for the induction of floor plate differentiation. They also provide evidence that motor neuron generation depends on two critical periods of long-range SHH signaling. During the early period, SHH derived from the notochord is required to convert naive neural plate cells into a ventralized progenitor state that permits the subsequent generation of motor neurons (Figure 8). Such ventralized progenitors, however, are able to generate motor neurons only when exposed to a second period of SHH signaling that persists late into the final division cycle of the progenitor cell. Ventralized progenitor cells generate interneurons rather than motor neurons when this late period of SHH signaling is blocked. Taken together, these results establish a key role for SHH in the induction and patterning of floor plate cells, motor neurons, and interneurons in the ventral neural tube.

The late dependence of motor neuron progenitors on SHH signaling argues against a simple cascade model (Roelink et al., 1995) in which SHH induces the expression of an intermediary diffusible factor that is sufficient to induce motor neurons. If such a factor were to exist, its expression in neuroepithelial cells would have to be dependent on maintained SHH signaling and its activity extremely labile. Our results do not preclude the existence of an intermediary factor that acts in cooperation with SHH to specify motor neuron identity.

Neural Plate Cells Require Early Exposure to SHH for Motor Neuron Generation

Our in vitro studies provide evidence that neural plate cells require exposure to SHH within a critical early period, soon after neural plate formation, in order to maintain their competence to generate motor neurons. The SHH required during this early period derives from the notochord, since SHH expression by cells at the ventral midline of the neural tube is not detected until after

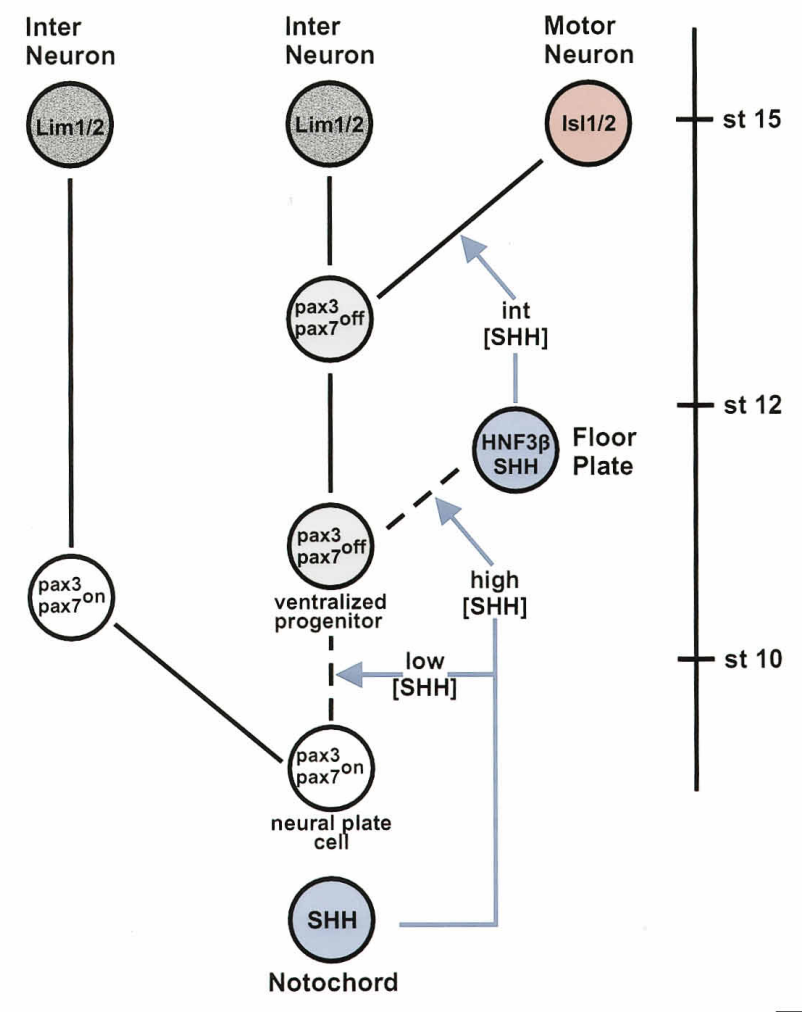

Figure 8. Sequential SHH-Dependent Steps in the Induction of Distinct Cell Types in the Ventral Neural Tube

The model summarizes the cellular source and threshold concentrations of SHH that regulate the differentiation of neural plate cells into floor plate cells, motor neurons, and Lim1/Lim2 interneurons.

SHH derived from the notochord acts at a low concentration threshold (~0.4 nM in vitro) to convert naive neural plate cells into ventralized progenitor cells. This conversion process is marked by the loss of *pax3* and PAX7 expression. Ventralized progenitors at the midline of the neural plate respond to a high local concentration of SHH from the notochord with the generation of floor plate cells (Roelink et al., 1995). The SHH-mediated conversion of naive neural plate cells into floor plate can occur in the absence of cell division (Placzek et al., 1993), as indicated by the broken lines. The generation of motor neurons from ventralized progenitors requires SHH signaling at an intermediate (int) concentration threshold (≥1.2 nM in vitro). The relevant source of SHH for converting ventralized progenitors into motor neurons is likely to be the floor plate. Ventralized progenitors differentiate into Lim1/Lim2 interneurons when the late period of SHH signaling is reduced below the threshold for motor neuron generation. Naive neural plate cells can, however, generate Lim1/Lim2 interneurons without being converted to a ventralized state.

The model depicts a common ventralized progenitor for floor plate cells, motor neurons, and Lim1/Lim2 interneurons. There is evidence that individual neural plate cells differentiate into floor plate cells or motor neurons as a function of the SHH concentration to which they have been exposed (Roelink et al., 1995) but it remains to be established whether motor neurons and Lim1/Lim2 interneurons derive from a common ventralized progenitor cell. The approximate time (HH stages) at which these inductive events occur (at cervical levels) is shown on the right hand side of the figure.

neural cells have acquired a stable ventralized progenitor state.

At present, the only functional indication that neural cells have attained a ventralized state is their ability to generate floor plate cells and motor neurons upon later exposure to SHH. However, the extinction of PAX7 expression by neural plate cells parallels closely their competence to generate ventral cell types, and thus PAX7 appears to provide a valid marker of the conversion of neural plate cells to a ventralized state. The expression of other homeobox genes, notably *pax3*, is also repressed by the notochord and by SHH (Goulding et al., 1993; Liem et al., 1995). Moreover, forced ventral expression of *pax3* in transgenic mice inhibits floor plate differentiation (Tremblay et al., 1996). Taken together, these observations suggest that a critical early step in the SHH-mediated induction of floor plate and motor neuron differentiation is the repression of expression of *pax3* and PAX7.

Ventralized Progenitors Require SHH Signaling to Generate Motor Neurons

The second critical period for SHH signaling in motor neuron generation occurs after naive neural plate cells have been converted into ventralized progenitor cells. Our in vitro studies show that ventralized progenitors deprived of SHH signaling are unable to generate motor neurons. Moreover, BrdU labeling studies indicate that ventralized progenitors maintain their dependence on SHH until late into S phase of the division cycle that precedes the generation of postmitotic motor neurons. The late-dependence on SHH signaling in motor neuron generation sets a constraint on the time at which motor neuron identity is determined, suggesting that it cannot be before late S phase of the final progenitor cell division. These observations are concordant with studies of neurogenesis in the mammalian cerebral cortex, which have shown that the laminar identity of cortical neurons is determined late in the final progenitor cell division cycle (McConnell and Kaznowski, 1991).

Our results suggest that in higher vertebrates the floor plate is the relevant source of SHH required for the conversion of ventralized progenitors into motor neurons. SHH expression is detected in the floor plate by stage 12, and some motor neuron progenitors remain SHH-dependent until at least stage 17, by which time the notochord has been displaced a considerable distance from the ventral neural tube. The late requirement for

SHH signaling in motor neuron generation also suggests a reason, at least in higher vertebrates, for the homeogenetic transfer of SHH expression from the notochord to floor plate cells. In lower vertebrates such as zebrafish, however, the first (primary) motor neurons are generated during gastrulation (Korzh et al., 1993; Kimmel et al., 1994), and the notochord retains contact with the ventral neural tube for a prolonged period. Thus, SHH derived from the axial mesoderm may be sufficient to generate motor neurons in lower vertebrates. Consistent with this idea, motor neurons are present in zebrafish embryos carrying the *cyclops* mutation (Hatta et al., 1991; Hatta, 1992), in which *HH* genes are not expressed at the midline of the neural plate (Krauss et al., 1993; Ekker et al., 1995).

Motor Neuron Generation Requires Long-Range SHH Signaling from the Notochord and Floor Plate

The present studies provide evidence that SHH signaling operates at a distance during both the early and late critical periods. During the early period, the domain of the neural plate over which PAX7 expression is repressed extends ~10 cell diameters from the notochord, a distance that may indicate the range of SHH action. In support of this, an elevated level of *patched* mRNA, diagnostic of exposure of cells to HH signaling in flies and vertebrates (Perrimon, 1995; Goodrich et al., 1996), is detected in the medial neural plate and ventral neural tube in a domain similar to that in which PAX7 expression is repressed (Goodrich et al., 1996; Marigo and Tabin, 1996).

Similarly, the late conversion of ventral progenitors into motor neurons appears to depend on long-range SHH signaling from the floor plate. Ventral neural tube explants isolated from floor plate cells retain a dependence on local SHH signaling for the generation of motor neurons. Since *Shh* mRNA is not expressed in ventral explants, the SHH protein present in explants appears to have derived by diffusion from a midline source, presumably the floor plate. Diffusible forms of SHH derived initially from the notochord and later from the floor plate are likely therefore to act at a distance to promote the generation of motor neurons.

SHH Signaling Directs Neuronal Fate

The state of SHH signaling during the final division of ventralized progenitor cells appears to determine whether motor neurons or interneurons are generated. Under conditions of SHH signaling, ventralized progenitors differentiate into motor neurons, but when SHH signaling is blocked, Lim1/Lim2 interneurons rather than motor neurons are generated. In vitro, ventralized progenitors appear to generate these two distinct neuronal cell types in response to changes in the SHH-N concentration of only ~3-fold. The establishment of distinct dorsoventral domains of the neural tube within which motor neurons and Lim1/Lim2 interneurons are generated is likely therefore to be controlled by the spatial extent of SHH signaling immediately prior to the onset of neuronal differentiation.

Our results have not resolved whether the SHH-dependent selection of distinct neuronal fates is exerted at the level of a common ventralized progenitor cell. Lineage tracing studies in the chick neural tube have shown that ventral progenitor cells frequently generate clones that include motor neurons and interneurons (Leber et al., 1990). Moreover, the coexpression by single neurons of LIM homeodomain protein markers of motor neuron and interneuron fates when the SHH concentration is at the threshold for motor neuron generation can most easily be explained if these distinct neuronal fates are normally available to an individual ventralized progenitor cell. In vivo, such double-positive cells are detected transiently at the junction of the motor neuron and interneuron populations, a position at which the local SHH concentration is likely to be close to the threshold for motor neuron generation.

Whether the Lim1/Lim2 interneurons that are generated in the intermediate region of the neural tube derive exclusively from ventralized progenitor cells remains unclear. Lim1/Lim2 interneurons can derive from ventralized progenitors if the late period of SHH signaling is blocked but can also be generated from naive neural plate cells that have not been exposed to SHH. Thus, the SHH-mediated conversion of naive neural plate cells to a ventralized progenitor state may not be a prerequisite for the generation of Lim1/Lim2 interneurons (Figure 8). Alternatively, the interneurons generated from naive and ventralized progenitors may represent distinct neuronal classes that share expression of Lim1/Lim2.

Finally, the present studies show that the SHH-N concentration threshold required to maintain the competence of neural plate cells for motor neuron generation is ~3-fold lower than the concentration threshold required later for the acquisition of motor neuron fate by ventralized progenitors. In turn, the SHH-N concentration threshold for induction of floor plate differentiation is ~3-fold greater than that for motor neuron generation (Roelink et al., 1995). Taken together, these findings suggest that different SHH-N concentration thresholds operate at sequential periods during the generation of a single neuronal cell type (Figure 8). They also support the idea that in vivo, SHH controls the identity and pattern of cell types generated in the ventral neural tube through actions at multiple concentration thresholds.

Experimental Procedures

Generation of Anti-SHH-N Antibodies

A cDNA clone encoding SHH-N (Porter et al., 1995) (residues 1–198 of rat SHH; Roelink et al., 1994) was cloned into a baculovirus expression vector (Invitrogen), and SHH-N protein derived from Tn-5B1–4 cells (Wickham et al., 1992) was purified (Porter et al., 1995). Monoclonal anti-SHH antibody 5E1 (IgG1 isotype) was obtained by fusion of spleen cells from SHH-N-immunized mice with NS1 myeloma cells. IgG fractions were obtained by affinity purification on protein A–agarose. H4 IgG fraction was used at a final concentration of 250 μg/ml, SHH-N affinity-purified H4 IgG at 50 μg/ml, and protein A–purified 5E1 IgG at 2.5 μg/ml, with similar results.

SHH-N Protein

SHH-N protein was purified as described above. The SHH-N concentration was determined by amino acid analysis after separation of the ~20 kDa SHH-N protein by SDS–polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose membranes. Protein concentrations indicated are based on a transfer efficiency of 100%. Average protein recovery after transfer, determined by comparison with Escherichia coli–derived mouse SHH-N (provided

by P. Beachy) is ≥50%; thus, the actual SHH-N protein concentration used in assays may be greater by a factor of ≤2.

Immunocytochemistry

Immunocytochemical localization of proteins was performed at the level of the twelfth somite as described (Yamada et al., 1991). SHH was detected with MAb 5E1, HNF3β with MAb 4C7 anti-chick HNF3β (A. Ruíz i Altaba, S. M., and T. J., unpublished data), PAX7 (Jostes et al., 1990) with a monoclonal anti-chick PAX7 antibody that does not react with PAX3 (A. K., unpublished data), Isl1/Isl2 with MAb 4D5 and rabbit antibody K5 (Tsuchida et al., 1994), and Lim1/Lim2 with MAb 4F2 and rabbit antibody T2 (Tsuchida et al., 1994). Coexpression of proteins was detected by using a Bio-Rad 600 confocal microscope.

Neural Plate Explant Assay

Neural plate, neural tube, and notochord tissue from Hamburger and Hamilton (1951) (HH) stage 10–17 embryos was isolated from the 12-somite level, independent of stage, and grown in vitro (Yamada et al., 1993). BMP4 protein was obtained as described (Liem et al., 1995).

BrdU Incorporation Studies

BrdU incorporation into neural plate cells was analyzed by labeling explants with 170 nM BrdU. Double-labeling experiments were performed with MAb 4D5 and Cy3-conjugated goat anti-mouse IgG (Jackson Labs) and FITC-conjugated anti-BrdU (Becton Dickinson) and analyzed on a Bio-Rad 600 confocal microscope.

RT–PCR Assay v

Analysis of *Isl1*, *Shh*, and *S17* expression was performed as described (Tanabe et al., 1995).

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Note Added in Proof

Analysis of the neural phenotype of mice lacking *Sonic Hedgehog* function has also provided evidence that SHH is required to initiate cell patterning in the ventral neural tube: Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy, P.A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic Hedgehog* gene function. Nature *383*, 407–413.