Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation

Yasuto Tanabe, Henk Roelink and Thomas M. Jessell

Howard Hughes Medical Institute, Deptartment of Biochemistry and Molecular Biophysics, Center for Neurobiology and Behavior, Columbia University, 701 West 168th Street, New York, New York 10032, USA.

Background: The differentiation of floor plate cells and motor neurons in the vertebrate neural tube appears to be induced by signals from the notochord. The secreted protein encoded by the *Sonic hedgehog* (*Shh*) gene is expressed by axial midline cells and can induce floor plate cells *in vivo* and *in vitro*. Motor neurons can also be induced *in vitro* by cells that synthesize Sonic hedgehog protein (Shh). It remains unclear, however, if the motor-neuron-inducing activity of Shh depends on the synthesis of a distinct signaling molecule by floor plate cells. To resolve this issue, we have developed an *in vitro* assay which uncouples the notochord-mediated induction of motor neurons from floor plate differentiation, and have used this assay to examine whether Shh induces motor neurons in the absence of floor plate differentiation.

Results: Floor plate cells and motor neurons were induced in neural plate explants grown in contact with the notochord, but only motor neurons were induced when explants were separated from the notochord. COS cells transfected with *Shh* induced both floor plate cells and motor neurons when grown in contact with neural plate explants, whereas only motor neurons were induced when the explants were grown at a distance from *Shh*-transfected COS cells. Direct transfection of neural plate cells with an *Shh*-expression construct induced both floor plate cells and motor neurons, with motor neuron differentiation occurring prior to, or coincidentally with, floor plate differentiation. The induction of motor neurons appears, therefore, not to depend on floor plate differentiation.

Conclusions: The induction of motor neurons by Shh does not depend on distinct floor-plate-derived signaling molecules. Shh can, therefore, initiate the differentiation of two cell types that are generated in the ventral region of the neural tube. These results show that the early development of motor neurons involves the inductive action of Shh, whereas the survival of motor neurons at later stages of embryonic development requires neurotrophic factors.

Current Biology 1995, **5**:651–658

Background

A central problem in vertebrate neural development is to define the cellular and molecular mechanisms that control the differentiation of distinct classes of cells at specific positions within the neural tube. The generation of cell types in the ventral half of the neural tube depends on signals provided by axial mesodermal cells of the notochord [1–6]. Floor plate differentiation at the ventral midline of the neural tube depends on a contact-mediated signal from the notochord, whereas the differentiation of motor neurons more laterally can be induced by a diffusible factor that does not elicit floor plate differentiation [2,7–9]. Floor plate cells subsequently acquire both these inducing activities [5,7,9].

Sonic hedgehog (Shh), also known as Vhh-1 or Hhg-1, is a vertebrate homologue of the secreted protein encoded by the *Drosophila* gene *hedgehog* [10,11], and is expressed by the notochord and floor plate at the time that these midline cell groups exhibit their inductive activities [12–16]. Misexpression of Shh in vertebrate embryos can induce the differentiation of floor plate cells at ectopic locations in the neural tube [13–15]. In addition, COS cells expressing Shh can induce floor plate differentiation *in vitro* when grown in contact with neural plate explants [15]. These findings suggest that Shh normally participates in floor plate induction.

Whether Shh is also the diffusible factor derived from the notochord and floor plate that induces motor neurons remains unclear. Motor neurons are induced in neural plate explants grown in contact with cells that express Shh [15], but this could reflect the activity of a distinct factor secreted by the floor plate cells that are also induced in these explants. To resolve this issue, we have established *in vitro* assay conditions (Fig. 1) in which the induction of motor neurons in neural plate explants in response to notochord-derived signals can be uncoupled from floor plate differentiation. We have then examined whether Shh expressed in COS cells or neural plate cells can mimic the ability of the notochord to induce motor neurons in the absence of floor plate cells.

Our results show that COS cells transfected with *Shh* acquire a diffusible activity that is sufficient to induce motor neurons in neural plate explants in the absence of floor plate differentiation. In addition, direct transfection of cells in neural plate explants with *Shh* induces, independently, motor neurons and floor plate cells. These findings suggest that Shh derived from the notochord normally initiates the differentiation of motor neurons in

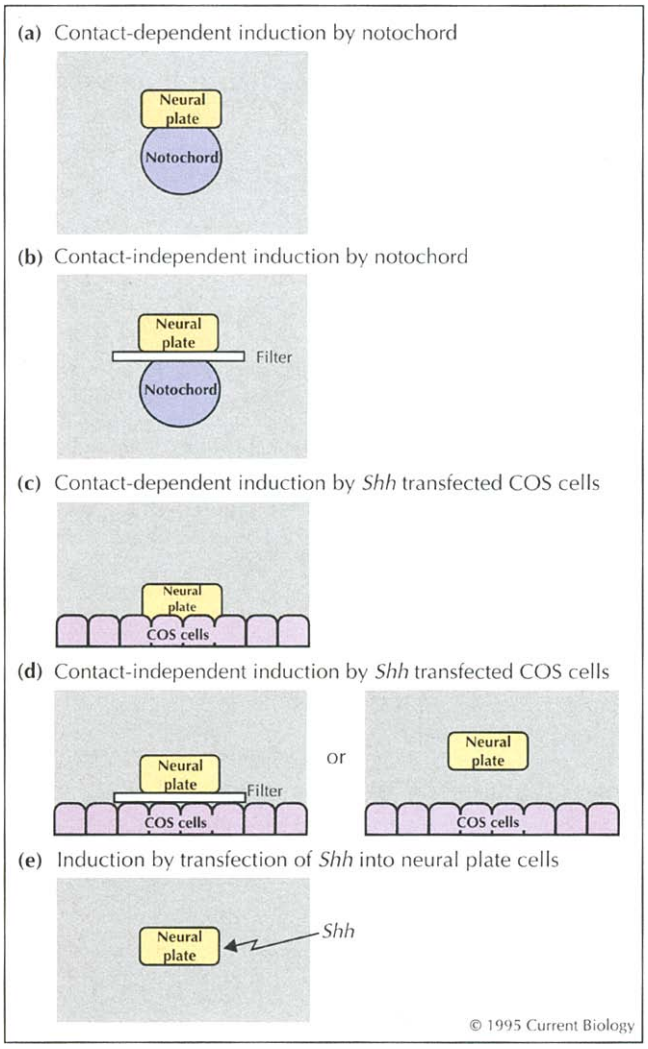

Fig. 1. Schematic outline of neural plate induction assays. Neural plate explants were embedded in a collagen gel (grey shading) and grown in contact with, or separated from, the notochord or cellular sources of Shh.

the ventral half of the neural tube by a mechanism that is independent of floor plate differentiation.

Results and discussion

Identification of floor plate cells and motor neurons in chick neural plate explants.

Floor plate and motor neuron differentiation was monitored in explants derived from the intermediate region of the neural plate of stage-10 chick embryos [8], using immunocytochemical and reverse transcription–polymerase chain reaction (RT–PCR) assays. Floor plate differentiation was assessed, primarily, by expression of the winged-helix transcription factor HNF3β (Table 1). HNF3β is an early marker of floor plate differentiation *in vivo* [17,18], and its transcription in neural plate cells *in vitro* appears to be a direct response to notochord-derived signals, as it can occur in the absence of protein synthesis [18]. Moreover, misexpression of HNF3β in the neural tube is sufficient to trigger ectopic floor plate cells [19,20] which, in turn, can induce ventral neurons in adjacent dorsal regions of the neural tube [19]. HNF3β expression provides, therefore, an early and reliable indicator of floor plate differentiation. As an independent marker of floor plate differentiation, we monitored the expression of the mRNA encoding the chemoattractant Netrin-1 (Table 1). Motor neuron differentiation was assessed by expression of the LIM homeodomain proteins Isl-1 and Isl-2 [21], by coexpression of SC1 with Isl-1 and Isl-2, and by expression of of *Isl-1*, *Isl-2* and *choline acetyltransferase* (*ChAT*) mRNAs (Table 1).

Table 1. Markers of floor plate and motor neuron differentiation in chick neural plate tissue.

Floor plate cells	Reference	Motor neurons	Reference
HNF3β	[17,18]	Isl-1/SC1	[5,8,21,38]
Netrin-1	[41,42]	Isl-2	[21]
		ChAT	[8]

Uncoupling motor neuron from floor plate differentiation in response to notochord signals

When neural plate explants were grown alone *in vitro* for 36 hours, floor plate and motor neuron markers were not expressed (Fig. 2a,e,f; Table 2a). In contrast, neural plate explants grown in contact with notochord (Fig. 1a) for 36 hours expressed *HNF3β* mRNA and protein (Fig. 2b, d,e) and *Netrin-1* mRNA (Fig. 2e), indicating the differentiation of floor plate cells. The same explants contained cells that expressed Isl-1 and/or Isl-2 (termed Isl^+ cells) in combination with SC1 (Fig. 2b–d,f), and in parallel assays explants expressed *Isl-1*, *Isl-2* and *ChAT* mRNAs (Fig. 2f), indicating the differentiation of motor neurons.

Table 2. Induction of floor plate and motor neuron markers in neural plate explants.

	$HNF3β^+$ cells/explant	Isl^+ cells/ explant	No. explants
(a) Induction by notochord			
Neural plate	0	<1	30
Notochord + neural plate	286 ± 40	215 ± 8	5,17
Notochord/filter/neural plate	0	38 ± 10	11
(b) Induction by Shh			
Antisense *Shh* + neural plate	0	0	25
Shh + neural plate	100 ± 23	182 ± 28	25
Shh/filter/neural plate	0	47 ± 8	30
Shh/collagen/neural plate	0	49 ± 5	9

Neural plate explants were grown for 36 h with **(a)** the notochord or **(b)** *Shh*-transfected COS cells, either in contact (indicated by + sign) or separated by membrane filters or by a strip of collagen gel (indicated by //). Values are mean ± s.e.m.

To separate experimentally the motor neuron- and floor plate-inducing activities of the notochord, we prevented contact between the notochord and neural plate explants by interposing a membrane filter (Fig. 1b). In the absence of contact, the notochord induced motor neuron differentiation (Fig. 2g,h), albeit less effectively as assessed by the number of Isl^+ neurons (Table 2a). In contrast, the notochord did not induce floor plate differentiation across a filter, as assessed by the absence of HNF3β expression at 24 hours (data not shown) or 36 hours (Fig. 2g; Table 2a). These results extend previous observations [7,8] in that they show that a notochord-derived diffusible factor can induce motor neurons in the absence of floor plate differentiation within the same neural plate explant.

Uncoupling motor neuron from floor plate differentiation in response to Shh-dependent signals

To examine whether Shh can mimic the contact-dependent and diffusible activities of the notochord, we grew neural plate explants for 36 hours, either in contact with

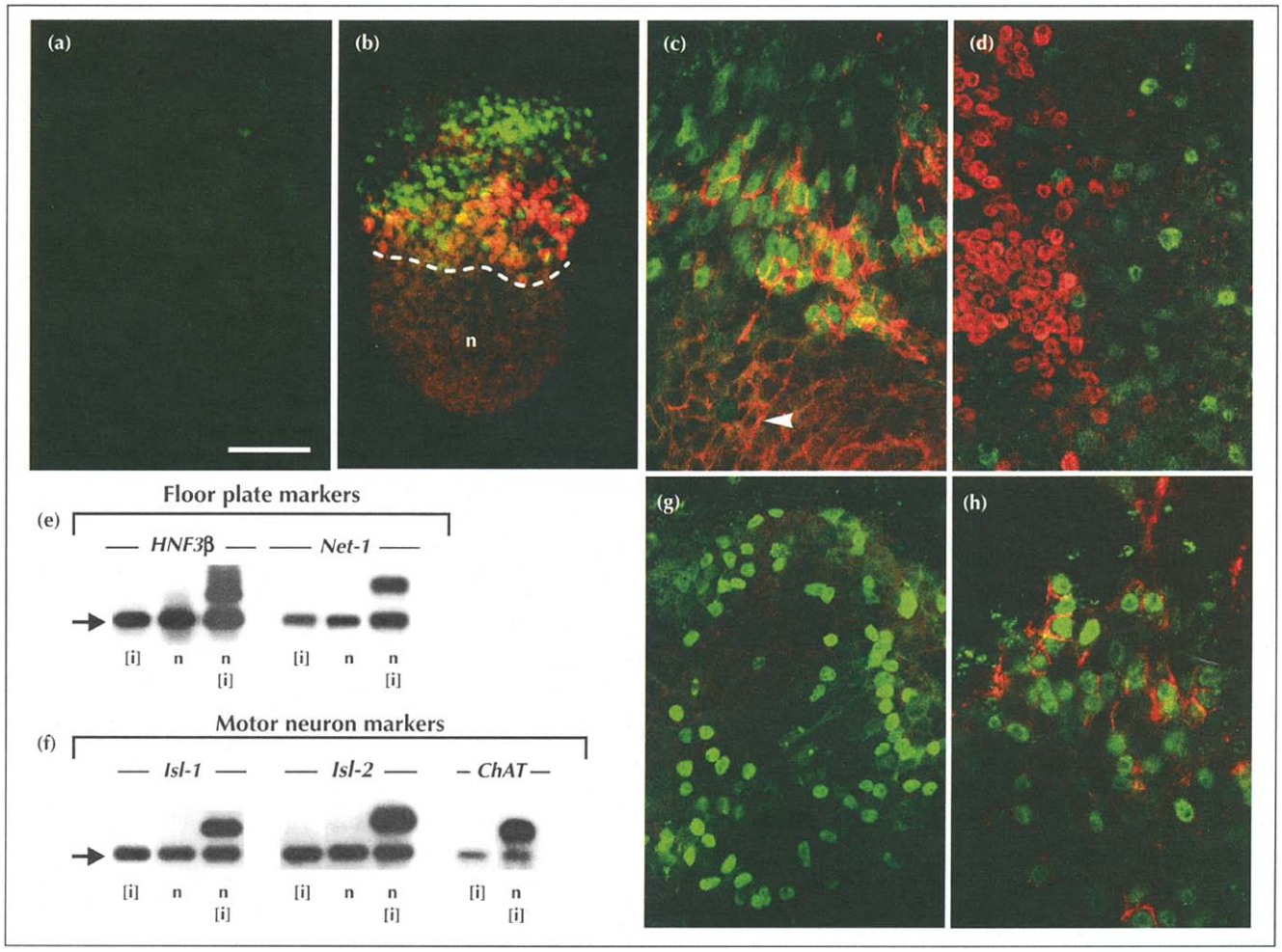

Fig. 2. Induction of floor plate and motor neuron differentiation by the notochord is distinguished by dependence on cell contact. **(a)** Neural plate explant grown for 36 h in the absence of the notochord and labelled with antibodies that detect HNF3β and Isl-1 and/or Isl-2 (Isl^+ cells). No $HNF3\beta^+$ or Isl^+ cells are detected. **(b)** Neural plate explant grown for 36 h in contact with notochord (n). $HNF3\beta^+$ (red) and Isl^+ (green) cells are induced. $HNF3\beta^+$ cells are located closer to the notochord/neural plate junction (white dotted line) than are Isl^+ cells. **(c)** Isl^+ cells (green) induced in neural plate explants by contact with the notochord coexpress the surface immunoglobulin-like protein SC1 (red). Patches of $SC1^+$ cells that do not express Isl proteins (arrowhead) correspond to floor plate cells [5]. **(d)** Contact with the notochord induces $Isl\text{-}2^+$ cells (green) in neural plate explants. $HNF3\beta^+$ cells (red) are also induced. **(e)** RT–PCR analysis of *HNF3β* and *Netrin-1* mRNA induction by contact with the notochord. Lower bands, marked by the arrow, indicate competitive templates introduced to control for the efficiency of the RT–PCR reactions. Intermediate neural plate explants ([i]) and notochord (n) do not express either gene when cultured alone for 36 h. Contact with the notochord (n + [i]) induces *HNF3β* and *Netrin-1* expression (upper bands). **(f)** RT–PCR analysis of *Isl-1*, *Isl-2* and *ChAT* mRNA induction by contact with the notochord. Intermediate neural plate explants ([i]) and notochord (n) do not express *Isl-1*, *Isl-2* or *ChAT* [8] when cultured alone for 36 h. Contact with the notochord (n + [i]) induces the expression of all three genes (upper bands). Lower bands, marked by the arrow, indicate internal standards introduced to control for the efficiency of the RT-PCR reactions. Results in (e) and (f) were obtained from RNA from the same set of explants. Similar results were obtained in 6 experiments. **(g)** Neural plate explants separated from the notochord by a Nucleopore filter and grown *in vitro* for 36 h contain Isl^+ (green) but not $HNF3\beta^+$ (red) cells. **(h)** Isl^+ cells (green) present in neural plate explants grown transfilter to the notochord express SC1 (red), indicating that they are motor neurons. Patches of $SC1^+/Isl^-$ cells were not detected, indicating the absence of floor plate differentiation. Similar results were obtained in 4 separate experiments using either Nucleopore or dialysis membrane filters. Scale bar: (a,c,h) = 20 μm; (b) = 100 μm; (d,g) = 33 μm.

(Fig. 1c), or separated from (Fig. 1d), COS cells transfected with sense or antisense rat *Shh* cDNA expression constructs [15]. Neural plate explants grown in contact with COS cells transfected with the sense *Shh* cDNA contained floor plate cells, assessed by expression of HNF3β (Fig. 3a and Fig. 3g, *HNF3β*, lane 1; Table 2b) and *Netrin-1* (Fig. 3g, *Net-1*, lane 1), and motor neurons, assessed by the presence of $Isl^+/SC1^+$ and Isl-2^+ neurons (Fig. 3a, b,c), and *Isl-1* and *ChAT* mRNA expression (Fig. 3h, lanes 1). Isl-2 was expressed in 74 ± 2 % (mean ± s.e.m., n = 6 explants) of neurons that expressed Isl-1 (Fig. 3c, and data not shown), which probably reflects the earlier expression of Isl-1 by embryonic motor neurons *in vivo* [21].

Neural plate explants grown in the absence of contact with COS cells transfected with the sense *Shh* cDNA did not express *HNF3β* mRNA or protein (Fig. 3d,g lane 3) or *Netrin-1* mRNA (Fig. 3g, lane 3), indicating that floor plate differentiation did not occur under these conditions. In contrast, motor neuron differentiation was induced in the absence of contact, as assessed by the presence of $Isl^+/SC1^+$ and Isl-2^+ neurons (Fig. 3d–f; Table 2b), and *Isl-1* and *ChAT* mRNA expression (Fig. 3h, lanes 3). Isl-2 was expressed in 74 ± 5 % (mean ± s.e.m., n = 8 explants) of neurons that expressed Isl-1 (Fig. 3f, and data not shown). Taken together, these results indicate that the diffusible activity derived from *Shh*-transfected COS cells induces the entire complement of motor neuron markers detected in response to induction by the notochord.

In previous studies, we have found that medium conditioned by *Shh*-transfected COS cells does not induce

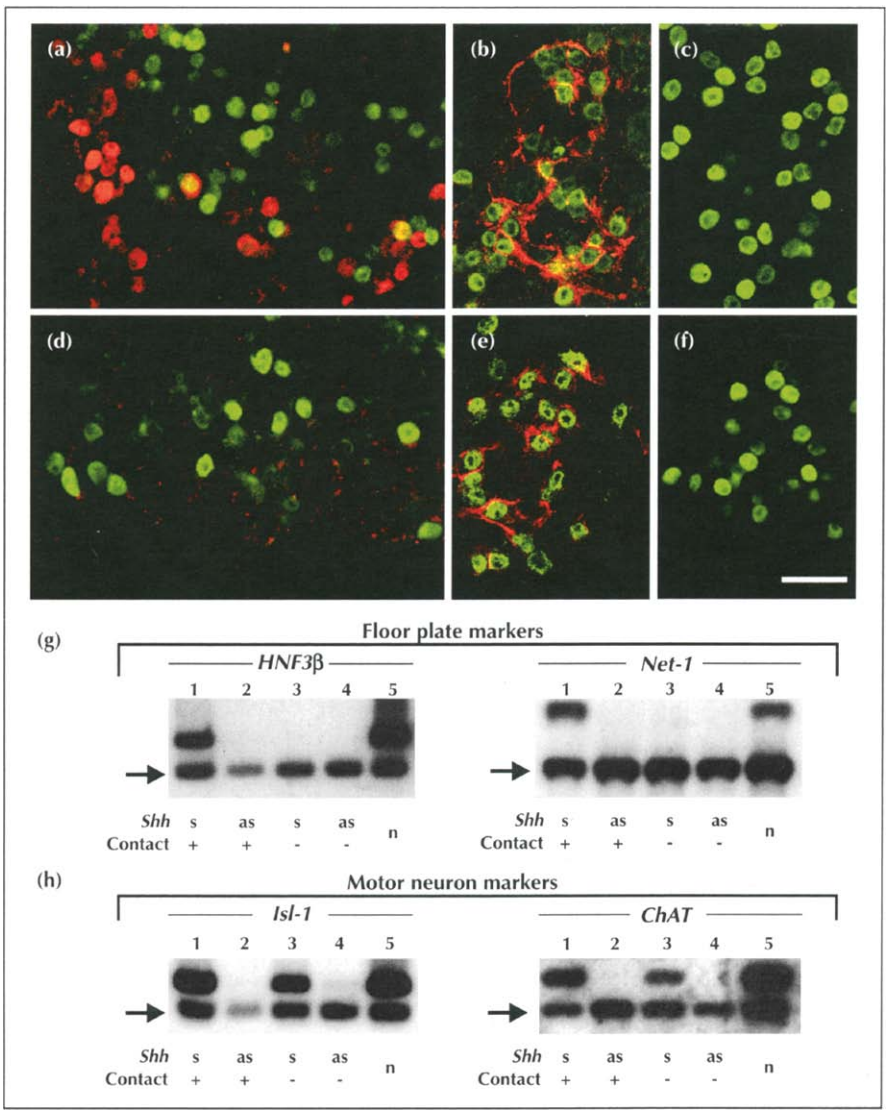

Fig. 3. COS cells that express Shh exhibit contact-dependent floor plate- and diffusible motor-neuron-inducing activities. **(a)** A neural plate explant grown in contact with *Shh*-transfected COS cells for 36 h contains $HNF3\beta^+$ (red) and Isl^+ (green) cells. The two cell groups are intermingled. Apparently yellow cells probably represent the superimposition of two distinct nuclei in the confocal section. **(b)** Isl^+ neurons (green) in neural plate explants grown in contact with *Shh*-transfected COS cells express SC1 (red). Isl^+ neurons that do not coexpress SC1 probably represent newly-differentiated motor neurons [21]. **(c)** Isl-2^+ neurons in intermediate neural plate explants grown in contact with *Shh*-transfected COS cells. **(d)** A neural plate explant separated from *Shh*-transfected COS cells in a collagen gel and grown for 36 h contains Isl^+ (green) but not $HNF3\beta^+$ (red) cells. **(e)** Isl^+ neurons (green) induced at a distance from *Shh*-transfected COS cells coexpress SC1 (red) and are motor neurons. **(f)** Isl-2^+ neurons induced at a distance from *Shh*-transfected COS cells. Intermediate neural plate explants grown in contact with or at a distance from COS cells transfected with antisense *Shh* cDNA did not contain $HNF3\beta^+$, Isl^+ or Isl-2^+ cells (Table 2, and data not shown). **(g)** RT–PCR analysis of floor plate induction by *Shh*-transfected COS cells (s, sense; as, antisense). *HNF3β* and *Netrin-1* expression is induced in neural plate explants grown in contact with *Shh*-transfected COS cells (lanes 1) but not with antisense *Shh*-transfected COS cells (lanes 2). *HNF3β* and *Netrin-1* expression is not induced in neural plate explants grown at a distance from *Shh*-transfected (lanes 3) or antisense *Shh*-transfected (lanes 4) COS cells. In the same experiment, notochord (n) grown in contact with neural plate explants induces both *HNF3β* and *Netrin-1* expression (lanes 5). **(h)** RT–PCR analysis of motor neuron induction by *Shh*-transfected COS cells (s, sense; as, antisense). *Isl-1* and *ChAT* expression is induced in neural plate explants grown in contact with *Shh*-transfected COS cells (lanes 2). *Isl-1* and *ChAT* expression are also induced in neural plate explants grown at a distance from *Shh*-transfected COS cells (lanes 3). *Isl-1* and *ChAT* expression is not induced in neural plate explants exposed to COS cells transfected with antisense *Shh* (lanes 2 and 4). Notochord (n) grown in contact with neural plate explants induces both *Isl-1* and *ChAT* (lanes 5). Results shown in Panels (a–h) have been replicated in 6 different experiments. Scale bar: (a,d) = 16 μm; (b,e) = 20 μm; (c,f) = 33 μm.

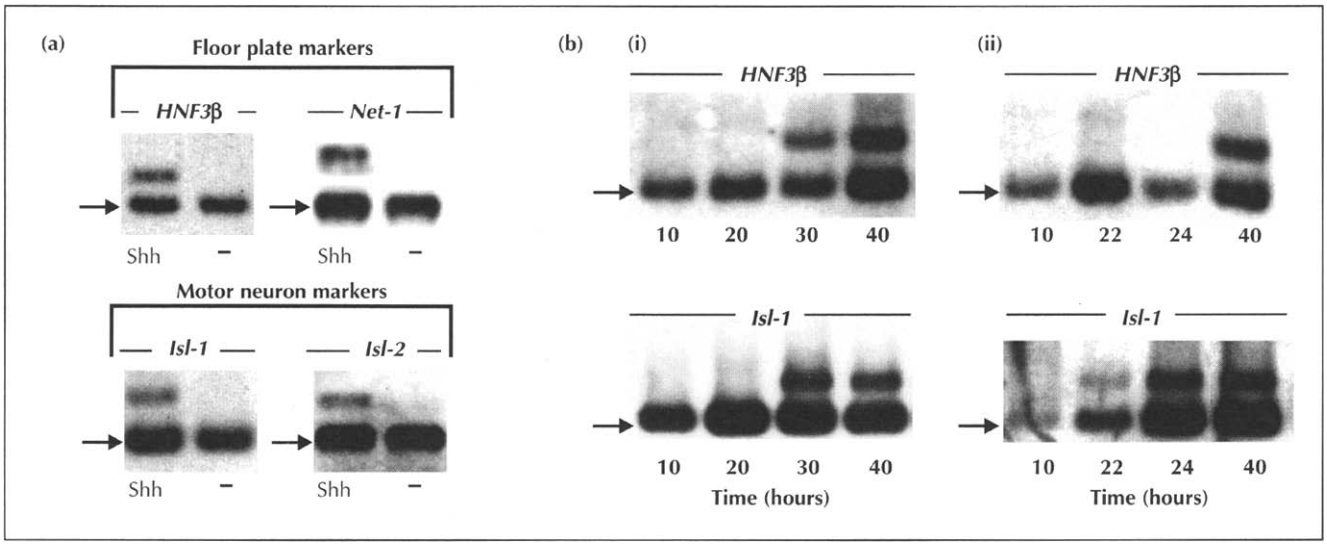

Fig. 4. Induction of floor plate and motor neuron differentiation by transfection of neural plate explants with *Shh*. **(a)** RT–PCR analysis of floor plate and motor neuron marker expression in neural plate explants analyzed 48 h after transfection with a CMV *Shh* cDNA expression construct. *HNF3β*, *Netrin-1*, *Isl-1* and *Isl-2* are induced in *Shh*-transfected explants but not in mock-transfected (–) explants. *Isl-1* was also detected albeit at lower levels in *Shh*-transfected neural plate explants grown in the absence of neurotrophin-3 (data not shown). Cells that expressed HNF3β and Isl immunoreactivity could also be detected (data not shown), although there was an extremely high background, possibly because of cell damage as a consequence of the transfection protocol. **(b)** Time-course of *HNF3β* and *Isl-1* expression in neural plate explants transfected with a CMV *Shh* cDNA expression construct. **(i)** In this experiment, neither *Isl-1* nor *HNF3β* are expressed 10 h or 20 h after transfection (lanes 1 and 2), but are detected at 30 h and 40 h (lanes 3 and 4). *Netrin-1* and *Isl-2* are also expressed after 30 h (data not shown). **(ii)** In this experiment, *Isl-1* expression is not apparent at 10 h (lane 1) and can first be detected at 22 h (lane 2). In contrast, *HNF3β* expression is not detected at either 22 h or 24 h (lanes 2 and 3, although the gene is expressed at 40 h (lane 4). Results showing that *Isl-1* expression occurs before or coincident with *HNF3β* expression were obtained in 4 separate experiments. In a further 3 experiments, *Isl-1* expression was detected, although *HNF3β* expression could not be detected.

floor plate or motor neuron differentiation in neural plate explants [15]. In the present experiments, the induction of motor neuron differentiation in neural plate explants grown at a distance from *Shh*-transfected COS cells might result from the provision of a higher concentration, or of a constant source, of Shh. COS cells transfected with antisense *Shh* did not induce floor plate or motor neuron differentiation under any condition (Fig. 3g,h, lanes 2 and 4, and data not shown).

Expression of Shh, therefore, confers COS cells with a contact-dependent floor-plate-inducing activity and a diffusible motor-neuron-inducing activity that does not elicit floor plate differentiation. The most likely explanation of these results is that Shh itself mediates both these activities. COS cells transfected with *Shh* also exhibit a diffusible activity that can induce ventral forebrain neurons [22] and ventralize segmental plate mesoderm [23].

Independent induction of motor neuron and floor plate differentiation after transfection of neural plate explants with *Shh*

To eliminate the possibility that COS cells transfected with *Shh* are induced to secrete a distinct factor that mediates the floor-plate- or motor-neuron-inducing activities described above, we tested whether Shh can itself induce motor neuron and floor plate differentiation. Cells within neural plate explants were transfected directly with an *Shh* expression construct (Fig. 1e). When assayed 48 hours after transfection with *Shh*, neural plate explants expressed *HNF3β*, *Netrin-1*, *Isl-1* and *Isl-2* mRNAs (Fig. 4a). Shh is, therefore, sufficient to induce floor plate and motor neuron differentiation in neural plate explants.

To determine whether the induction of motor neurons in neural plate explants transfected with *Shh* occurs independently of floor plate differentiation, we analyzed the time-course of expression of *HNF3β* and *Isl-1*. Expression of *Isl-1* in neural plate explants transfected with *Shh* was first detected after about 22 hours, and either preceded (Fig. 4b(ii)) or occurred coincidentally (Fig. 4d(i)) with that of *HNF3β*, depending on the particular experiment. Thus, motor neuron differentiation in neural plate explants transfected with *Shh* occurs prior to, or synchronously with, floor plate differentiation.

These results indicate that Shh acts on neural plate cells to induce the differentiation of motor neurons in a manner that does not require the prior differentiation of floor plate cells. Previous studies in chick embryos have shown that cells in lateral regions of the neural plate are exposed to a motor-neuron-inducing signal from the notochord prior to the differentiation of floor plate cells [8]. Our results support the idea that Shh mediates this notochord-derived signal.

Shh as an inducer of motor neurons

The present findings provide evidence that the ability of the notochord to induce motor neuron differentiation via a diffusible factor can be attributed to the activity of

Shh. Importantly, the induction of motor neuron differentiation in response to Shh does not appear to require the prior differentiation of floor plate cells. This finding provides direct evidence on the identity of an inductive factor responsible for the differentiation of motor neurons in the embryonic central nervous system. Our results are consistent with the idea that, *in vivo*, Shh diffuses from the notochord and acts on lateral neural plate cells to induce the expression of transcription factors, including Isl-1 and Isl-2, that commit neural progenitor cells to a motor neuron fate. We cannot, however, exclude the possibility that the initiation of motor neuron differentiation by Shh is mediated solely by the contact-dependent induction of a distinct secreted factor in neural plate cells. If this factor exists, our results would indicate that its induction in neural plate cells by Shh is independent of floor plate differentiation.

The mechanism by which Shh induces the differentiation of two distinct ventral cell types, floor plate cells and motor neurons, is not resolved by the present studies. *Drosophila* and vertebrate hedgehog proteins undergo autoproteolysis to generate an amino-terminal cleavage product, which is associated with the cell surface, and a carboxy-terminal cleavage product, which is diffusible [24–26]. The induction of motor neuron and floor plate differentiation, however, does not appear to result from distinct biological activities that reside in the two cleavage products of Shh. The amino-terminal autoproteolytic cleavage product of *Drosophila* hedgehog has been shown to mediate the biological activities of the hedgehog precursor [25,27], and in recent studies we have found that the amino-terminal cleavage product of Shh has both floor-plate- and motor-neuron-inducing activity [28]. Taken together with the present finding, these results raise the possibility that, *in vivo*, motor neuron and floor plate fates are specified by different concentrations of the amino terminal cleavage product of Shh, in a manner similar to that proposed for transforming growth factor β (TGFβ)-related proteins in the patterning of mesodermal tissues [29–31].

The ability to induce motor neuron differentiation distinguishes Shh from other factors that are known to regulate the embryonic development of motor neurons (Fig. 5). Members of the neurotrophin, TGFβ and fibroblast growth factor (FGF) families, and neurally active cytokines such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), support the survival of embryonic motor neurons [32,33], but do not elicit the differentiation of motor neurons from progenitor cells in the neural plate (Fig. 4a, and data not shown). Neurotrophin-3 (NT3) has been shown to increase the number of motor neurons in dissociated neural tube cultures, possibly by enhancing the survival and/or proliferation of progenitor cells [34–36]. The initial induction of motor neuron differentiation, therefore, appears to be mediated by Shh, whereas the later progression of motor neuron development may involve the actions of distinct families of secreted signaling proteins.

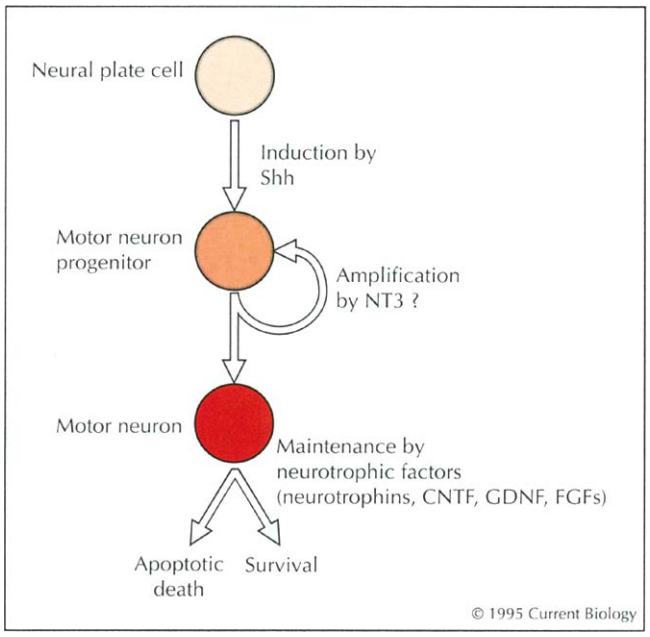

Fig. 5. Sequential actions of secreted signaling factors during embryonic motor neuron development. The diagram illustrates the distinction between the activities of Shh and those of other factors that regulate motor neuron development. Shh appears to initiate the differentiation of motor neurons in neural plate cells, whereas neurotrophins and other neurotrophic factors regulate later steps in the embryonic development of motor neurons. (GDNF is glial-derived neurotrophic factor.)

Conclusions

The data in this paper show that Shh can induce motor neurons in a manner that is independent of floor plate differentiation. The independent induction by Shh of two distinct cell types generated in the ventral region of the neural tube suggests that the differentiation of other classes of ventral neurons may also be dependent on Shh-mediated signaling. Previous studies have shown that dorsalin-1, a member of the bone morphogenic protein (BMP) family, can suppress motor neuron differentiation and promote neural crest cell differentiation. The opposing actions of Shh and BMPs may, therefore, control the induction and patterning of distinct cell types along the dorsoventral axis of the neural tube.

Materials and methods

Neural plate induction assays

Intermediate neural plate explants were dissected from the caudal region of the neural plate of Hamburger-Hamilton [37] (HH) stage-10 chick embryos as described [8]. Notochord explants were dissected after dispase treatment from the caudal region of HH stage-10 chick embryos. Conjugates between notochord and neural plate explants were prepared in collagen gels. When required, notochord and neural plate explants were separated by Nucleopore polycarbonate (pore size 0.1 μm, COSTAR) or dialysis membrane (Spectrum, Spectra/Por membrane, molecular weight cut off: 50 kD) filters. Explants were grown in defined medium as described [8].

For the induction of floor plate cells and motor neurons by *Shh*-transfected COS cells, intermediate neural plate explants were placed on a monolayer of transfected COS cells, embedded in the collagen gel and cultured for 36 h in F12/N3 medium. To prepare transfilter assays, intermediate neural plate explants were separated from COS cells by a polymerized collagen gel, by Nucleopore Polycarbonate filter or by dialysis membrane (see Fig. 2 legend).

Detection of neural markers

HNF3β was detected with rabbit antibodies [17,18], Isl-proteins were detected by antibodies that recognize both Islet-1 and Islet-2 (Isl^+ cells), and by Isl-2-specific monoclonal antibodies [21,38] (S. Morton, unpublished). The SC1 glycoprotein was detected with SC1-specific monoclonal antibody [39]. Neural plate explants were fixed with 4 % paraformaldehyde at 4 °C for 1–2 h and washed with phosphate-buffered saline (pH 7.4) at 4 °C for 1–2 h. Explants were incubated with primary antibodies overnight at 4 °C, then with FITC-conjugated goat anti-mouse lgG (Boehringer Mannheim) or Texas red-conjugated goat anti-rabbit lgG (Molecular Probes) for 1–2 h at 22 °C. The explants were then washed and mounted on slides in 50 % glycerol:50 % 0.2 M carbonate buffer, pH 9.0 containing paraphenylene diamine (0.4 mg ml^{-1}). Explants were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics. Optical sectioning of explants was performed on a Bio-Rad MRC-500 confocal microscope.

Competitive PCR analysis

RT-PCR analysis was performed essentially as described [8]. Total RNA was extracted from 10–20 explants cultured in collagen gel with 5 μg of glycogen as carrier [40]. An internal standard for competitive PCR analysis was prepared by deleting (in *HNF3β*, *Isl-1*) or inserting (in *Isl-2*, *Netrin-1*, *ChAT*) a 200–300 base-pair fragment within the sequence to be amplified. Plasmid DNAs were linearized and transcribed *in vitro* to prepare sense-oriented RNA. 100 fg of competitive template RNA was added to the total RNA of each sample and was reverse transcribed using MoMLV-RT (Gibco BRL). One tenth of each reaction product was subjected to PCR using specific primers flanking the deleted or inserted site of each clone. The primers used were:

HNF3β: 5′-TCACCATGGCCATCCAGCAGTCG and
5′–CAGCAGGTGCTGCGCTGGAGAGG;
Netrin-1: 5′-TGGGCAGCACCGAGGAC and
5′–CCTTCCATCCCTCAATA;
Isl-1: 5′–TCAAACCTACTTTGGGGTCTTA and
5′–ATCGCCGGGGATGAGCTGGCGGCT;
Isl-2: 5′–TGCTGAACGAGAAGCAG and
5′–TGGTAGGTCTGCACCTCCA;
ChAT: 5′–TCCATACGCCGATTTGATGAGGGC and
5′–CTATTGCTTGTCAAATAGGTCTCA.

Each PCR cycle was at 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min. Twenty two cycles were used for amplifying *Isl-2*, *Isl-1*, *HNF3β* and *Netrin-1*, and twenty cycles for *ChAT*. The PCR products were detected by Southern-blot hybridization with ^{32}P-labeled DNA probes. Blots are aligned such that the tissue-derived band is above the internal standard. Sizes of tissue-derived PCR bands are: *HNF3β* 510 bp, *Netrin-1* 232 bp, *Isl-1* 427 bp, *Isl-2* 304 bp and *ChAT* 283 bp.

COS cell transfections

Transfections with sense or antisense *Shh* expression plasmids were performed as described [15]. Briefly, 1 μg of DNA and 12 μg ml^{-1} of Lipofectamine (Gibco BRL) in Dulbecco's modified Eagles medium (DMEM) supplemented with 1 % glutamine was added to the 80–90 % confluent COS cells in 35 mm dishes. After 5 h of incubation, the transfection reaction was stopped by replacing the medium with DMEM supplemented with 10 % calf serum. Induction assays were carried out after 36 h of incubation.

Neural plate transfections

CMV- or RSV-LTR-based *Shh* expression plasmids were transfected directly into intermediate neural plate explants using Lipofectamine (Gibco BRL). 400 ng of DNA and 2 μg of Lipofectamine were mixed in 100 μl of F12/N3 and added to neural plate explants. The explants were incubated for 5 h, rinsed and cultured in collagen gels as described [8]. In experiments on *Shh*-transfected explants, 28 cycles of amplification were used on 1/100th of the tissue-derived cDNA product. The viability of neural plate explants subjected to the transfection protocol was impaired (data not shown). We therefore supplemented the culture medium with neurotrophin-3 (10 ng ml^{-1}, Genentech, Inc.), which has no floor-plate- or motor-neuron-inducing activity (Fig. 4a and data not shown), but which enhances the number of motor neurons that differentiate in dissociated neural tube cultures [34].

Acknowledgements: We thank T. Yamada for help in establishing *in vitro* assays, S. Morton, S. Pfaff and T. Tsuchida for Isl-2-specific antibodies, A. Ruiz i Altaba for the HNF3β antibody and chick *HNF3β* primer sequences, A. Rosenthal (Genentech) for neurotrophin-3 and M. Tessier-Lavigne for *Netrin-1* primer sequences. We also thank R. Axel, J. Dodd, T. Edlund, S. Pfaff, G. Struhl and A. Tomlinson for comments on the manuscript, and I. Schieren and C. Bireline for help in preparing the text and figures. This work was supported by N.I.M.H. grant MH50733-01. Y.T. is an Associate and T.M.J. an Investigator of the Howard Hughes Medical Institute.

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Received: **10 March 1995**; revised: **13 April 1995**.
Accepted: **13 April 1995**.