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The seven-transmembrane receptor Smoothened cell-autonomously induces multiple ventral cell types

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Sonic Hedgehog (Shh) is a secreted protein that controls cell fate and mitogenesis in the developing nervous system. Here we show that a constitutively active form of Smoothened (Smo-M2) mimics concentration-dependent actions of Shh in the developing neural tube, including activation of ventral marker genes (*HNF3β*, *patched*, *Nkx2.2*, *netrin-1*), suppression of dorsal markers (*Pax-3*, *Gli-3*, *Ephrin A5*) and induction of ventral neurons (dopaminergic, serotonergic) and ventrolateral motor neurons (Islet-1+, Islet-2+, HB9+) and interneurons (Engrailed-1+, CHX10+). Furthermore, Smo-M2's patterning activities were cell autonomous, occurring exclusively in cells expressing *Smo-M2*. These findings suggest that Smo is a key signaling component in the Hh receptor and that Shh patterns the vertebrate nervous system as a morphogen, rather than through secondary relay signals.

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Sonic hedgehog (Shh) is a vertebrate homolog of the secreted segment-polarity gene, Hedgehog (Hh), which is produced by the embryonic notochord and floor plate. Genetic studies in human[1,2] and mouse[3] as well as studies with cultured chick and rodent neural-tube explants[4] revealed that Shh controls multiple cell-patterning events along the ventral aspect of the neural tube. An early activity of Shh is to restrict the expression of multiple transcription factors (for example, *Pax3*, *Pax7*, *Msx1* and *Msx2*)[4] and secreted proteins (for instance, *Ephrin-A5*)[5] to the dorsal aspect of the neural tube. Concomitant with the exclusion of future dorsal cell markers, Shh upregulates the expression of ventral transcription factors [for example, HNF3β, Nkx 2.2 (ref. 6) Pax 6 (ref. 7) and Gli-1 (ref. 5)] as well as cell surface (Ptc)[8] and secreted (for example, Netrin-1)[9,10] proteins in the ventral neural tube, thus committing the affected neural precursors to a general ventral cell fate. Shh further induces the specification of these 'generic' ventral progenitors to distinct neuronal and non-neuronal cell types that typify the ventral aspect of the mature vertebrate nervous system. These include floor plate cells, Islet-1, 2 and HB9-positive motor neurons, Chx10+ and Engrailed-1+ (En-1) interneurons[4], serotonergic (5HT)[5,11], dopaminergic (DA)[12,13] and multiple forebrain[6] neurons.

The mechanism of Shh signal transduction is not fully understood. However, biochemical studies demonstrate that Shh binds the 12-transmembrane (TM) protein Ptc[14,15], and that Ptc in turn forms a physical complex with Smo[15]. Gene ablation and overexpression experiments in *Drosophila* as well as gene ablation of *Ptc* in mice further suggest that Smo is required for Hh signal transduction[16,17]. In contrast, Ptc seems to be a negative regulator in this signaling system[18–20]. Consistent with these suggestions, *Ptc* is inactivated in hereditary basal cell nevus syndrome (BCNS), a disease associated with developmental abnormalities and high incidence of medulloblastomas and basal cell carcinomas[21]. In contrast, *Smo* acquires activating mutations in sporadic forms of this disorder[22].

Taken together, these findings lead to the hypothesis that the receptor for Shh is composed of both Ptc and Smo[15,20,23]. However, although Smo mediates simple mitogenic signals of Shh in vertebrate skin[22], the question of whether it is involved in the coordination of the complex patterning actions of Shh in the neural tube remains open. To directly examine this issue, we expressed a constitutively active form of *Smo* (*Smo-M2*) in the neural tubes of mice and chick embryos. We show that ectopic expression of *Smo-M2* resulted in downregulation of dorsal cell markers, induction of ventral cell markers and the ectopic formation of ventral neurons.

Having obtained evidence that Smo-M2 is a key signaling component of the Shh receptor, we asked whether Shh induces distinct cell types in the neural tube directly or through a second wave of secreted inducers. We assumed that if Shh specified neural progenitors directly, its activated receptor would suppress dorsal cell markers and upregulate ventral cell markers only in the cells in which it was expressed. On the other hand, if a relay signal were involved, cells that did not express Smo-M2 would also be affected, as they would respond to secreted relay signals produced by Smo-M2-expressing cells. Studies in non-neuronal tissues in *Drosophila* and vertebrates suggest that, depending on the particular system, Hh can mediate its activities directly or via a relay mechanism. For example, the mitogenic and patterning effects of Shh in the vertebrate limb are executed via members of both the fibroblast growth factor (FGF) and the bone morphogenic factors (BMP) protein families[24,25], which act as relay signals. Likewise, the cell-patterning effects of Hh in the anterior compartment of the *Drosophila* leg imaginal disc are thought to be mediated indirectly via the induction of BMP, dorsally, and wingless, ventrally[26]. In addition, Hh seems to influence cell polarity in the *Drosophila*

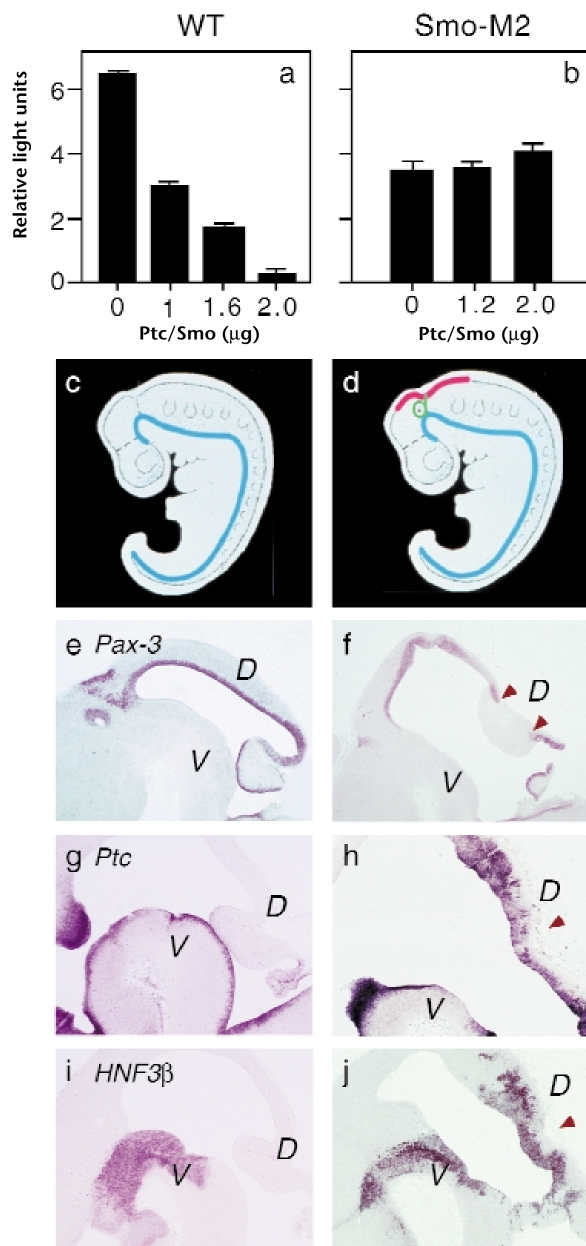

Fig. 1. Smo-M2 ventralizes the neural tube in transgenic mice. (**a**, **b**) Induction of Shh-responsive reporter gene by Smo and Smo-M2. WT Smo is inhibited by Ptc (**a**), whereas Smo-M2 is not (**b**). Smo activity is indicated by the luciferase reporter gene. Data represent the mean ± s.d. of duplicate determinations from one of three representative experiments. (**c**, **d**) Schemata for transgenic mice. The region in which WT Smo and Smo-M2 were expressed in the transgenic mice is indicated in pink. Blue line indicates the ventral midline. (**e**, **f**) *Pax-3*, which is normally expressed along the length of the dorsal midbrain and hindbrain (**e**) is suppressed in the region flanked by the red arrowheads in the *Smo-M2* TG embryos (**f**). Anterior is to the left. (**g–j**) *Ptc* (**g**, **h**) and *HNF3β* (**i**, **j**), normally present at high levels only in the ventral mid-/hindbrain of E12 WT embryos, are each ectopically induced (marked by red arrowheads) in the dorsal mid-/hindbrain of *Smo-M2* transgenic embryos. D, dorsal; V, ventral brain. Scale bar, ~1.0 mm (**e–i**).

abdomen indirectly, through the induction of additional secreted factors[27]. On the other hand, Hh directly specifies multiple types of cuticular structures and cell types in the *Drosophila* abdomen[27] and dorsal epidermis[28]. Moreover, Shh seems to have direct, long-range actions on sclerotome differentiation[24].

We found that Smo-M2 induced ventral markers and cell types requiring high concentrations of Shh[29] that develop adjacent to Shh-producing floor plate and notochord cells, as well as cell types induced by low concentrations of Shh[4] that develop at a distance from the Shh source. Each of the examined cell types were induced cell autonomously in Smo-M2-expressing cells but not in adjacent or distant cells. These findings strongly support the hypothesis that Smo-M2-expressing cells pattern the ventral neural tube directly, and that Shh therefore, normally acts as a morphogen.

Results

Smo-M2 alters cell pattern in the neural tube

We expressed cDNAs encoding wild-type (WT) Smo[15] or a constitutively active form (Trp 535 to Leu)[22] of Smo (Smo-M2) in transgenic mice under the En-2 promoter-enhancer[30] (**Fig. 1c** and **d**). These two receptor forms seemed to have similar signaling properties but differed in their susceptibility to negative regulation. Thus, in the absence of Ptc, both WT Smo and Smo-M2 were capable of activating a Gli-BS luciferase reporter assay[31] (**Fig. 1a** and **b**). However, whereas Ptc suppressed the activity of WT Smo[31] (**Fig. 1a**), Smo-M2 was refractory to this suppression (**Fig. 1b**).

Visual inspection of *Smo-M2* embryos at days 12 and 14 (E12 and E14) revealed excessive overgrowth of dorsal neural tissue and displacement of the skull bones in the midbrain and hindbrain region (data not shown). A similar phenotype is reported for transgenic embryos that ectopically express *Shh*[32], *Shh-N*[5], *HNF-3β* (an inducer of Shh)[30] or the zinc-finger transcription factor *Gli-1*(a candidate mediator of the Shh signal)[5]. Consistent with the aberrant overgrowth, the expression domain of the homeobox-containing transcription factors *En-1* and *En-2*, normally found in regions of midbrain and hindbrain[33,34], was expanded in these animals (data not shown). In contrast, transgenic mice that expressed the WT Smo protein appeared normal (data not shown).

We next examined whether ectopic expression of Smo-M2 affected dorsal gene expression normally inhibited by Shh. Dorsally, WT embryos strongly expressed *Pax-3* and *Ephrin-A5* (refs. 35 and 36, respectively; **Fig. 1e** and data not shown). In Smo-M2 E12 and E14 embryos, these markers were not expressed in dorsal midbrain and hindbrain regions where Smo-M2 was overexpressed (**Fig. 1f** and data not shown). Downregulation of the zinc-finger transcription factor *Gli3*, which takes place in response to Shh signaling in the chick limb[14] and in the mouse and frog neural tube[5,37], also occurred here (data not shown).

Another important function of Shh in the developing neural tube is the induction of early ventral cell markers, such as *Ptc*, *HNF3β* [4] and *Shh* itself. At E12, *Ptc*[8,15,38,39], *HNF3β* and *Shh*[32,40–42], normally expressed at high levels only in the ventral aspect of the neural tube, were ectopically induced in the dorsal midbrain/hindbrain of the *Smo-M2* embryos (**Fig. 1g–j** and data not shown). Ectopic induction of these genes was maintained in the E14 *Smo-M2* embryos (data not shown), indicating that dorsal progenitors in the midbrain and hindbrain retained a ventral cell fate. In contrast, no ectopic induction of ventral genes was detected in transgenic embryos expressing WT Smo (data not shown).

Smo-M2 specifies ventral cell types

We next examined whether Smo-M2 could mimic the ability of Shh to specify multiple ventral cell types. Three classes of neu-

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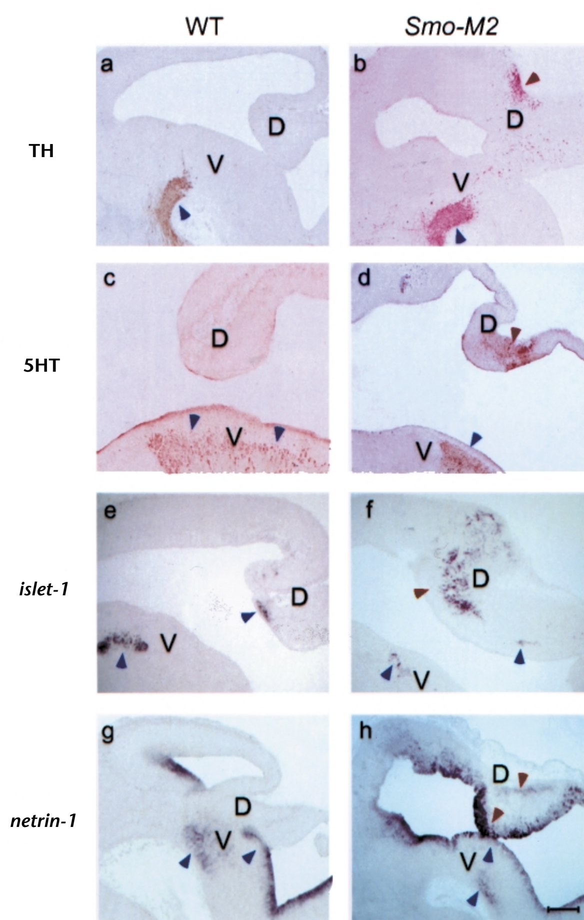

Fig. 2. Induction of multiple neuronal classes and *netrin-1*, in *Smo-M2* TG mice. (**a–h**) Sections of E14 WT (**a**, **c**, **e** and **g**) and *Smo-M2* TG (**b**, **d**, **f** and **h**) embryos stained for TH (**a**, **b**) or 5HT (**c**, **d**), or probed for *islet-1* (**e**, **f**) or *netrin-1* (**g**, **h**). TH immunoreactivity, 5HT immunoreactivity, *islet-1* and *netrin-1* are all induced dorsally in the *Smo-M2* TG mice. Blue arrowheads point to ventral expression and in the case of *islet-1*, normal ventral and dorsal expression. Red arrowheads point to ectopic expression. Scale bars, ~0.65 mm (**a**, **b**), ~0.3 mm (**c**), ~0.8 mm (**d**), ~1.1 mm (**e**), ~0.9 mm (**f**), ~1.1 mm (**g**, **h**).

rons were examined, DA neurons[5,12,13,43,44], located in the ventral midbrain[45], 5HT neurons, present in the ventral hindbrain[5,11,44,46] and *islet-1*-expressing, putative motor neurons in the midbrain, hindbrain and spinal cord[11,47,48]. In addition, we tested for ectopic induction of the ventral marker and axon-guidance protein, Netrin-1 (refs. 9, 10), by Smo-M2.

In WT animals, tyrosine hydroxylase-positive DA neurons were restricted to the ventral part of the midbrain (**Fig. 2a**). In contrast, *Smo-M2* embryos showed two regions of tyrosine hydroxylase immunoreactivity, one ventral and the second in an ectopic, dorsal midbrain location expressing Smo-M2 (**Fig. 2b**). Further, whereas WT embryos developed 5HT neurons only in the ventral hindbrain (**Fig. 2c**), large numbers of ectopic 5HT neurons were observed in the *Smo-M2*-transgenic dorsal hindbrain (**Fig. 2d**). *Islet-1* was also ectopically induced in the dorsal midbrain, showing expanded expression in the dorsal mid- and hindbrain of *Smo-M2* animals (**Fig. 2e** and **f**). Examination of *netrin-1* in WT embryos revealed intense expression in the superficial aspect of the ventral hindbrain and a site of lower expression in the deep hindbrain (**Fig. 2g**). In *Smo-M2*-transgenic (TG) embryos, *netrin-1* was ectopically induced in the dorsal midbrain and hindbrain, with the two regions of expression in the hindbrain faithfully duplicated in mirror image (**Fig. 2h**).

Thus, Smo-M2 reproduced multiple actions of Shh in the developing nervous system, including induction of cell proliferation, suppression of dorsal markers, induction of ventral markers and formation of mature ventral neurons.

Mid-/hindbrain Smo-M2 activity is cell autonomous

Having established that Smo-M2 can reproduce multiple actions of Shh, we next used this receptor to examine whether Shh patterns the neural tube directly or through a relay mechanism. Examination of WT embryos revealed endogenous *Smo* expression along the length of the dorsal midbrain and hindbrain at a consistent, low level (data not shown). In contrast, transgenic *Smo-M2* was intensely expressed in an irregular pattern (**Fig. 3a**). The ectopic induction of ventral (for instance, *HNF3β*, *Shh* and *Ptc*) and the suppression of dorsal (for example, *Ephrin-A5*) markers seemed restricted to *Smo-M2*-expressing cells (**Fig. 3b–d** and data not shown), indicating that Smo-M2, and therefore Shh, patterned the neural tube directly, and not via a relay mechanism.

Because it was impossible to determine the precise boundary of the *Smo-M2* expression domain at the single-cell level in the transgenic mice, it remained possible that cells at the border of *Smo-M2*-expressing cells were patterned via a short acting or membrane-bound relay signal. To address this question, stage-10 chick embryos were electroporated with a single DNA construct to co-express *Smo-M2* and green fluorescent protein (GFP; via an internal ribosomal entry site; **Fig. 3e–g**). The chicks were allowed to develop for three days *in ovo*, and adjacent sections from the midbrain/hindbrain region were analyzed for ectopic expression of ventral cell markers. *Netrin-1* (**Figs. 3h** and **4f**), *Ptc* (**Fig. 3i**) and *HNF3β* (**Figs. 3j** and **4i**) were each induced and co-expressed in ectopic locations. More importantly, ectopic expression of each of these ventral markers was observed only in *Smo-M2*/GFP-expressing cells.

We next studied whether Smo-M2 could cell-autonomously specify ventral midbrain/hindbrain (motor) neurons. Islet-1+ motor neurons are thought to be induced in response to low concentrations of Shh *in vivo* at a distance from the Shh-producing floorplate and notochord[4]. Individual sections from *Smo-M2*/GFP-expressing chick embryos were immunostained for Islet-1 (red fluorescence) and examined for co-expression of Smo-M2/GFP (green fluorescence). In all cases, Islet-1 was induced exclusively in cells that co-expressed Smo-M2/GFP (**Fig. 4a–c**). Individual cells expressed different combinations of markers; some expressed Smo-M2/GFP, *netrin-1* and Islet-1 (**Fig. 4d–f**; arrows), Smo-M2/GFP and Islet-1 but not *netrin-1* (**Fig. 4d–f**; arrowheads) or Smo-M2/GFP and *netrin-1* but not Islet-1. Cells co-expressing Islet-1 and *HNF-3β* were not observed (**Fig. 4g–i**). Taken together, these findings show that Smo induces stereotypic combinations of ventral markers in a cell-autonomous manner.

Smo-M2 induces ventral cell types in the spinal cord

The ventral markers and cell types examined in midbrain/hindbrain normally reside close to the source of Shh. Thus, it remained possible that neurons, which develop in more dorsal aspects of the ventral neural tube, were induced by relay signals. We therefore examined the consequences of overexpressing Smo-M2 in the spinal cord where multiple, well defined cell types are induced in distinct positions along the dorsoventral axis of the neural tube in response to different concentrations of Shh[4].

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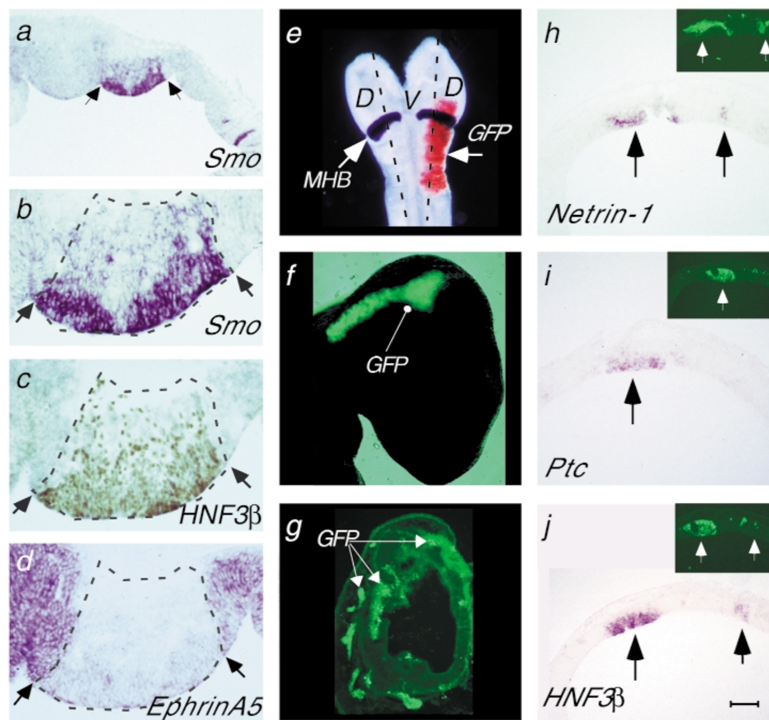


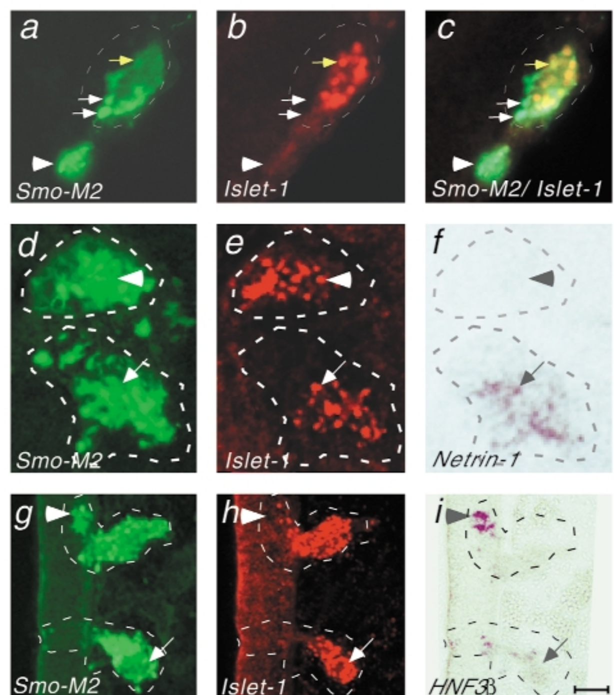
Fig. 3. Changes in gene expression in the midbrain/hindbrain region are confined to the Smo-M2 expression domain. (**a–d**) Photomicrographs of adjacent sections from an E14 Smo-M2 transgenic embryo showing expression of *Smo*/*Smo-M2* (**a**, **b**), HNF-3β (**c**) and *Ephrin-A5* (**d**). Note that the induction and inhibition of the Shh-dependent genes seem restricted to the domain of *Smo-M2*-expressing cells. (**e–g**) Visualization of *GFP* signal in electroporated chick embryos. (**e**) Chick embryos electroporated with *GFP* show high expression of *GFP* mRNA (pink) spanning the midbrain and hindbrain one day after electroporation, as detected by *in-situ* hybridization. Blue signal marks the midbrain/hindbrain boundary (MHB; *in-situ* hybridization with *fgf8*). (**f**) High expression of *Smo-M2*/GFP is retained in the midbrain and hindbrain three days after electroporation. (**g**) Sagittal cryostat sections show GFP expression in patches (arrows point to some) along the length of the midbrain and hindbrain. Clustering of GFP-positive cells was not seen following electroporation of *WT Smo*/GFP or GFP alone or in animals injected with five other genes, suggesting that these patches may result from proliferative effects of Smo-M2. (**h–j**) The Shh-dependent genes *netrin-1* (**h**), *Ptc* (**i**) and *HNF3β* (**j**) were induced in the dorsal hindbrain (black arrows) within a region of *Smo-M2*/GFP-expressing cells (see inserts; white arrows). These genes were always induced in a subset of the *Smo-M2*/GFP-expressing cells. Scale bars, 0.3 mm (**a**), 0.1 mm (**b–d**), 0.12 mm (**e**), 1.0 mm (**f**), 1.2 mm (**g**), 0.3 mm (**h–j**).

Specifically, we followed the expression of Nkx2.2, Islet 1 and 2 and HB9 (expressed in different subtypes of motor neurons or their progenitors), the Chx10 protein (a marker of the more dorsal V2 interneurons) and the homeodomain protein En-1 (expressed even further dorsal in V1 interneurons). These markers, characteristic of cell types lying close the source of Shh as well as those at a distance from the floor plate and notochord, all showed cell-autonomous, ectopic induction in Smo-M2-expressing cells (**Fig. 5**). Interestingly, induction of the characteristically more dorsal markers Chx10 and En-1 was more robust in Smo-M2-transgenic chick embryos, and expression of these markers in cells that expressed lower amounts of Smo-M2/GFP was more prevalent. Thus, overexpression of a single gene, Smo-M2, resulted in cell-autonomous induction of multiple, distinct, ventral markers characteristic of cells induced over a wide concentration range of Shh activity.

Fig. 4. *Smo-M2* induces Islet-1+ motor neurons cell autonomously in the mid/hindbrain region. (**a**–**c**) Sagittal section through the chick dorsal midbrain showing ectopic Islet-1 expression that is completely confined to Smo-M2/GFP+ cells (ectopic Islet-1 expression was never observed in Smo-M2/GFP negative cells in multiple sections, derived from 6 animals). By confocal analysis, cytoplasmically localized GFP was seen surrounding nuclear Islet-1 in a single cell (not shown). (**d**–**f**) Sequential photography of a single section shows that the ectopic induction of Islet-1 and *netrin-1* are both confined to *Smo-M2*/GFP+ cells. In some cases, Islet-1 colocalizes with *netrin-1* (arrows), but in other cases Islet-1 is induced in the absence of *netrin-1* (arrowheads). Likewise, *netrin-1* can be detected in some cells that are Islet-1-negative. (**g**–**i**) Sequential photography of a single section for Smo-M2/GFP, Islet-1 and *HNF3β* shows that Islet-1 (arrows) and *HNF3β* (arrowheads) are both induced in subsets of Smo-M2/GFP+ cells, but co-expression of Islet-1 and *HNF3β* is not seen. Scale bar, 0.35 mm (**a**–**c**), 0.4 mm (**d**–**f**), 0.5 mm (**g**–**i**).

Discussion

By ectopically expressing Smo-M2 in the dorsal mid- and hindbrain of transgenic mice and chicks, we demonstrated that this protein could mimic multiple actions of Shh. These include the induction of Shh-responsive genes *Ptc*, *HNF3β*, *Shh*, *Nkx2.2* and *netrin-1*, the suppression of dorsally restricted markers *Pax-3*, *Ephrin-A5* and *Gli-3* and the induction of DA-, 5HT-, d-MN and v-MN motor neu-



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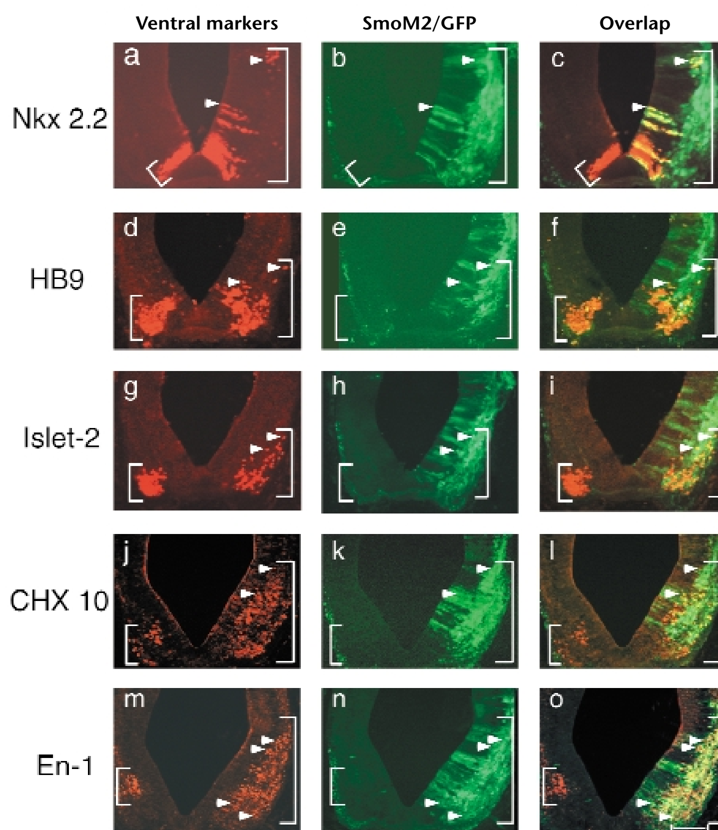

Fig. 5. *Smo-M2* induces ventral and ventrolateral markers and cell types in the spinal cord. (**a**–**o**) Smo-M2 induces the marker for ventral spinal cord and d-MN progenitor, Nkx2.2 (**a**–**c**), the v-MN motor neuron markers HB9 (**d**–**f**) and islet-2 (**g**–**i**), and the progressively more dorsal V1 and V2 interneuron markers Chx10 (**j**–**l**) and En-1 (**m**–**o**). Each of these markers and cell types is induced in a cell-autonomous manner in the electroporated (right) but not control (left) side of the neural tube (examples marked by arrowheads). Scale bar, ~0.5 mm (**a**–**o**).

rons as well as V1 and V2 interneurons (for nomenclature, see ref. 49). We further show that these patterning activities are cell autonomous, occurring only in cells that express Smo-M2. Together with the available genetic and biochemical data, these findings support the ideas that Smo is the key signaling component of the Shh receptor and that Shh patterns the entire ventral aspect of the neural tube directly as a morphogen, and not via relay signals.

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Smo as a signaling coreceptor for Shh

Genetic studies in *Drosophila* as well as biochemical studies in vertebrates prompted the hypothesis that Smo is a signaling subunit in a multi-component receptor for Shh. However, biological evidence for this hypothesis in vertebrates has been diffuse. Known activities of Smo included its ability to activate a single reporter gene in culture and to elicit basal cell carcinoma in the skin of transgenic mice, but the relationship between Smo and the patterning activities of Shh in the developing nervous system were not examined. Thus, the Shh signal in the neural tube could be mediated by a Shh receptor other than Smo and/or indirectly by an entirely distinct relay signal. Here we show that Smo can coordinate complex patterning signals involving the activation or suppression of at least 15 different genes including *Ptc, HNF3β, Shh, netrin-1*, Nkx2.2, En1, Chx10, HB9, Islet-1 and 2, TH, tryptophan hydroxylase, *Pax-3*, *Ephrin-A5* and *Gli3*. These findings demonstrate that Smo can transduce the full spectrum of Shh responses in the vertebrate nervous system and can interpret and execute a graded Shh signal.

Unlike *Smo-M2*, WT *Smo* did not elicit any Shh response in transgenic mice or in chick embryos. Similarly, whereas expression of *Smo-M2* in the skin of transgenic mice under the Keratin-5 promoter leads to basal cell carcinoma-like skin lesions[22], expression of WT *Smo* under the same promoter produced no detectable phenotype (F. de S., A.R.; unpublished observations). These differences seem to stem from differential susceptibility to negative regulation by Ptc[31] (**Fig. 1**), the Shh coreceptor, which is present[15] and active[19] throughout the mouse nervous system.

Smo and Shh pattern the neural tube directly

The availability of Smo-M2 allowed us to directly discern whether Shh patterns the vertebrate neural tube as a morphogen or via additional patterning molecules. Whereas non-cell-autonomous activities would indicate relay signals, cell-autonomous actions of Smo-M2 demonstrated that Shh patterned the neural tube directly. We cannot exclude the possibility that ventral markers and neurons not examined here arise by non-cell-autonomous actions of Smo. However, all examined changes in gene expression and cell fate, including induction of motor neurons and interneurons that normally arise in response to low concentrations of Shh and at a distance from its sources, were strikingly restricted to cells expressing Smo-M2. This, combined with the induction of multiple cell types by Shh in a concentration-dependent manner, in explant culture, strongly argues that Shh acts as a morphogen to pattern the ventral aspect of the neural tube.

All the observed activities of Smo-M2 seemed cell autonomous, even though *Shh* was induced and conceivably could have acted on neighboring cells. One possible explanation for the failure of the induced Shh to act at a distance is that its induction occurred after the developmental period during which neural progenitors can change their fate. Alternatively, Shh may have failed to diffuse in the dorsal neural tube. Additionally, it is formally possible that Shh does not normally diffuse but instead only patterns cells in which it is transiently expressed. In sum, our findings support the hypotheses that Smo is the key signaling coreceptor for Shh and that Shh is a morphogen in the neural tube.

Methods

Transgenic mice. Complementary cDNAs encoding either the WT Smo or Smo-M2 were placed under the control of the En-2 enhancer promoter[30,43], and the resulting constructs were injected into SJL/C57b/6 zygotes. Embryos were harvested at either E12 or E14 (plug, D0) and were tested for the transgene by PCR. Twenty-seven WT Smo and seven Smo-M2 mutant animals were identified and analyzed.

Histology and *in-situ* hybridization. Embryos were fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose in 0.12 M phosphate buffer, pH 7.4, embedded in the sagittal plane and stored frozen until sectioning on a cryostat (14-µM sections). Prepared sections were dried (50°C, 30 min) and re-stored at –70°C until further use. Sections were subjected to *in-situ* hybridization performed with 500–700 bp digoxygenin-labeled probes, as described[12,43]. Immunohistochemistry was carried out as described[43] using antisera to HNF-3β (provided by A. Ruiz i Altaba), rabbit anti-Islet-1, 4G11 (anti-En-1, anti-HB9 and anti-CHX-10, provided by T. Jessell and S. Brenner-Morton), 39.4D5 (mouse anti-Islet-1), 51.4H9 (mouse anti-Islet-2 and anti-Nkx 2.2; purchased from the Developmental Studies

Hybridoma Bank, University of Iowa), anti-rabbit TH (1:500; Chemicon, Temecula, California) and anti-5HT (Incstar, Stillwater, Minnesota).

Luciferase assay. The luciferase experiments were reported as described[31,50]. C3H10T1/2 cells were seeded in 15-cm plates and transfected 24 h after plating with different ratios of gD-WT-Smo or gD-M2-Smo to Ptc-GFP (normalized to 8 μg of total DNA with control GFP vector), 8 μg of reporter plasmid, and 0.01 μg Renilla Luciferase reference plasmid (pRL-TK). Forty-eight hours after transfection, 1×10^6 cells were labeled for FACS analysis with an anti-gD monoclonal antibody and gD/GFP double-positive cells were sorted with an Epics Elite cell sorter (Coulter, Hialeah, Florida). Equal numbers of double-positive cells were then directly lysed and processed for luciferase activity[31]. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin), and values were normalized using Renilla Luciferase activity. All the experiments were repeated at least three times.

Electroporation. Fertilized E0 chicken eggs were incubated at 38°C in a humidified incubator for 20 hours until the embryos reached HH stages 8–10. A small window was made in the egg shell, and DNA (5–8 mg per ml) encoding Smo-M2/GFP or WT-Smo/GFP with an internal ribosomal entry site or GFP alone, was microinjected into the central canal of the neural tube at the mid-/hindbrain or spinal cord levels. A pair of platinum electrodes flanking the neural tube delivered 6 pulses (28 V; duration, 50 ms; interpulse interval, 100 ms). DNA was introduced into one side of the neural tube after the electric shock. The manipulated egg was then sealed with Scotch tape (3M), and the embryos were allowed to develop for three days before harvesting and processing for immunohistochemistry and *in-situ* hybridization. For colocalization of GFP, *in-situ* hybridization and antibody staining signals, photos of the GFP signal were obtained before antibody incubation and *in-situ* hybridization.

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