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Sonic Hedgehog Induces the Differentiation of Ventral Forebrain Neurons: A Common Signal for Ventral Patterning within the Neural Tube

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Summary

The vertebrate *hedgehog*-related gene *Sonic hedgehog* (*Shh*) is expressed in ventral domains along the entire rostrocaudal length of the neural tube, including the forebrain. We show here that SHH induces the differentiation of ventral neuronal cell types in explants derived from prospective forebrain regions of the neural plate. Neurons induced in explants derived from both diencephalic and telencephalic levels of the neural plate express the LIM homeodomain protein Isl-1, and these neurons possess distinct identities that match those of the ventral neurons generated in these two subdivisions of the forebrain in vivo. A single inducing molecule, SHH, therefore appears to mediate the induction of distinct ventral neuronal cell types along the entire rostrocaudal extent of the embryonic central nervous system.

Introduction

In vertebrate embryos, the patterning of the nervous system is initiated by inductive signals that direct the fate of neural progenitor cells. The complex pattern of cell types generated within the neural tube is thought to involve the action of signals that impose regional character on cells at different rostrocaudal positions within the neural plate (Doniach et al., 1992; Ruiz i Altaba, 1992) and that define the identity of cells along the dorsoventral axis of the neural tube (Jessell and Dodd, 1992; Basler et al., 1993; Smith, 1993). Thus, the fate of neural progenitor cells depends on their position along the rostrocaudal and dorsoventral axes of the neural tube (Roach, 1945; Jacobson, 1964; Simon et al., 1995).

The mechanisms that control the differentiation of cell types along the dorsoventral axis of the neural tube have been examined in most detail at caudal levels of the neuraxis. In the spinal cord, the differentiation of ventral cell types is initiated by signals transmitted from axial mesodermal cells of the notochord to overlying neural plate cells, inducing the differentiation of floor plate cells at the ventral midline and motor neurons more laterally within the neural tube (van Straaten et al., 1988; Placzek et al., 1990, 1991; Yamada et al., 1991, 1993; Goulding et al., 1993). At later stages, similar or identical signaling properties are acquired by floor plate cells (Hatta et al., 1991; Yamada et al., 1991; Placzek et al., 1993). The identity of the ventral neuronal cell types that are generated in response to midline-derived signals, however, appears to depend on the position of origin of neuronal progenitor cells along the rostrocaudal axis. For example, serotonergic neurons are induced at the level of the rostral rhombencephalon (Yamada et al., 1991), whereas dopaminergic neurons are induced at the level of the mesencephalon (Hynes et al., 1995).

At caudal levels of the neuraxis, a vertebrate homolog of the secreted protein encoded by the Drosophila gene *hedgehog* (Nüsslein-Volhard and Wieschaus, 1980; Lee et al., 1992), Sonic hedgehog (SHH), also termed Vhh-1 or Hhg-1, has been implicated in the induction of ventral cell types. *Shh* is expressed by the notochord and floor plate at the time that these two cell groups exhibit their inductive activities (Riddle et al., 1993; Krauss et al., 1993; Echelard et al., 1993; Chang et al., 1994; Roelink et al., 1994). Furthermore, exposure of neural plate explants to SHH leads to the differentiation of motor neurons in addition to floor plate cells (Roelink et al., 1994, 1995; Tanabe et al., 1995), suggesting that SHH participates in the induction of ventral neurons at caudal levels of the neuraxis.

At most levels of the embryonic forebrain, the notochord and floor plate are absent (Kingsbury, 1930; Puelles and Rubenstein, 1993), and neither the identity nor the source of inductive signals that trigger the differentiation of ventral forebrain neurons has been established. However, *Shh* is expressed by cells at the ventral midline of the embryonic forebrain (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994; Roelink et al., 1994), raising the possibility that this gene participates in the specification of neuronal identity within the forebrain as well as at more caudal levels of the neuraxis. To address this issue, we first defined transcription factors that permit the identification of ventral neuronal cell types generated in diencephalic and telencephalic subdivisions of the forebrain. We then used these markers to assess the ability of SHH to induce the differentiation of distinct ventral neuronal classes in explants derived from levels of the neural plate fated to give rise to the forebrain.

Our results show that SHH induces in neural plate explants neuronal cell types normally generated in the ventral diencephalon and telencephalon. A single inducing molecule, SHH, therefore appears to be responsible for inducing ventral neuronal cell types along the entire rostrocaudal extent of the neuraxis. Our results also suggest that the differentiation of neuronal cell types generated in the ventral region of the telencephalon is induced by an

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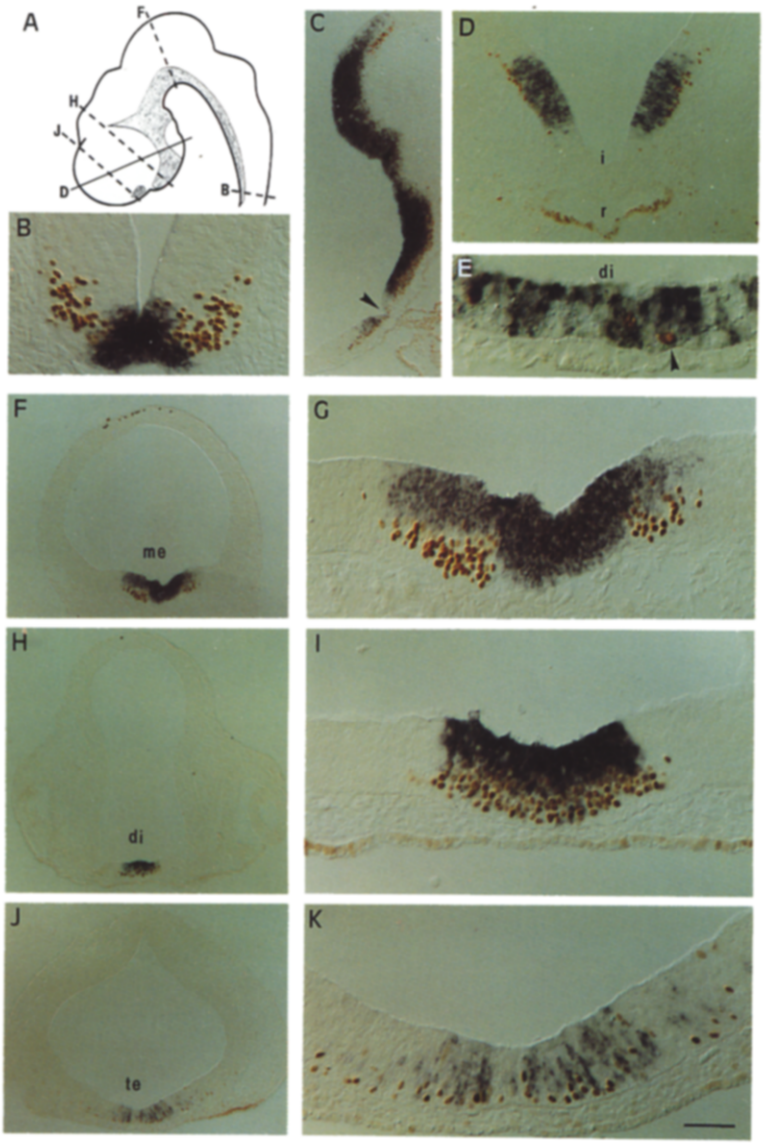

Figure 1. *Shh* and Isl-1 Are Expressed in Adjacent Ventral Domains in the Embryonic Chick Central Nervous System

(A) Sagittal view showing the domain of neural *Shh* expression in a stage 18–19 chick embryo (shaded area). Broken lines indicate the levels and planes of the sections shown in (B)–(K).

(B–K) *Shh* (blue-black) and Isl-1 (brown) expression in adjacent domains of the ventral central nervous system.

(B) A transverse section through the caudal rhombencephalon, showing *Shh* expression at the ventral midline in the floor plate and Isl-1 expression laterally, in motor neurons.

(C) A sagittal section of the neural tube, showing *Shh* and Isl-1 expression in the ventral mesencephalon, diencephalon, and telencephalon. *Shh* expression is detected in the basal telencephalon, rostral to the optic chiasm (arrowhead). Note that there is a region at the rostralmost tip of the ventral diencephalon, abutting the optic chiasm, that does not express *Shh*.

(D) A transverse section through the middiencephalon at the level of infundibulum (i). Cells that express Isl-1 are located at the lateral edge of the domain of *Shh* expression. Isl-1$^+$ cells are absent from the ventral midline at the level of the infundibulum. Cells in Rathke's pouch (r) express Isl-1.

(E) In the rostral diencephalon at stage 13, Isl-1$^+$ cells are interspersed with cells that express *Shh*. The double labeling method does not resolve whether cells coexpress *Shh* and Isl-1 at this stage.

(F) A transverse section through the mesencephalon, showing ventral midline expression of *Shh* and Isl-1. At this axial level, a small number of Isl-1$^+$ sensory neurons can also be detected dorsally, in the trigeminal mesencephalic nucleus.

(G) Higher magnification of (F), showing that Isl-1$^+$ cells are located lateral to the midline domain of *Shh* expression.

(H) A transverse section at the level of the rostral diencephalon, showing ventral midline expression of *Shh* and Isl-1.

(I) Higher magnification of (H). *Shh* is expressed in the ventricular zone, whereas Isl-1$^+$ cells are located basally.

(J) A transverse section at the level of the caudal telencephalon, showing *Shh* and Isl-1 cells in the floor of the telencephalon.

(K) Higher magnification of (J). In the ventral telencephalon, cells that express *Shh* and Isl-1 are more dispersed than at caudal regions of the ventral central nervous system.

Abbreviations: i, infundibulum; di, diencephalon; me, mesencephalon; te, telencephalon.

Scale bar in (B), (G), (I), and (K), 50 μm; in (C), (F), (H), and (J), 200 μm; in (D), 100 μm; in (E), 25 μm.

SHH-mediated signal that originates at the ventral midline of the rostral diencephalon.

Results

Shh and Isl-1 Occupy Adjacent Ventral Domains in the Embryonic Central Nervous System

To examine the involvement of SHH in the patterning of the embryonic forebrain, we first identified early markers of ventral forebrain neurons. At caudal levels of the neuraxis, motor neurons can be identified by expression of Islet-1 (Isl-1) (Karlsson et al., 1990), a LIM homeodomain protein expressed as motor neuron progenitors leave the cell cycle (Ericson et al., 1992; Korzh et al., 1993; Inoue et al., 1993; Tsuchida et al., 1994). In the forebrain, motor neurons are absent, but Isl-1 is expressed by ventral neurons (Thor et al., 1991). We therefore examined the pattern of expression of Isl-1 in the embryonic chick brain and compared it with that of *Shh*.

At stage 18, Isl-1$^+$ cells were found in ventral regions of the telencephalon, diencephalon, and mesencephalon (Figure 1). At each axial level, ventral Isl-1$^+$ cells abutted the domain of expression of *Shh* (Figure 1; see Figure 2Ai for a summary). Isl-1 expression, therefore, defines ventral

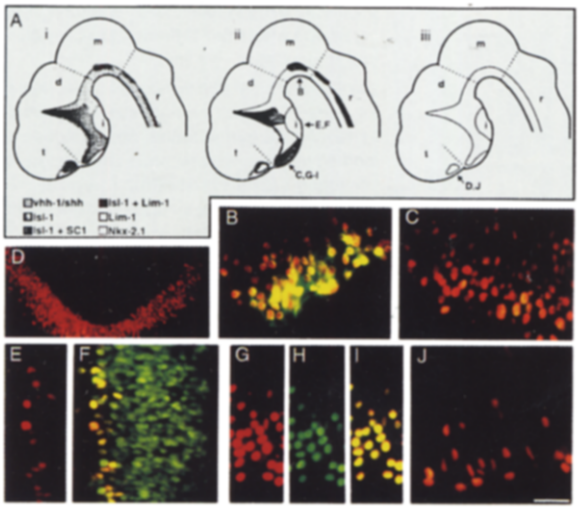

Figure 2. Isl-1 Expression Defines Distinct Populations of Ventral Neurons at Different Rostrocaudal Levels of the Neuraxis

(A) Diagram of a sagittal section of the neural tube of a stage 18–19 chick embryo, showing the domains of expression of cell type markers. Small arrows indicate the plane of sections shown in (B)–(J).

(i) Expression of *Shh* (stippled) and Isl-1 (red), derived from Figure 1.

(ii) Coexpression of markers in Isl-1$^+$ neurons. In the rhombencephalon (r) and mesencephalon (m), ventral Isl-1$^+$ neurons coexpress SC1 (green). In the ventral diencephalon, Isl-1$^+$ neurons are absent from the most caudal region, although Lim-1$^+$ cells (brown) are expressed. In the region of the mid-diencephalon at the zona limitans interthalamica (Puelles et al., 1987), and also at the ventral midline of the rostral diencephalon, most Isl-1$^+$ neurons coexpress Lim-1 (blue). In the intervening region, Isl-1 and Lim-1 are expressed in separate but intermingled neuronal populations (brown and red stripes). In the ventral telencephalon, Isl-1$^+$ neurons (red) do not express SC1 or Lim-1. For simplicity, the domain of neuroepithelial Lim-1 expression that occupies the entire dorsoventral extent of the mid-diencephalon is not depicted in this diagram.

(iii) Ventral domain of expression of Nkx-2.1.

(B) Ventral detail of a transverse section through the mesencephalon, showing that motor neurons of the oculomotor nucleus coexpress Isl-1 (red) and SC1 (green).

(C) Ventral detail of a transverse section through the rostral diencephalon, showing that Isl-1$^+$ neurons do not express SC1.

(D) Detail of a transverse section through the ventral telencephalon, showing expression of Nkx-2.1 in virtually all cells.

(E and F) Detail of a transverse section through the lateral region of the mid-diencephalon dorsal to the infundibulum (see Figure 1D for a low power view), showing that virtually all undifferentiated neuroepithelial cells express Lim-1 at low levels (F) and that Isl-1$^+$ neurons (E) (red) also coexpress Lim-1 (yellow cells in [F]).

(G–I) Ventral detail of a transverse section through the rostral diencephalon, showing that Isl-1$^+$ neurons (I) (red) express Lim-1 (H) (green). (I) shows a double exposure of (G) and (H) to indicate the overlap of labeled cells.

(J) Detail of a coronal section through the ventral telencephalon, showing that Isl-1$^+$ neurons do not express Lim-1, as shown by the absence of yellow cells in this double exposure of Isl-1 (red) and Lim-1 (green).

Abbreviations: r, rhombencephalon; m, mesencephalon; d, diencephalon; t, telencephalon; i, infundibulum.

Scale bar in (B), 160 μm; in (C) and (E)–(I), 25 μm; in (D) and (J), 20 μm.

cell types at forebrain as well as at more caudal levels of the neural tube. At all axial levels, Isl-1$^+$ cells expressed a neuron-specific β-tubulin isoform (data not shown), showing that they were neurons.

In the diencephalon, mesencephalon, and rhombencephalon, the expression of *Shh* preceded the differentiation of Isl-1$^+$ cells. Between stages 6 and 10, midline expression of *Shh* extended from the mesencephalon rostrally into the diencephalon and caudally into the rhombencephalon (see Figure 3A; data not shown). The onset of Isl-1 expression at diencephalic, mesencephalic, and rhombencephalic levels occurred between stages 13 and 15 (Figure 1E; Ericson et al., 1992; Tsuchida et al., 1994; data not shown), 18–24 hr after the onset of *Shh* expression at the same axial levels. In the telencephalon, however, expression of *Shh* was not detected until late stage 17 (Figures 1J and 1K), about 30 hr after the gene was first expressed in ventral midline cells of the rostral diencephalon (data not shown). Thus, in the ventral telencephalon, the initial expression of Isl-1 and SHH occurs synchronously.

Neurons That Express Isl-1 at Different Axial Levels Have Distinct Identities

To determine whether Isl-1$^+$ neurons found at different rostrocaudal positions have distinct identities, we examined the expression of other homeodomain proteins and cell surface markers.

SC1 Expression Defines Isl-1$^+$ Neurons Caudal to the Forebrain

In the rhombencephalon and mesencephalon, ventral Isl-1$^+$ neurons are motor neurons (Simon et al., 1994) and coexpress the SC1 protein (Figures 2Aii and 2B; data not shown), in common with spinal motor neurons (Yamada et al., 1991; Ericson et al., 1992). In contrast, neither diencephalic nor telencephalic Isl-1$^+$ neurons expressed SC1 (Figure 2C; data not shown).

Nkx-2.1 Expression Defines Isl-1$^+$ Neurons in the Ventral Forebrain

Expression of the homeodomain protein Nkx-2.1 (Lazzaro et al., 1991; Price et al., 1992) was used to distinguish cells in diencephalic and telencephalic regions from those found more caudally (Rubenstein et al., 1994). In chick embryos examined at stages 14–18, Nkx-2.1 was expressed by virtually all cells in a broad ventral domain of the mid- and rostral diencephalon and telencephalon that encompassed the region in which Isl-1$^+$ neurons were generated (Figures 2Aiii and 2D; data not shown). Nkx-2.1$^+$ cells were not detected in the rhombencephalon (Figure 2Aiii; data not shown). The onset of expression of Nkx-2.1 occurred prior to that of Isl-1$^+$, at stage 9 in the diencephalon and at stages 13–14 in the telencephalon (data not shown), and in both regions, expression was transient. Because of this, it was difficult to determine whether all Isl-1$^+$ neurons derived from Nkx-2.1$^+$ precursors. Nevertheless, when examined at stage 18, about 10% of Nkx-2.1$^+$ cells coexpressed Isl-1 (data not shown), supporting the idea that Isl-1$^+$ neurons derive from Nkx-2.1$^+$ cells. Thus, the expression of Nkx-2.1 provides a marker of ventral forebrain cells, and coexpression of Nkx-2.1 can be used to distinguish Isl-1$^+$ neurons generated in the diencephalon and telencephalon from those in more caudal regions of the neural tube.

Lim-1 Expression Distinguishes Isl-1$^+$ Neurons in the Diencephalon from Those in the Telencephalon

Expression of the LIM homeodomain protein Lim-1 (Taira

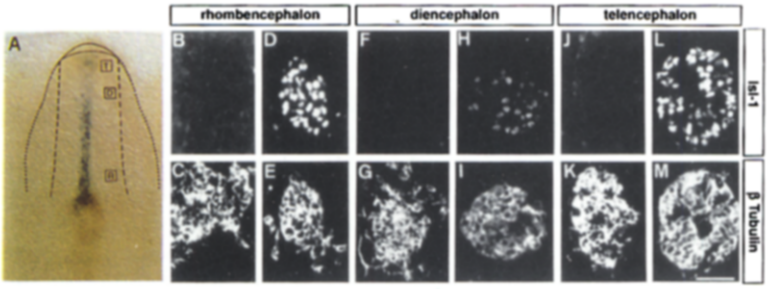

Figure 3. SHH Induces Isl-1$^+$ Neurons in Explants Derived from Different Axial Levels of the Neural Plate

(A) Expression of *Shh* in the cells at the midline of a stage 6 chick embryo shown by whole-mount in situ hybridization. *Shh* is expressed both in neural ectoderm and in the underlying mesoderm (data not shown). The position of the prospective telencephalic (T), diencephalic (D), and rhombencephalic (R) regions of the neural plate isolated for in vitro assays is indicated. The head fold is at the top, and the approximate neuroectodermal–ectodermal border is indicated by a broken line. Dotted line indicates approximate border of the epiblast.

(B)–(M) show explants grown for ~65 hr on COS cells transfected with antisense or sense *Shh*.

(B, C, F, G, J, and K) Sections of rhombencephalic (B and C), diencephalic (F and G), and telencephalic (J and K) level explants grown on COS cells transfected with antisense *Shh*. No Isl-1$^+$ cells are detected, even though β-tubulin$^+$ neurons have differentiated.

(D, E, H, I, L, and M) Sections of rhombencephalic (D and E), diencephalic (H and I), and telencephalic (L and M) level explants grown on COS cells transfected with sense *Shh*. Numerous Isl-1$^+$ cells are detected, virtually all of which coexpress β-tubulin.

Scale bar in (A), 250 μm; in (B)–(M), 25 μm.

et al., 1992) was used to distinguish Isl-1$^+$ neurons in the diencephalon from those in the telencephalon (Barnes et al., 1994; Fujii et al., 1994). In chick embryos examined from stages 14–18, Lim-1$^+$ cells were detected in the diencephalon but not in the telencephalon (Figure 2A; data not shown). In the mid-diencephalon, Lim-1 was expressed by most neuroepithelial cells (Figures 2Aii and 2F) as well as by postmitotic neurons that coexpressed Isl-1$^+$ (Figures 2E and 2F). In the rostral diencephalon, Lim-1 expression was confined to neurons at the ventral midline, all of which expressed Isl-1 (Figures 2G–2I). In the intervening region of the diencephalon, Lim-1 was expressed in a population of neurons distinct from, but intermingled with, Isl-1$^+$ neurons (Figure 2Aii). Importantly, in the telencephalon, Isl-1$^+$ neurons did not express Lim-1 (Figure 2J). Thus, Lim-1 expression distinguishes diencephalic from telencephalic cells, and coexpression of Lim-1 indicates the diencephalic origin of Isl-1$^+$ neurons in the forebrain.

SHH Induces Isl-1$^+$ Neurons in Prospective Forebrain Regions of the Neural Plate

To examine the influence of SHH on cell differentiation in regions of the neural plate that give rise to the forebrain, we constructed a coarse fate map of the neural plate of stage 6 chick embryos (M. P., unpublished data). This map, together with a stage 8 fate map (Couly and Le Douarin, 1987), was used as a guide to isolate explants from lateral regions of the neural plate fated to give rise to the telencephalon and diencephalon and, as a control, to the rhombencephalon (Figure 3A). We then used the markers described above to examine whether SHH can induce the differentiation of ventral neurons in these explants.

Numerous Isl-1$^+$ cells were induced in explants derived from each of the three axial levels of the neural plate grown on COS cells transfected with sense *Shh* (Figure 3), whereas explants grown on COS cells transfected with antisense *Shh* did not contain Isl-1$^+$ cells (Figure 3; Table

Table 1. Induction of Isl-1$^+$ Cells by SHH in Neural Plate Explants

		Contact			Transfilter	
Region of Neural Plate	Transfection Construct	Percent Isl-1$^+$ Explants	Percent Isl-1$^+$ Neurons per Explant	Percent Isl-1$^+$ Neurons That Express Lim-1	Percent Isl-1$^+$ Explants	Isl-1$^+$ Neurons per Explant
Rhombencephalic	Antisense *Shh*	0 (49)	0	0	0 (18)	0
	Sense *Shh*	57 (45)	39 (11)	0 (16)	8 (36)[a]	1 ± 2 (10)
Diencephalic	Antisense *Shh*	0 (28)	0	0	0 (22)	0
	Sense *Shh*	57 (30)	35 (9)	22 (11)	64 (28)	33 ± 8 (5)
Telencephalic	Antisense *Shh*	0 (46)	0	0	0 (25)[b]	0[b]
	Sense *Shh*	78 (42)	96 (7)	0 (15)	76 (25)	39 ± 10 (6)

Neural plate explants isolated from telencephalic, diencephalic, and rhombencephalic levels of stage 6 chick embryos were cultivated for 60–66 hr in contact with or transfilter to COS cells transfected with an *Shh* expression construct in sense or antisense orientation, and the proportion of explants that express Isl-1 was determined by whole-mount immunohistochemistry. The number of expants is indicated in brackets. Values represent mean ± SEM.

[a] Of 36 rhombencephalic explants cultured on sense *Shh* COS cells, three contained between one and five Isl-1$^+$ cells.

[b] Of 25 telencephalic explants cultured on antisense *Shh* COS cells, one contained <5 weakly Isl-1$^+$ cells.

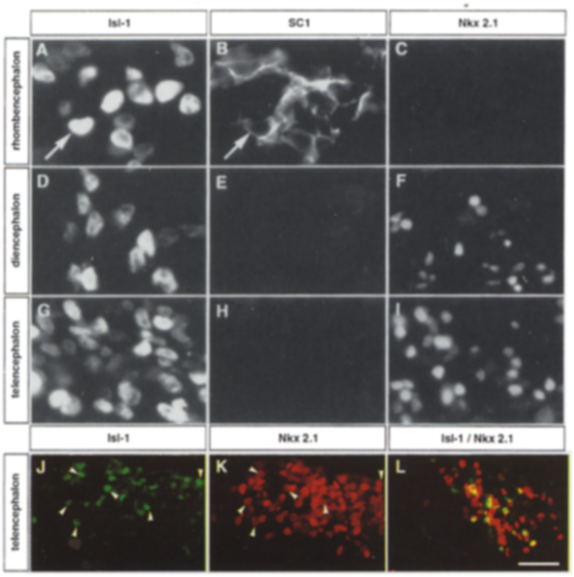

Figure 4. Expression of Ancillary Markers Distinguishes Isl-1$^+$ Neurons Induced by SHH in Rhombencephalic, Diencephalic, and Telencephalic Level Neural Plate Explants

(A and B) Section through a rhombencephalic level explant exposed to SHH. Double-label images of the same section show that Isl-1$^+$ cells (A) express SC1 (B). Arrows in (A) and (B) indicate the same cell.

(C) No Nkx-2.1$^+$ cells are detected in rhombencephalic level explants exposed to SHH.

(D and E) Section through a diencephalic level explant exposed to SHH. Isl-1$^+$ cells (D) do not coexpress SC1 (E).

(F) Section through a diencephalic level explant exposed to SHH, showing induction of Nkx-2.1$^+$ cells.

(G and H) Section through a telencephalic level explant exposed to SHH. Isl-1$^+$ cells (G) do not express SC1 (H).

(I) Section through a telencephalic level neural plate explant exposed to SHH, showing induction of Nkx-2.1$^+$ cells.

(J and K) Double-label immunofluorescence micrograph derived from a telencephalic level explant exposed to SHH for 48 hr, showing Isl-1$^+$ (J) and Nkx-2.1$^+$ (K) cells in the same section. Over 70% of Isl-1$^+$ cells express Nkx-2.1 (arrows point to some of the same cells).

(L) Superimposition of Isl-1$^+$ (green) and Nkx-2.1$^+$ (red) cells in a telencephalic level explant. Yellow cells coexpress Isl-1 and Nkx-2.1. Similar results were obtained in diencephalic level explants.

Scale bar in (A)–(E) and (G)–(I), 10 μm; in (F), 15 μm; in (J)–(K), 25 μm; in (L), 50 μm.

1). The proportion of Isl-1$^+$ neurons in induced explants derived from the three axial levels differed markedly. In telencephalic level explants, 96% of cells exposed to SHH expressed Isl-1 (Table 1), whereas only 35% of cells in diencephalic level explants and 39% of cells in rhombencephalic level explants expressed Isl-1 (Table 1).

Isl-1$^+$ Neurons Induced by SHH Have Distinct Identities

To define the identity of induced Isl-1$^+$ neurons, we examined the expression of SC1, Nkx-2.1, and Lim-1.

SC1 Expression

To define Isl-1$^+$ neurons generated at levels caudal to the forebrain, we examined the coexpression of SC1 and Isl-1. Rhombencephalic level explants exposed to SHH contained Isl-1$^+$ neurons that coexpressed SC1 (Figures 4A and 4B), indicating that these cells are motor neurons.

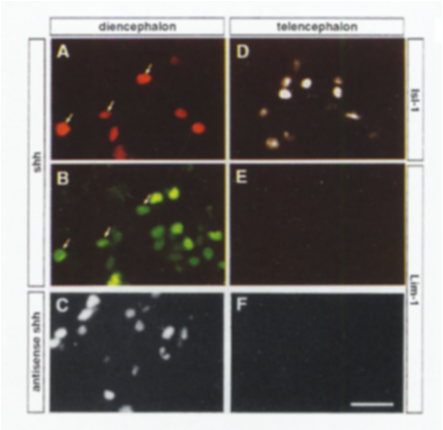

Figure 5. Expression of Lim-1 Distinguishes Isl-1$^+$ Neurons Induced by SHH in Diencephalic and Telencephalic Level Neural Plate Explants

(A and B) Many Isl-1$^+$ cells (A) in diencephalic level explants exposed to SHH express Lim-1 (B). Arrows indicate some of the cells that coexpress Isl-1 and Lim-1. Isl-1$^+$/Lim-1$^-$ and Isl-1$^-$/Lim-1$^+$ cells are also present.

(C) Lim-1$^+$ cells are present in diencephalic level neural plate explants grown on antisense *Shh* COS cells.

(D and E) Isl-1$^+$ cells (D) in telencephalic level explants exposed to SHH do not express Lim-1 (E). No Lim-1$^+$ cells are present in telencephalic level explants after exposure to SHH. Similar results were obtained in over 20 explants.

(F) Lim-1$^+$ cells are not detected in telencephalic level explants grown on antisense *Shh* COS cells.

Scale bar, 20 μm.

Isl-1$^-$/SC1$^+$ cells and FP1$^+$ cells were also detected (data not shown), showing that floor plate cells are also induced (Yamada et al., 1991). Isl-1$^+$ neurons induced in diencephalic (Figure 4D) and telencephalic (Figure 4G) level explants by exposure to SHH did not coexpress SC1 (Figures 4E and 4H), providing evidence that they are not motor neurons. Floor plate cells, defined by expression of FP1 and SC1, were not detected in diencephalic or telencephalic level explants exposed to SHH (data not shown).

Nkx-2.1 Expression

To determine whether Isl-1$^+$ neurons induced in diencephalic and telencephalic level explants had properties of ventral forebrain neurons, we examined the coexpression of Nkx-2.1 and Isl-1. Over 80% of cells in diencephalic and telencephalic level explants exposed to SHH for 24 hr expressed Nkx-2.1 (Figures 4F and 4I), whereas induced rhombencephalic level explants did not contain Nkx-2.1$^+$ cells (Figure 4C). Moreover, about 70% of Isl-1$^+$ cells coexpressed Nkx-2.1 in explants after 48 hr exposure to SHH (Figures 4J, 4K, and 4L), confirming the forebrain identity of these Isl-1$^+$ neurons.

Lim-1 Expression

To distinguish diencephalic from telencephalic Isl-1$^+$ neurons in induced explants, we monitored the coexpression of Lim-1 and Isl-1. In diencephalic level explants exposed to SHH, 22% of Isl-1$^+$ neurons expressed Lim-1 (Figures 5A and 5B; Table 1), consistent with a diencephalic identity (see Figure 2Aii). In telencephalic level explants, however, the Isl-1$^+$ neurons induced by SHH did not express Lim-1 (Figures 5D and 5E; Table 1). Lim-1$^+$ cells were detected

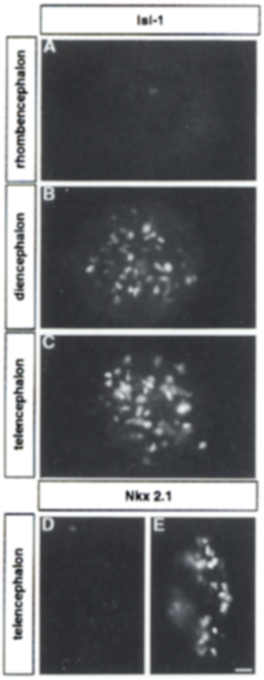

Figure 6. Transfilter Induction of Ventral Forebrain Neurons by Shh

(A) Whole mount of a rhombencephalic level explant grown for 60 hr transfilter to COS cells expressing SHH. Very few Isl-1$^+$ neurons are induced.

(B and C) Whole mounts of diencephalic (B) and telencephalic (C) level explants grown for 60 hr transfilter to COS cells expressing SHH. Numerous Isl-1$^+$ cells are induced.

(D and E) Sections through telencephalic level explants grown for 48 hr transfilter to COS cells expressing antisense (D) or sense (E) *Shh*. Numerous Nkx-2.1$^+$ cells are induced by sense (E) but not by antisense (D) *Shh*.

Scale bar, 20 μm.

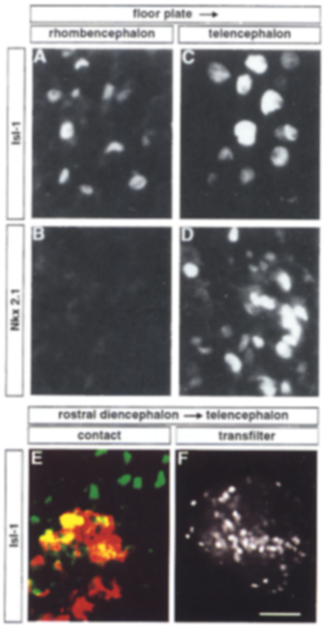

Figure 7. Floor Plate and Ventral Cells of the Rostral Diencephalon Induce Ventral Neurons at Different Levels of the Neuraxis

(A) Isl-1$^+$ neurons are induced by floor plate in rhombencephalic level explants. These cells coexpress SC1 (data not shown).

(B) Nkx-2.1$^+$ cells are not induced by floor plate in rhombencephalic level explants.

(C) Isl-1$^+$ neurons are induced by floor plate in telencephalic level explants. These neurons do not coexpress SC1 (data not shown).

(D) Nkx-2.1$^+$ cells are induced by floor plate in telencephalic level explants.

(E) Rostral diencephalic tissue induces Isl-1$^+$ cells (green) in telencephalic level neural plate explants. Diencephalic tissue of murine origin is delineated by anti-nestin immunoreactivity (red) and contains a few Isl-1$^+$ neurons (yellow cells). The induced telencephalic Isl-1$^+$ neurons do not express SC1 (data not shown). About 15% of cells in telencephalic explants were induced to express Isl-1.

(F) Induced Isl-1$^+$ cells in telencephalic level neural plate explants grown transfilter to explants derived from the ventral midline region of the rostral diencephalon. A total of 83% of explants (n = 12) contained Isl-1$^+$ cells (46 ± 14 cells per explant; mean ± SEM; n = 6). No induction of Isl-1$^+$ cells was observed with dorsal rostral diencephalic tissue (n = 8).

Scale bar in (A) and (B), 15 μm; in (C) and (D), 10 μm; in (E), 25 μm; in (F), 45 μm.

in rhombencephalic and diencephalic (Figure 5C) but not telencephalic (Figure 5F) level explants grown on COS cells transfected with antisense *Shh*.

These results indicate that a rostrocaudal character of neural cells that has been established at the neural plate stage is maintained in vitro, in the absence and presence of ventralizing signals mediated by SHH. Thus, an early and stable restriction in the potential of cells located at different rostrocaudal positions within the neural plate appears to define the repertoire of ventral neuronal cell types that can be generated upon exposure of cells to SHH.

SHH Can Induce Ventral Forebrain Neurons in a Contact-Independent Manner

In neural plate explants derived from spinal cord levels, the induction of motor neurons by the notochord or by cells expressing SHH can be achieved in the absence of contact (Tanabe et al., 1995; Roelink et al., 1995). We examined whether SHH can similarly induce Isl-1$^+$ neurons in forebrain level neural plate explants in a contact-independent manner. Diencephalic and telencephalic level neural plate explants grown transfilter to COS cells expressing SHH contained Isl-1$^+$ neurons (Figures 6B, 6C, and 6D; Table 1) and Nkx-2.1$^+$ cells (Figure 6E). These results show that ventral forebrain Isl-1$^+$ neurons can be induced by cells expressing SHH in the absence of contact with forebrain level explants. In contrast, few, if any, Isl-1$^+$ neurons were induced in rhombencephalic level neural plate explants (Figure 6A; Table 1). At rhombencephalic levels, efficient induction of Isl-1$^+$ motor neurons, therefore, appears to depend on contact with cellular sources of SHH. Similarly, the induction of dopaminergic neurons in the mesencephalon depends on contact with the floor plate (Hynes et al., 1995).

Inductive Activity of Floor Plate and Rostral Diencephalic Cells

We next examined whether the inductive actions of SHH reflect those of endogenous neural sources of SHH present at different levels of the neuraxis.

Chick floor plate was used as a source of SHH implicated in the induction of ventral cell types at rhombencephalic levels (see Figure 1). Rhombencephalic level explants grown in contact with floor plate tissue contained Isl-1$^+$/SC1$^+$ neurons (Figure 7A; data not shown) but not Nkx-2.1$^+$ cells (Figure 7B). In telencephalic level explants, floor plate tissue induced Isl-1$^+$/SC1$^-$ and Isl-1$^+$/Lim-1$^-$ neurons (Figure 7C; data not shown) and Nkx-2.1$^+$ cells (Figure 7D). Thus, the profile of markers induced in neural plate explants exposed to floor plate and to SHH is identical.

The activity of cells at the ventral midline of the rostral diencephalon (see Figure 1) was assayed as a neural source of SHH implicated in patterning the diencephalon (Hatta et al., 1994) and ventral telencephalon (see Discussion). Since the ventral region of the rostral diencephalon itself contains Isl-1$^+$ neurons, conjugate experiments were performed with tissue obtained from E11 mouse embryos and identified by antibodies to mouse nestin (Dahlstrand et al., 1992). Cells at the ventral midline of the rostral diencephalon induced numerous Nkx-2.1$^+$ cells (data not shown) and expression of Isl-1 in about 15% of cells in telencephalic level explants (Figure 7E). The decreased number of induced Isl-1$^+$ neurons is likely to result from the provision of lower levels of SHH by diencephalic tissue than by *Shh*-transfected COS cells. These Isl-1$^+$ neurons did not express SC1 or Lim-1 (data not shown), characteristic of a telencephalic phenotype. Furthermore, chick ventral rostral diencephalic tissue was able to induce Isl-1$^+$ neurons when grown transfilter to telencephalic level explants (Figure 7F; Table 1). In contrast, diencephalic tissue isolated from the dorsal midline of the rostral diencephalon or the ventral midline at the level of the infundibulum, regions that do not express *Shh* (see Figure 1), did not induce Isl-1$^+$ neurons either in contact with or transfilter to telencephalic level explants (data not shown).

Thus, signals derived from the ventral midline of the rostral diencephalon appear able to act at a distance to induce Isl-1$^+$ neurons characteristic of the ventral telencephalon. These results show also that the identity of ventral neurons induced by neural sources of SHH appears to depend on rostrocaudal restrictions in the response properties of neural plate cells and not on the axial level of origin of the inducing tissue.

Discussion

A vertebrate homolog of the Drosophila *hedgehog* gene, *Shh*, is expressed by the notochord and floor plate and can mimic the ability of these two midline cell groups to induce motor neuron differentiation (Roelink et al., 1994, 1995; Tanabe et al., 1995). On this basis, SHH has been implicated in the induction of ventral neuronal types at caudal levels of the neural tube. *Shh* is also expressed by cells in the ventral neural tube rostral to the floor plate, raising the question of whether SHH also participates in the induction of ventral neurons in the forebrain. Our results show that SHH induces ventral neurons characteristic of the diencephalon and telencephalon in regions of the neural plate that normally give rise to these two subdivisions of the forebrain. Thus, a single inducing molecule, SHH, appears to participate in the differentiation of ventral neuronal cell types along the entire rostrocaudal extent of the neural tube, inducing distinct cell types through its actions on neural plate cells of predetermined rostrocaudal character.

Although the final identity of the embryonic forebrain neurons induced by SHH has not been resolved by these studies, in the adult forebrain, Isl-1$^+$ neurons are found in several ventral diencephalic nuclei and in the basal telencephalon (Thor et al., 1991). It is likely that neurons in these ventral forebrain nuclei represent the mature derivatives of the Isl-1$^+$ neurons that are induced by SHH at prospective forebrain levels of the neural plate.

SHH as an Inducer of Ventral Forebrain Neurons

In telencephalic level neural plate explants, SHH induced virtually all cells to differentiate into Isl-1$^+$ neurons of telencephalic character. Moreover, exposure of telencephalic level explants to SHH does not induce endogenous *Shh* expression (J. E. and J. M., unpublished data). These results suggest that at telencephalic levels, SHH induces ventral neurons by an action on neural plate cells that is independent of the induction of *Shh* or of intermediary cell types. Similarly, in neural plate explants derived from spinal cord levels, the induction of motor neurons in response to SHH does not depend on the induction of floor plate differentiation (Tanabe et al., 1995; Roelink et al., 1995). Thus, at many levels of the neuraxis, the induction of ventral neurons by SHH does not depend on the prior differentiation of specialized midline cells. It remains possible that in the diencephalon, SHH induces midline cells that secrete a distinct factor that is responsible for inducing ventral Isl-1$^+$ neurons. Since in the rostral diencephalon, the domains of expression of *Shh* and Isl-1 overlap at the midline, it is also possible that Isl-1$^+$ neurons differentiate from cells that have expressed *Shh* at an earlier stage.

Induction of Ventral Telencephalic Neurons by Signals from the Rostral Diencephalon

One important issue that is raised by our studies is the source of the inductive signal that triggers the differentiation of neurons in the ventral telencephalon. Our results suggest that the induction of Isl-1$^+$ neurons in the ventral telencephalon depends on cell groups distinct from those that induce ventral cell types at more caudal levels of the neuraxis.

Caudally, ventral Isl-1$^+$ neurons appear to be induced by a signal, presumably SHH, that derives initially from the notochord and later from floor plate cells. Two lines of evidence suggest that at telencephalic levels, the induction of ventral neurons appears not to depend on the axial mesoderm. First, cells fated to give rise to the floor of the telencephalon are located in the lateral margins of the neural plate and are never in proximity to the prechordal mesoderm (Couly and Le Douarin, 1987; M. P., unpublished data). Second, prospective ventral telencephalic tissue isolated as late as stage 12 does not give rise to Isl-1$^+$ neurons when grown in vitro (J. M., unpublished data). Thus, signals that commit telencephalic cells to a ventral neuronal fate are required at a stage when the prechordal

mesoderm is even farther removed from the prospective telencephalon (M. P., unpublished data).

If the axial mesoderm does not represent a source of signals involved in the induction of neurons in the ventral telencephalon, from where do these signals originate? Ventral telencephalic cells are unlikely to provide this signal, since *Shh* is not expressed by cells at the floor of the telencephalon until stages 17–18, coincident with the appearance of telencephalic Isl-1$^+$ neurons and after the specification of ventral forebrain Isl-1$^+$ neurons. Cells at the ventral midline of the rostral diencephalon, however, represent a potential source of SHH involved in inducing Isl-1$^+$ neurons in the ventral telencephalon. *Shh* is expressed at the midline of the rostral diencephalon from stage 9, prior to the onset of expression of Nkx-2.1 and to the specification of Isl-1$^+$ neurons in the telencephalon. Furthermore, our in vitro studies show that midline rostral diencephalic cells that express SHH can act at a distance to induce Isl-1$^+$ neurons in telencephalic regions of the neural plate. It remains possible that rostral diencephalic cells secrete additional factors that cooperate with SHH to define the number and diversity of ventral cell types generated within the floor of the telencephalon.

Thus, in vivo, a SHH-mediated signal from ventral midline cells of the rostral diencephalon might act in a planar manner to induce the differentiation of neurons in the ventral telencephalon. Studies of the zebrafish mutant *cyclops* (Hatta et al., 1991) have provided evidence that cells at the ventral midline of the embryonic diencephalon also have a role in patterning the diencephalon (Hatta et al., 1994; Macdonald et al., 1994).

Homeobox Gene Expression and a Common Program for the Generation of Ventral Neurons

The detection of Isl-1 in ventral neuronal cell types induced by SHH at different positions along the rostrocaudal extent of the neural tube suggests that Isl-1 expression is more closely associated with the differentiation of neurons of ventral character than with the generation of any specific class of ventral neuron. Moreover, Isl-1, although a prominent marker of ventral neuronal differentiation, is not always expressed by ventral neurons that differentiate in response to notochord- and floor plate–derived signals. For example, at rhombencephalic and mesencephalic levels, serotonergic and dopaminergic neurons differentiate ventrally in response to signals from the notochord and floor plate but do not express Isl-1 (Yamada et al., 1991; Hynes et al., 1995; our unpublished data).

Nevertheless, the expression of Isl-1 by many distinct classes of ventral neurons raises the possibility that elements of the response of neural plate cells to SHH may be conserved along the rostrocaudal axis. In support of this, members of the *Nkx-2* family of homeobox genes, notably *Nkx-2.1* and *Nkx-2.2*, are expressed in the ventral neural tube at all rostrocaudal levels, in a domain that overlaps closely with that of *Shh* (Price et al., 1992; Lazzaro et al., 1991; Rubenstein et al., 1994). Moreover, at forebrain levels, the expression of both Nkx-2.1 and Nkx-2.2 (Barth and Wilson, 1995) is induced by SHH. Thus, the Nkx-2 and Isl homeodomain proteins might represent elements of a common SHH-response program that is activated in neural plate cells independent of their rostrocaudal position.

Experimental Procedures

Animals

Fertilized white leghorn chicken eggs were obtained from Agrisera AB, Umeå, Sweden. Chick embryos were staged according to the protocols of Hamburger and Hamilton (1951). Time-mated mouse embryos were obtained from the animal facility of Umeå University.

In Situ Hybridization and Immunohistochemistry

In situ hybridization analysis using a chick *Shh* probe (T. L. and J. Dodd, unpublished data) was performed essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Immunohistochemical localization of antigens was performed as described (Yamada et al., 1991). Double-label immunohistochemistry and in situ hybridization was performed as described by Tsuchida et al. (1994). Whole-mount in situ hybridization was performed as described (Francis et al., 1994).

Isl-1 was detected by using rabbit anti-Isl-1 antibodies (Thor et al., 1991; Ericson et al., 1992) or monoclonal antibody (MAb) 4D5 (Roelink et al., 1994). Lim-1 (Taira et al., 1992) was detected with MAb 4F2, which also recognizes Lim-2 (Tsuchida et al., 1994). Lim-1 and Lim-2 have similar patterns of expression in the forebrain (data not shown). SC1 was detected with MAb SC1 (Tanaka and Obata, 1984), the homeodomain protein Nkx-2.1 with rabbit antibodies (Lazzaro et al., 1991), floor plate cells with MAb FP1 (Yamada et al., 1991), nestin with antisera 129/130 (Dahlstrand et al., 1992), and acetylated β-tubulin with MAb T6793 (Sigma Immunochemicals). The number of Isl-1$^+$ and Lim-1$^+$ cells in explants was determined by sectioning explants and counting the number of labeled cells in every fifth section. The total number of cells was determined by nuclear labeling using DAPI (Boehringer Mannheim).

Isolation and Culture of Neural Plate Explants

Neural plate explants (Yamada et al., 1993) corresponding to presumptive telencephalic, diencephalic, and rhombencephalic regions were dissected from stage 6 chick embryos. Floor plate tissue was isolated from stage 25 chick embryos as described (Yamada et al., 1993). Midline rostral diencephalic tissues expressing *Shh* were dissected from E11 mouse embryos and from stage 17 chick embryos. The infundibulum and roof of the diencephalon, tissues that do not express *Shh*, were also dissected. Neural plate explants were cultured for 24–66 hr in contact with or transfilter to COS cell aggregates, floor plate tissue, or diencephalic tissue as described (Roelink et al., 1994; Tanabe et al., 1995). For transfection, COS cells were grown until 90% confluency, transfected with 1 μg of DNA per 35 mm dish with 12 μg/ml lipofectamine reagent (GIBCO BRL) in Dulbecco's modified Eagle's medium (DMEM), and processed as described (Roelink et al., 1994).

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