

# The teratogenic *Veratrum* alkaloid cyclopamine inhibits Sonic hedgehog signal transduction

John P. Incardona<sup>1</sup>, William Gaffield<sup>2</sup>, Raj P. Kapur<sup>3</sup> and Henk Roelink<sup>4,\*</sup>

<sup>1</sup>Department of Pediatrics, University of Washington, Seattle, WA 98195, USA

<sup>2</sup>Western Regional Research Center, ARS, USDA, Albany, CA 94710, USA

<sup>3</sup>Department of Pathology, University of Washington, Seattle, WA, 98195, USA

<sup>4</sup>Department of Biological Structure, University of Washington, Seattle, WA 98195, USA

\*Author for correspondence (e-mail: roelink@u.washington.edu)

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## SUMMARY

The steroidal alkaloid cyclopamine produces cyclopia and holoprosencephaly when administered to gastrulation-stage amniote embryos. Cyclopamine-induced malformations in chick embryos are associated with interruption of Sonic hedgehog (Shh)-mediated dorsoventral patterning of the neural tube and somites. Cell types normally induced in the ventral neural tube by Shh are either absent or appear aberrantly at the ventral midline after cyclopamine treatment, while dorsal cell types normally repressed by Shh appear ventrally. Somites in cyclopamine-treated embryos show Pax7 expression throughout, indicating failure of sclerotome induction. Cyclopamine at concentrations of 20-100 nM blocks the response of neural plate explants to recombinant Shh-N in a dose-dependent manner. Similar concentrations have no effect on the post-translational modification of Shh by cholesterol in transfected COS-1 cells. Comparison of the

effects of cyclopamine to those of the holoprosencephaly-inducing cholesterol synthesis inhibitor AY-9944 shows that cyclopamine does not induce malformations by interfering with cholesterol metabolism. Although AY-9944 does not interrupt Shh signaling in ovo, it blocks the response to Shh-N in explants cultured without an exogenous cholesterol source. As predicted by current models of the regulation of cholesterol metabolism, the response to Shh-N in AY-9944-treated explants is restored by providing exogenous cholesterol. However, exogenous cholesterol does not restore Shh signaling in cyclopamine-treated explants. These findings suggest that cyclopamine-induced teratogenesis is due to a more direct antagonism of Shh signal transduction.

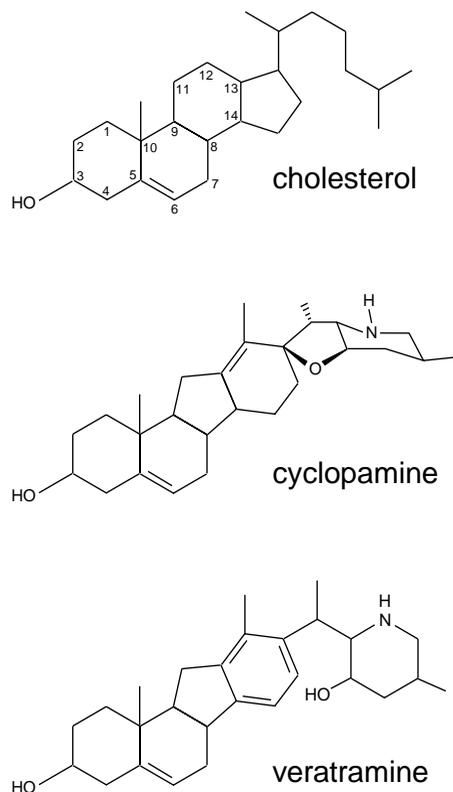
Key words: Sonic hedgehog, Cyclopamine, Cholesterol, Holoprosencephaly, Chick development, Teratogenesis, *Veratrum*

## INTRODUCTION

Cyclopia is a complex birth defect characterized by absence of median facial structures and an undivided forebrain, or holoprosencephaly, and arises from abnormal patterning of the ventral neural tube (Siebert et al., 1990). This malformation was an endemic, but unpublicized, birth defect in sheep herds of the western United States, until an epidemic in the 1950s prompted investigation (Binns et al., 1959). The etiology proved to be ingestion of a lily common in subalpine meadows, *Veratrum californicum*, from which the teratogenic steroidal alkaloid cyclopamine was purified (Fig. 1) (Binns et al., 1963; Keeler, 1969). Administration of cyclopamine or the related compound jervine to gastrulation-stage amniote embryos induces cyclopia at high frequency (Gaffield and Keeler, 1996; Keeler and Binns, 1968). Mutations in *Sonic hedgehog* (*Shh*) also cause holoprosencephaly in mice (Chiang et al., 1996) and humans (Roessler et al., 1996).

Shh secreted from the notochord and floor plate acts as a morphogen that is necessary and sufficient for patterning the ventral neural tube (Tanabe and Jessell, 1996), and is involved

in patterning the ventral somites (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). In vitro experiments indicate that distinct concentrations of Shh induce different ventral neural tube cell types, while repressing formation of dorsal cell types (Ericson et al., 1997; Roelink et al., 1995; Tanabe et al., 1995). Floor plate cells (Shh<sup>+</sup>, HNF-3β<sup>+</sup>) are induced by high concentrations of notochord-derived Shh (Roelink et al., 1995; Tanabe et al., 1995). Lower concentrations of Shh, probably representative of long-range Shh signal derived from notochord and/or floor plate in vivo, is required for induction of motor neurons (Isl1/2<sup>+</sup>) (Ericson et al., 1992; Tsuchida et al., 1994) dorsolateral to the floor plate (Roelink et al., 1995). Ventral motor neurons are derived from Nkx-2.2<sup>+</sup> ventral progenitor cells (Barth and Wilson, 1995) which appear in a bilateral domain immediately adjacent to the floor plate, while dorsal motor neurons are derived from precursors that express Pax6 (Ericson et al., 1997). Lim1/2<sup>+</sup> interneurons are restricted from the ventral neural tube by Shh-mediated suppression (Tsuchida et al., 1994). Probably the earliest events in ventral neural tube patterning are the Shh-mediated repression of Pax3, Pax6 and Pax7, which initially are expressed throughout the neural plate.



**Fig. 1.** The structures of cholesterol, cyclopamine and veratramine. Note that the C3-OH group is present in all molecules, as is the C5-C6 double bond, which is required for teratogenicity of cyclopamine and related steroidal alkaloids (Gaffield and Keeler, 1996). Acid aromatization of the cyclopamine furan ring converts it to veratramine, eliminating its potential to induce cyclopia. Jervine, the other major teratogenic alkaloid produced by *Veratrum* species, is identical to cyclopamine except for a keto group at C-11 (Gaffield and Keeler, 1996).

Pax7 is repressed in the medial neural plate by very low concentrations of Shh (Ericson et al., 1997) and its expression is thus a very sensitive measure of Shh activity.

Perturbations in cholesterol metabolism are also associated with holoprosencephaly in mammalian embryos. Holoprosencephaly is produced in the offspring of pregnant rats treated with AY-9944, an inhibitor of the final step of cholesterol synthesis catalyzed by  $\Delta^7$ -dehydrocholesterol (7-DHC) reductase (Roux and Aubry, 1966). Less severe forms of holoprosencephaly can be a feature of Smith-Lemli-Opitz syndrome, a human disorder that results from genetic reduction in 7-DHC reductase activity (Kelley et al., 1996; Tint et al., 1994). These conditions result in both low levels of tissue cholesterol and accumulation of 7-DHC and other precursor sterols (Kelley et al., 1996; Roux and Aubry, 1966; Tint et al., 1994; Wolf et al., 1996). A mild form of holoprosencephaly also resulted in mouse embryos null for *gp330/megalin*, which encodes a member of the low density lipoprotein (LDL) receptor family expressed in neuroepithelium (Willnow et al., 1996).

Shh is synthesized as a 45 kDa precursor which is cleaved autoproteolytically to generate a 19 kDa amino-terminal fragment possessing the signaling activity (Marti et al., 1995; Porter et al., 1995; Roelink et al., 1995). During cleavage,

cholesterol is covalently attached to the N-terminal fragment and anchors mature Shh in the plasma membrane of cells that synthesize the protein (Porter et al., 1996). Membrane cholesterol also has a potential role in reception and transduction of the Shh signal. Patched, the receptor for Hedgehog proteins (Marigo et al., 1996; Stone et al., 1996), possesses multiple transmembrane domains and forms a non-covalent receptor complex with Smoothened, which is similar to serpentine transmembrane receptors (van den Heuvel and Ingham, 1996). Five of the Patched transmembrane segments are homologous to sterol-sensing domains present in several proteins involved in regulating cholesterol metabolism (Hua et al., 1996). Patched also has additional homology to NPC1, a protein involved in the intracellular transport of LDL-derived cholesterol and lacking in patients with Niemann Pick Type C disease (Carstea et al., 1997; Loftus et al., 1997). Although a role for the putative sterol-sensing domain in Patched has yet to be demonstrated, it is unlikely to be involved in Shh binding: the amino-terminal fragment of Shh expressed from a truncated cDNA (Shh-N) lacks cholesterol but is able to induce the full repertoire of Shh-dependent cell types in neural plate explants (Ericson et al., 1996, 1997; Roelink et al., 1995).

Shh-dependent patterning of the ventral neural tube is interrupted in cyclopamine-treated embryos, indicating that cyclopamine teratogenesis is mediated through interruption of the Shh signaling pathway. The response of neural plate explants to recombinant Shh-N is blocked by cyclopamine in the nM range. The same concentrations of cyclopamine do not affect the cholesterol-dependent biogenesis of Shh in transfected COS-1 cells. Comparison of the effects of cyclopamine to those of AY-9944 shows that, although both compounds interrupt Shh signaling, they do so by different mechanisms.

## MATERIALS AND METHODS

### Drug treatment of chick embryos

Cyclopamine and veratramine were obtained from *V. californicum* as described previously (Gaffield et al., 1986). Fertilized White Leghorn eggs (H & N International, Redmond, WA) were treated either at stage 1 (Hamburger and Hamilton, 1951) or incubated to stage 9-10 before treatment. Cyclopamine was delivered to stage 1 embryos through windows cut in the shell as 5  $\mu$ g complexed with 2-hydroxypropyl- $\beta$ -cyclodextrin (HBC; Sigma) in PBS. Veratramine was delivered as 5  $\mu$ g complexed with HBC. Cyclopamine-HBC (1  $\mu$ g) or veratramine-HBC (5  $\mu$ g) was delivered to stage 9-10 embryos through a small hole torn in the vitelline membrane with a tungsten needle. Cyclopamine- and veratramine-HBC were produced by suspending 1 mg of each compound in 1 ml 45% HBC in sterile PBS and stirring for 1-2 hours at 65°C. Control embryos were treated with HBC alone. For treatment with AY-9944, stage 9-10 embryos were prepared as above. 15 eggs each were treated with 5  $\mu$ l AY-9944 in PBS at concentrations of 220  $\mu$ M, 22  $\mu$ M, 2.2  $\mu$ M and 220 nM. Embryos treated with 220  $\mu$ M AY-9944 failed to establish circulation and arrested development, but 33/45 embryos treated with the three lower concentrations survived to harvesting 60 hours after treatment.

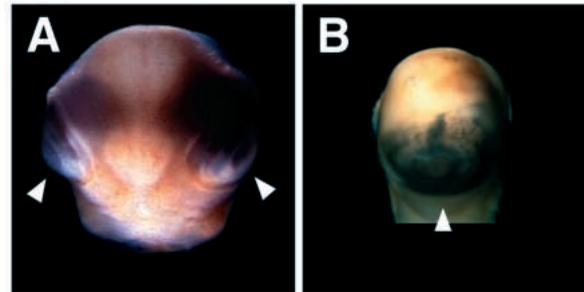
### Immunocytochemistry

Treated embryos were collected at stage 18-19, rinsed in PBS, fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and cryosectioned. The following primary antibodies were used on sections or neural plate explants: anti-Shh polyclonal H4 (Ericson et al., 1996) (1:1000), anti-HNF3 $\beta$  monoclonal (Ruiz i Altaba et al.,

1995) (1:10), anti-Nkx-2.2 mAb 745-A5 (Ericson et al., 1997) (1:100), anti-isl1/2 polyclonal K5 (Ericson et al., 1992) (1:1000), anti-Lim1/2 mAb 4F2 (Tsuchida et al., 1994) (1:10), monoclonal anti-Pax6 and anti-Pax7 (Kawakami et al., 1997) (each 1:10), and monoclonal anti-Msx-1/2 (Liem et al., 1995). Sections were incubated in primary and secondary antibodies, each for 1 hour at room temperature, while explants were immunolabeled as whole mounts, with overnight incubations in primary and secondary antibodies at 4°C, and extensive washing in PBS+0.1% Triton X-100+2% normal goat serum. Secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG (1:200) and fluorescein-conjugated goat anti-mouse IgG (1:500; both from Cappel). All specimens were mounted in 0.1 M carbonate buffer pH 9.5, 50% glycerol and a trace of N-phenylenediamine. Immunofluorescence was viewed on a Nikon Microphot-SA and video images were collected using an Optronics frame integrator. Dual-color images were generated using Adobe Photoshop.

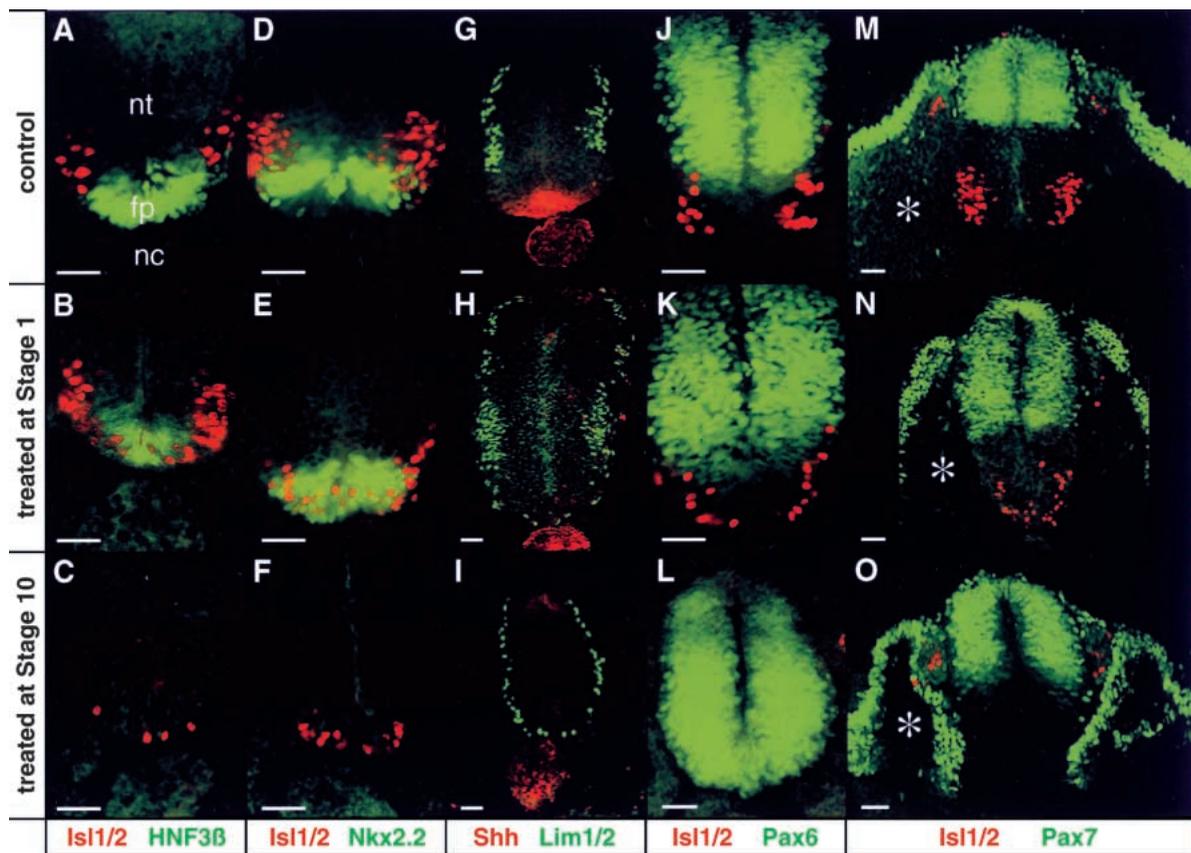
### Transfections and western blots

COS-1 cells were transfected with a full-length Shh cDNA in pMT21 (Roelink et al., 1994; Tanabe et al., 1995) using Lipofectamine (BRL) according to the manufacturer's directions. A single transfected 100 mm dish was divided into a 6-well plate and individual wells were



**Fig. 2.** (A) Frontal view of the head of a 6-day-old chick embryo, treated with HBC alone. Arrowheads indicate the bilateral eyes. (B) Frontal view of the head of a 6-day-old cyclopic, holoprosencephalic embryo generated by administration of 5 µg cyclopamine-HBC at stage 1. Arrowhead indicates the single midline eye.

incubated in medium containing cyclopamine or ethanol solvent alone. After 6 or 36 hours, cells were lysed at 37°C in 150-300 µl buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 4



**Fig. 3.** Analysis of the distribution of neural tube cell types in cyclopamine-treated embryos by immunofluorescence. (A-F,J-L) Transverse sections showing a ventral portion of the neural tube (nt) and a portion of the notochord (nc) beneath. The floor plate (fp) lies at the ventral midline of the neural tube. (G-I,M-O) Lower magnifications of the entire neural tube and a portion of the notochord, and portions of the somites, marked with an asterisk in M-O, lateral to the neural tube. In all sections dorsal is at top. Untreated embryos (top row), embryos treated at stage 1 (middle row) and embryos treated at stage 9-10 (bottom row) were labeled with (A-C) anti-isl1/2 (red) and anti-HNF-3β (green); (D-F) anti-isl1/2 (red) and anti-Nkx2.2 (green); (G-I) anti-Shh (red) and anti-Lim1/2 (green); (J-L) anti-isl1/2 (red) and anti-Pax6 (green); (M-O) anti-isl1/2 (red) and anti-Pax7 (green). In O note the abnormal appearance of the somites in the stage 10-treated embryo in comparison to the control embryo in M; instead of a solid block of tissue with dorsal Pax7<sup>+</sup> cells, the somites of the cyclopamine-treated embryo are hollow in cross-section, with near-circumferential Pax7 expression. Scale bars are 100 µm.

mM N-ethylmaleimide, 10 mM Tris pH 7.5, and 1 µg/ml each leupeptin and pepstatin. Lysates were boiled in SDS sample buffer, subjected to SDS-PAGE in 15% acrylamide. Separated proteins were electroblotted onto nitrocellulose and Shh was detected by polyclonal anti-Shh and peroxidase chemiluminescence.

### Chick neural plate explants

Intermediate neural plate explants were dissected from stage 9-10 chick embryos and cultured in collagen gels (Yamada et al., 1993). Shh-N and BMP4 were obtained as 48 hour collections of serum-free conditioned medium from COS-1 cells transiently transfected with pShh-N (Roelink et al., 1995) expressing truncated Shh-N, and pMT21 expressing a full-length BMP4 cDNA (Liem et al., 1995). Levels of Shh-N in conditioned medium were determined by titration of the response of chick neural plate explants, where floor plate cells are induced by approx. 20 nM Shh-N. The Shh-N conditioned medium was aliquoted, stored at -80°C and fresh frozen aliquots used for each experiment. Explants were always cultured in a 1:1 mixture of Shh-N-conditioned medium and defined medium (Yamada et al., 1993) containing a 2× concentration of the test compound. Cyclopamine and veratramine were added to culture medium dissolved in ethanol; AY-9944 and lovastatin were added to culture medium dissolved in PBS. Free cholesterol (Sigma) was added to culture medium in ethanol. Control explants were cultured in equivalent concentrations of ethanol. The LDL fraction was purified from fetal calf serum by isopycnic ultracentrifugation in NaBr (Havel et al., 1955), dialyzed extensively against DMEM and purity was verified by SDS-PAGE. The LDL preparation was added to explant cultures at 4%. To determine if cyclopamine inactivated Shh-N, 1 ml each of Shh-N conditioned medium was mixed with cyclopamine to 1 µg/ml or ethanol solvent to 1%, incubated for 1 hour at 37°C, then dialyzed against 2 liters of PBS for 24 hours. The dialysates were tested on neural plate explants at a 1:5 dilution with defined medium. All explants were cultured for 24-29 hours at 37°C, fixed in 4% paraformaldehyde and processed for indirect immunofluorescence as described above.

## RESULTS

### Cyclopamine interrupts Shh-dependent patterning in the chick neural tube and somites

We administered cyclopamine to chick embryos at stage 1 and observed a range of facial malformations associated with holoprosencephaly, the most severe of which was cyclopia (Fig. 2 and data not shown). An undivided, monoventricular telencephalon (alobar holoprosencephaly) occurred with more severe facial anomalies, consistent with interruption of inductive events between prechordal mesoderm and the anterior neural plate (Chiang et al., 1996). Examination of 56 embryos viable 48 hours after treatment at stage 1 revealed 24 that were grossly affected: 5 with cyclopia and holoprosencephaly, 6 with synophthalmia and holoprosencephaly, and the remainder with ocular hypotelorism and/or fusion of the olfactory placodes with and without alobar holoprosencephaly. Caudal regions of embryos treated at stage 1 appeared normal. To determine if cyclopamine treatment could affect posterior inductive events, embryos were treated at stage 9-10. This resulted in grossly evident malformation of trunk somites caudal to those already formed prior to treatment: affected somites were translucent and had rounded rather than square lateral margins (data not shown, and Fig. 3). Treatment of stage 1 embryos produced malformations in 40% (24/56) of embryos viable 48 hours after treatment, while greater than 80% (16/19) of embryos treated at stage 9-10 were affected. Importantly, embryos treated at

either stage with veratramine (Fig. 1), which is formed by acid aromatization of cyclopamine and does not cause cyclopia in sheep (Keeler, 1978), did not develop similar malformations.

Malformations caused by cyclopamine were associated with a disturbance of Shh-dependent patterning events in the ventral neural tube and somites. The normal distributions of Shh-induced cell types are best defined for levels of the neural tube that give rise to spinal cord. Focusing on cervical neural tube in embryos with craniofacial malformations due to treatment at stage 1 and thoracic neural tube in embryos treated at stage 9-10 (Fig. 3), we observed a failure of dorsoventral patterning consistent with attenuation of Shh signaling. Shh was always detected in the notochord of treated embryos (Fig. 3G-I and data not shown), where its initial expression is dependent on factors other than Shh itself (Chiang et al., 1996). However, Shh<sup>+</sup>, HNF-3β<sup>+</sup> floor plate cells (Fig. 3A-C,G-I) were always absent in cervical regions of cyclopic embryos produced by stage 1 treatment. In embryos with less severe craniofacial defects, such as ocular hypotelorism, a reduced number of Shh<sup>+</sup> (data not shown) or HNF-3β<sup>+</sup> floor plate cells was observed (Fig. 3B), often interspersed with midline *isl1/2*<sup>+</sup> cells. Floor plate cells were also absent in trunk regions of embryos treated at stage 9-10 (Fig. 3C,H). Similarly, Nkx2.2<sup>+</sup> ventral progenitor cells (Fig. 3D-F) were always found aberrantly at the ventral midline in cervical regions of embryos treated at stage 1 (Fig. 3E), while they were often absent in trunk regions of embryos treated at stage 10 (Fig. 3F). In embryos treated at stage 1, *isl1/2*<sup>+</sup> motor neurons were usually found in the normal dorsolateral location, but also aberrantly at the ventral midline (Fig. 3B,E,K,N). In embryos treated at stage 10, *isl1/2*<sup>+</sup> motor neurons were absent or reduced to a few ventrally placed midline cells (Fig. 3C,F,L,O). These findings are consistent with a reduction of HNF-3β expression previously observed in the ventral neural tube of cyclopamine-treated hamster embryos (Coventry et al., 1998).

Shh suppresses the development of *Lim1/2*<sup>+</sup> cells within the ventral neural tube (Tsuchida et al., 1994), and repression of Pax6 and Pax7 ventrally is an early event in Shh signaling (Chiang et al., 1996; Ericson et al., 1997; Macdonald et al., 1995). In cyclopamine-treated embryos, *Lim1/2*<sup>+</sup> interneurons (Fig. 3G-I) appeared in the normal dorsal location, but they also appeared aberrantly in the ventral neural tube, including the ventral midline (Fig. 3H,I). Embryos treated at stage 1 often showed a ventrally expanded domain of Pax6<sup>+</sup> cells in cervical regions of neural tube (Fig. 3K), while Pax6<sup>+</sup> cells were found throughout the ventral neural tube in trunk regions of embryos treated at stage 10 (Fig. 3L). Finally, although we never observed complete failure of Pax7 repression in the neural tube, the Pax7 domain often extended ventrally (Fig. 3O). These patterns of Pax6 and Pax7 expression in the neural tube of cyclopamine-treated embryos are consistent with *in vitro* experiments showing that Pax7 is repressed by very low Shh concentrations and that Pax6 repression requires a concentration 8-fold higher (Ericson et al., 1997).

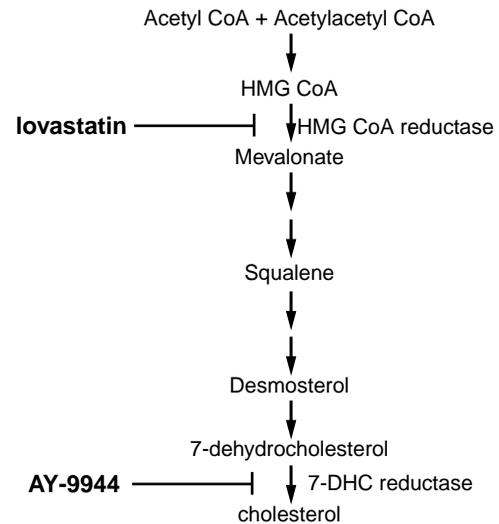
In somites, Shh signaling mediates the repression of dorsal cell types such as dermatome, which express Pax7 (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). In embryos treated at stage 10, Pax7<sup>+</sup> cells were found throughout the somites (Fig. 3O), indicating development of dermatome at the expense of sclerotome and producing the gross hollow appearance of the

somites. The greater severity of patterning defects observed in embryos treated at stage 10 is probably a result of a higher local concentration of cyclopamine due to easier access to the embryo.

Because a normal *Shh*<sup>+</sup> notochord was always found adjacent to abnormal regions of neural tube, cyclopamine causes neural tube patterning defects without disruption of mesodermal sources of *Shh*. However, extirpation of prechordal mesoderm can produce cyclopia in the chick embryo (Pera and Kessel, 1997) and a failure of neural plate tissue to migrate into the ventral midline was associated with cyclopia in a zebrafish mutant (Heisenberg and Nusslein-Volhard, 1997). Although we have not determined if prechordal mesoderm is intact in embryos treated at stage 1, in analogy with the dorsoventral patterning defects caused by cyclopamine at caudal levels of the neural tube, the most likely explanation for holoprosencephaly in cyclopamine-treated embryos is a failure of *Shh* signaling and subsequent ventralization of the rostral neural tube. Together, the results obtained by cyclopamine treatment at either stage are consistent with attenuation of *Shh* signaling, but cannot resolve an effect on sources of *Shh* or an effect on *Shh* signaling in responding cells.

#### Cyclopamine blocks the response to recombinant *Shh*-N in neural plate explants in a dose-dependent manner

To determine if cyclopamine interferes with *Shh* signal transduction in responding tissue, we used intermediate neural plate explants from stage 10 chick embryos (Yamada et al., 1993). Recombinant *Shh*-N induces ventral cell types in explants in a concentration-dependent manner: ventral motor neurons develop in response to 4 nM *Shh*-N, while development of floor plate cells requires 3- to 5-fold higher concentrations (Ericson et al., 1996, 1997; Roelink et al., 1995). When explants were cultured with *Shh*-N in the presence of cyclopamine, induction of markers for ventral cell types and repression of dorsal markers were blocked in a dose-dependent manner (Fig. 5). At 24 nM cyclopamine, Pax7 repression was blocked by 50%, while induction of *is11/2*<sup>+</sup> motor neurons and *HNF-3β*<sup>+</sup> floor plate cells was blocked by 50% and 65%, respectively (Fig. 5C). 48 nM cyclopamine reduced motor neuron induction by approximately 75% and floor plate induction by approximately 95%, while at 120 nM cyclopamine Pax7 repression and induction of both floor plate cells and motor neurons were completely blocked. Induction of ventral neural tube cell types in explants cannot be secondary to explant-derived *Shh* expression, because the earliest expression of *HNF-3β* and *Shh* occurs at 16 hours and 20 hours of culture, respectively (Roelink et al., 1995). Because the explant cultures were discontinued at 24 hours, only effects on the response to applied *Shh*-N are measured. Importantly, veratramine (Fig. 1) at 240 nM had no effect on the response to *Shh*-N (data not shown). Furthermore, the induction of *Msx1/2*<sup>+</sup> cells in explants by BMP4 (Liem et al., 1995) was unaffected by 120 nM cyclopamine (data not shown). Cyclopamine also does not bind to and inactivate *Shh*, because preincubation of *Shh*-N with cyclopamine followed by dialysis had no effect on the ability of *Shh*-N to induce motor neurons or repress Pax7 in explants (data not shown). The effects of different cyclopamine concentrations correlate well with the



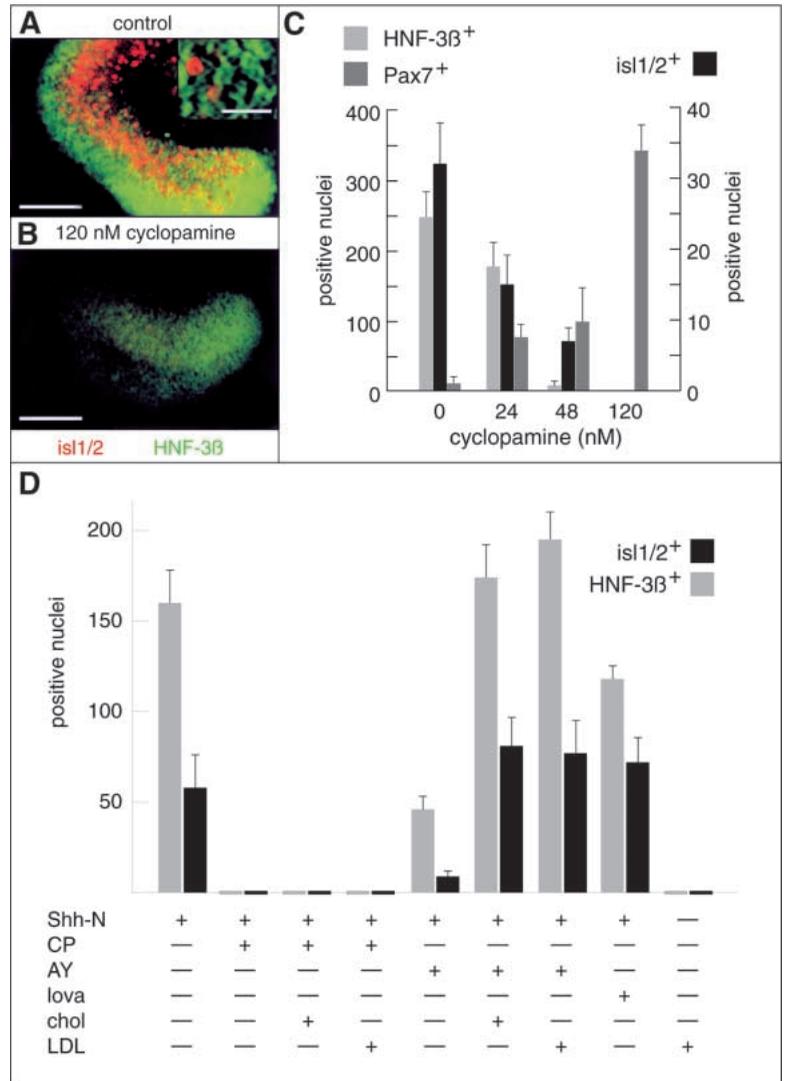
**Fig. 4.** Schematic representation of the cholesterol biosynthetic pathway, with only a portion of the intermediates identified. The synthesis of the six-carbon compound mevalonate catalyzed by HMG-CoA reductase is the rate-limiting step. A series of isoprenoid intermediates leads to squalene, which is cyclized into the first sterol precursor. A series of demethylations and reductions leads to 7-dehydrocholesterol. 7-DHC reductase catalyzes the final rearrangement of the double bond to the 5-6 position of cholesterol (see Fig. 1). This final step is inhibited by AY-9944, and 7-DHC reductase activity is reduced in patients with Smith-Lemli-Opitz syndrome.

range of changes observed in the neural tubes of embryos treated in ovo (Fig. 3), where induction of floor plate is more severely affected than motor neuron induction. However, these experiments do not demonstrate whether cyclopamine inhibits *Shh* signal transduction directly by interacting with an element in the pathway or indirectly by affecting another cellular process required for the response.

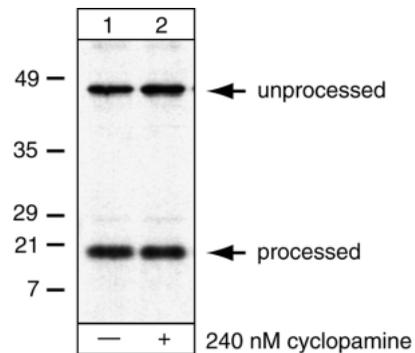
#### The effect of cyclopamine is not due to inhibition of cholesterol metabolism

The association of holoprosencephaly with reduced cholesterol levels in mammalian embryos described above suggests that cyclopamine could produce holoprosencephaly by interfering with cholesterol synthesis or uptake. Cholesterol is synthesized from mevalonate via a series of intermediates (Fig. 4). The enzymes of the biosynthetic pathway are strongly down-regulated by cholesterol both transcriptionally and by a reduction in protein half-life, such that little *de novo* synthesis occurs in the presence of exogenous cholesterol (Brown and Goldstein, 1997; Goldstein and Brown, 1990). Although at later stages the embryonic chick brain synthesizes cholesterol *de novo* to a significant degree, the embryo utilizes yolk-derived cholesterol at early stages (Conner et al., 1969), when *Shh*-dependent patterning occurs. In contrast, explants are routinely cultured in the absence of exogenous cholesterol, so any cholesterol required beyond that present in cells at the time of dissection must be synthesized *de novo*. If cyclopamine were acting by inhibition of cholesterol synthesis, then its effects should be mimicked by known cholesterol synthesis inhibitors.

**Fig. 5.** Effects of teratogens on intermediate neural plate explants. (A) An explant in which *isl1/2*<sup>+</sup> motor neurons (red) and HNF-3 $\beta$ <sup>+</sup> floor plate cells (green) were induced by recombinant Shh-N. Inset shows higher magnification of positive nuclei. Scale bars represent 50  $\mu$ m and 10  $\mu$ m (inset). (B) An explant incubated with Shh-N and 120 nM cyclopamine showing failure of induction of both floor plate cells and motor neurons. Similar explants show Pax7<sup>+</sup> cells throughout. (C) Quantification of the inhibitory effect of cyclopamine on the induction of HNF-3 $\beta$ <sup>+</sup> floor plate cells and *isl1/2*<sup>+</sup> motor neurons, and repression of Pax7. Explants were incubated for 24 hours in equal concentrations of Shh-N and the indicated concentrations of cyclopamine. Values represent the mean number of positive nuclei counted for each antigen. Error bars represent s.e.m. (D) Comparison of the effects of cyclopamine and known cholesterol synthesis inhibitors on induction of HNF-3 $\beta$ <sup>+</sup> floor plate cells and *isl1/2*<sup>+</sup> motor neurons by recombinant Shh-N in explants. The data are derived from one experiment, which is a representative example of at least four independent observations. Values are the mean and s.e.m. of positive nuclei for each antigen counted in at least 8 explants. Explants were cultured for 29 hours in equal concentrations of Shh-N. Cyclopamine was added to 120 nM, free cholesterol to 13  $\mu$ M, and purified LDL-cholesterol to 4%. AY-9944 was added to 440 nM, and lovastatin was added to 300 nM. AY-9944 treatment resulted in a significant reduction in the induction of HNF-3 $\beta$ <sup>+</sup> ( $P < 0.001$ ) and *isl1/2*<sup>+</sup> cells ( $P < 0.02$ ), and also a failure of Pax7 repression (data not shown). Addition of 13  $\mu$ M cholesterol to AY-9944-treated explants resulted in a significant increase in the number of HNF-3 $\beta$ <sup>+</sup> cells ( $P < 0.001$ ) and a significant increase in *isl1/2*<sup>+</sup> cells ( $P < 0.002$ ) to levels slightly beyond those obtained with Shh-N alone. Addition of LDL-cholesterol to 4% also resulted in both a significant rescue of HNF-3 $\beta$  induction ( $P < 0.001$ ) and *isl1/2* induction ( $P < 0.001$ ). Lovastatin had no effect on the induction of *isl1/2*<sup>+</sup> cells compared to control ( $P = 0.3$ ); however, there was a significant reduction in HNF-3 $\beta$ <sup>+</sup> cells ( $P < 0.01$ ). However, with lovastatin treatment considerable cell death occurred at the periphery of explants, where most HNF-3 $\beta$ <sup>+</sup> cells are induced (see A above). In similar experiments, there was no effect of lovastatin on Pax7 repression (data not shown). The addition of LDL-cholesterol alone resulted in no HNF-3 $\beta$  or *isl1/2* induction. Abbreviations: CP, cyclopamine; AY, AY-9944; lova, lovastatin; chol, free cholesterol; LDL, LDL-cholesterol.



We tested two cholesterol synthesis inhibitors in the chick embryo system: the teratogenic 7-DHC reductase inhibitor AY-9944 and lovastatin. Lovastatin is a potent inhibitor of the rate-limiting step of cholesterol synthesis catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Alberts et al., 1980), blocking de novo synthesis without accumulation of sterol precursors. Lovastatin and related HMG-CoA reductase inhibitors are not associated with holoprosencephaly and are largely non-teratogenic. When stage 9-10 embryos were treated in ovo with a range of AY-9944 concentrations up to 220  $\mu$ M, we observed no effects on Shh-dependent patterning in the neural tube and somites of 30 viable embryos. We did observe non-specific toxic effects resulting in growth-retarded embryos with disorganized somites, suggesting that the drug was able to penetrate embryonic tissue. However, immunostaining of cryosections with the antibodies indicated in Fig. 3 revealed normal patterning of the neural tube and somites (data not shown). On the contrary, when explants were



**Fig. 6.** Analysis of Shh processing in the presence of cyclopamine. Lane 1 shows protein from cells treated with ethanol alone; lane 2 shows protein from cells treated with 240 nM cyclopamine. Unprocessed Shh migrates at 45 kDa, while the cholesterol-linked cleavage product migrates at 19 kDa. Migration of molecular weight standards is indicated at the left.

treated with 440 nM AY-9944, a concentration two-fold above that necessary for maximal inhibition of cholesterol synthesis in cultured rat embryos (Llirbat et al., 1997), we observed a marked reduction in the response to Shh-N (Fig. 5D). Moreover, the response in AY-9944-treated explants was restored to normal levels by the addition of either free cholesterol or LDL-cholesterol to the culture medium (Fig. 5D), supplements that should suppress cholesterol biosynthesis and prevent the accumulation of AY-9944-induced sterol intermediates. Although there is circumstantial evidence that LDL may bind and neutralize AY-9944 (Rampini et al., 1989), the restoration of Shh responsiveness with free cholesterol was equally robust. It is also notable that the response to Shh-N was never completely blocked by AY-9944, as it was by cyclopamine, even at 2.2  $\mu$ M (data not shown). This effect of AY-9944 on explants is probably not due to cholesterol deficiency alone, since a normal response to Shh-N was observed in explants treated with lovastatin at either 300 nM (Fig. 5D) or 1.2  $\mu$ M (data not shown), concentrations well above the IC<sub>50</sub> for cholesterol synthesis in a wide range of cultured cells (Corsini et al., 1995).

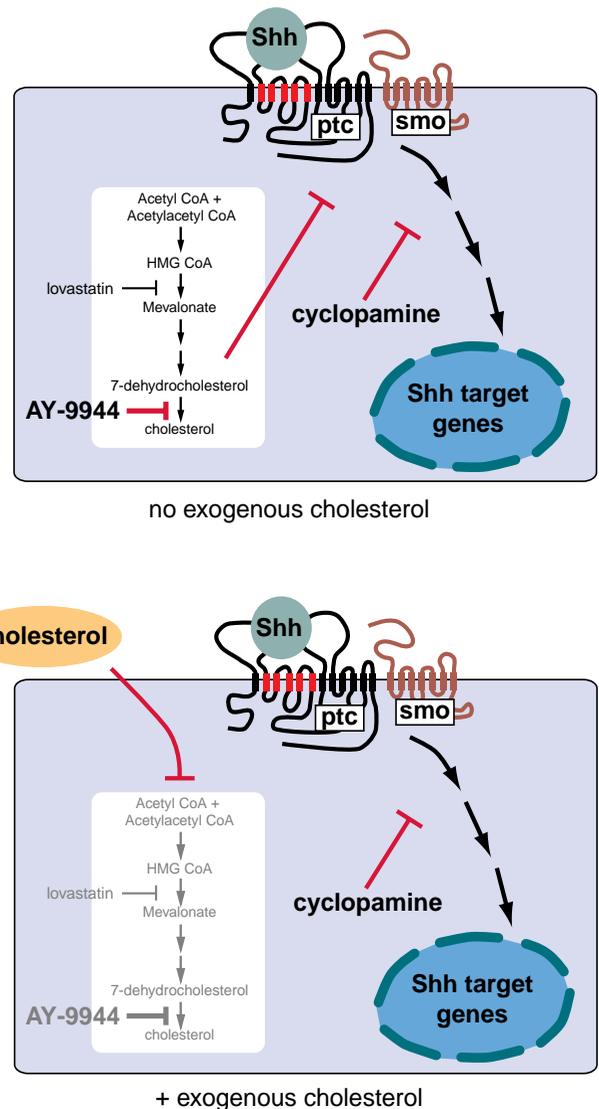
In contrast to AY-9944, the inhibition of Shh signaling by cyclopamine was unaffected by the presence of either free cholesterol or LDL (Fig. 5D). This indicates that the failure to respond to Shh-N in the presence of cyclopamine is not secondary to inhibition of cholesterol synthesis. Furthermore, cyclopamine is unlikely to block uptake or trafficking of lipoprotein-derived cholesterol; genetic or pharmacologic blockade of cholesterol trafficking can be bypassed by free sterols in organic solvent (Goldstein and Brown, 1990; Metherall et al., 1996).

### Shh biogenesis in COS-1 cells is unaffected by concentrations of cyclopamine that block the Shh response in explants

The association of holoprosencephaly with perturbations in cholesterol metabolism has been hypothesized to be due to interruption of the post-translational modification of Shh by cholesterol (Kelley et al., 1996). To determine if Shh biogenesis is affected by cyclopamine, we exposed transfected COS-1 cells expressing a full-length *Shh* cDNA to increasing concentrations of cyclopamine. COS-1 cells can process transfected *Shh*, acquiring the same inductive properties as the notochord on neural plate explants (Roelink et al., 1994; Tanabe et al., 1995). We observed no effect on the levels of cholesterol-linked Shh produced by cells treated with cyclopamine at 240 nM (Fig. 6). However, we did observe a reduction in cholesterol-linked Shh beginning at 1.2  $\mu$ M cyclopamine, with a maximal effect at 24  $\mu$ M (data not shown). Although these data suggest that high concentrations of cyclopamine can inhibit the post-translational modification of Shh by cholesterol, it is unclear if these levels are achieved in vivo. Maximal inhibition of Shh signaling was obtained with cyclopamine at a concentration 200-fold lower than that significantly affecting Shh processing in COS-1 cells.

## DISCUSSION

Examination of a suite of Shh-dependent cell types in the neural tube and somites in embryos with cyclopamine-induced



**Fig. 7.** Model describing the differences between the teratogenic mechanisms of cyclopamine and AY-9944. Shh binds to the Patched/Smoothed receptor complex (*ptc* and *smo*) and ultimately affects the transcription of Shh-responsive genes through an incompletely understood signal transduction cascade. The relative position of the putative sterol-sensing transmembrane domains of Patched are indicated in red. Shh-responsive cells that do not have an exogenous supply of cholesterol are represented by the top panel. The cholesterol biosynthetic pathway is active, the target for AY-9944 (7-DHC reductase) is present, and AY-9944 treatment interrupts the response to Shh, probably through the action of a sterol precursor or metabolite thereof. Cyclopamine blocks the response to Shh under these conditions as well. The bottom panel represents Shh-responsive cells that have an exogenous supply of cholesterol, such as yolk, LDL or free cholesterol dissolved in ethanol. Under these conditions, cholesterol biosynthesis is downregulated, the target of AY-9944 is at low levels or absent, and AY-9944 treatment no longer interrupts Shh signaling. However, the potential target(s) of cyclopamine must be present when there is adequate exogenous cholesterol, because cyclopamine still completely blocks Shh signaling. This indicates the effects of cyclopamine are not secondary to alteration of cholesterol metabolism. Lovastatin does not affect Shh signaling in either situation.

malformations shows that essentially all aspects of Shh signaling in these tissues are interrupted by cyclopamine treatment. Cyclopamine treatment thus produces a phenocopy of a *Shh* loss-of-function mutation, with features similar to the mouse null mutant. The correlation of the severity of malformations with severity of defects in neural tube patterning is consistent with both a dose-dependent effect of cyclopamine and the action of Shh as a morphogen. For example, the successful repression of Pax7 observed in the ventral neural tube of embryos most severely affected by cyclopamine is consistent with *in vitro* studies indicating that Pax7 repression in neural plate cells is the most sensitive measure of Shh activity (Ericson et al., 1997). This also suggests that tissue levels of cyclopamine producing malformations *in ovo* are less than 120 nM. The ability of cyclopamine to block the response to Shh-N in neural plate explants shows that its teratogenic effects are mediated through inhibition of Shh signaling, rather than by affecting Shh biogenesis. This is confirmed by the absence of an effect on Shh biogenesis in COS-1 cells transfected with a full-length *Shh* cDNA and treated with a concentration of cyclopamine sufficient to completely block Shh signaling in explants.

In our studies of neural plate explants, we examined the effects of three agents: cyclopamine and AY-9944, which cause holoprosencephaly, and lovastatin, which is not associated with holoprosencephaly. Cyclopamine, which interrupts Shh signaling *in ovo*, also blocks the response to Shh-N in explants, independent of exogenous cholesterol supplementation. AY-9944, which inhibits the terminal step of cholesterol biosynthesis leading to an accumulation of various sterol intermediates (Wolf et al., 1996), also inhibits the response to Shh-N in explants, but not in the presence of exogenous cholesterol. Furthermore, AY-9944 does not disrupt Shh signaling *in ovo*. Lovastatin, which inhibits cholesterol synthesis without the accumulation of precursor sterols (Alberts et al., 1980), does not interfere with the response to Shh-N in explants.

These findings suggest a model for the teratogenicity of cyclopamine and AY-9944 that is summarized in Fig. 7. AY-9944 induces holoprosencephalic malformations in rat embryos when administered to pregnant dams (Roux and Aubry, 1966), but Shh signaling is intact in chick embryos treated *in ovo* with widely different concentrations of AY-9944. This is consistent with differences in cholesterol metabolism between avian and mammalian embryos, the latter depending predominantly on *de novo* synthesis (Belknap and Dietschy, 1988). However, routine chick neural plate explant cultures are performed in medium without cholesterol, so cells within the explants must satisfy the requirement for cholesterol by induction of cholesterol biosynthetic enzymes and *de novo* synthesis. Because membrane cholesterol is the main regulator of the synthesis and half-lives of cholesterol biosynthetic enzymes (Brown and Goldstein, 1997; Goldstein and Brown, 1990), under these culture conditions the target for AY-9944, 7-DHC reductase, would be present. In contrast, the early chick embryo *in ovo* has a large pool of exogenous cholesterol supplied by endocytosis of yolk (Bellairs, 1958) and, when AY-9944-treated explants are provided with exogenous cholesterol, Shh signaling is restored.

Cyclopamine interferes with Shh signaling by a mechanism

in which inhibition of cholesterol synthesis does not play a major role. This is suggested by (1) the high frequency of malformations associated with disruption of Shh signaling reproducibly found in chick embryos treated with cyclopamine *in ovo* and (2) the failure of either free cholesterol or LDL to rescue Shh signaling in explants treated with cyclopamine. If the target of cyclopamine were within the cholesterol biosynthetic pathway, such a target would be absent or at low levels under conditions of adequate exogenous cholesterol. A blockade of uptake and delivery of LDL-derived cholesterol by cyclopamine would be bypassed by providing free cholesterol (Goldstein and Brown, 1990). These predictions are met by the ability of both free cholesterol and LDL to rescue the response in AY-9944-treated explants. Furthermore, the teratogenicity of AY-9944 and the non-teratogenicity of lovastatin are mirrored by their respective effects on neural plate explants, suggesting that membrane sterol composition is more important for Shh signal transduction than overall cellular cholesterol levels. A sterol precursor or metabolite that accumulates in AY-9944-treated tissue may have effects similar to those of cyclopamine. The failure to rescue Shh signaling with exogenous cholesterol in cyclopamine-treated explants suggests that cyclopamine teratogenesis results from a more direct interaction with some element in the Shh signal transduction cascade.

The putative sterol-sensing domain of Patched is a possible target for cyclopamine or abnormal membrane sterols. The sterol-sensing domains of HMG-CoA reductase and the sterol response element binding protein (SREBP) cleavage activating protein (SCAP) have been shown to be involved in the sterol-regulated reduction of protein half-life (Gil et al., 1985; Kumagai et al., 1995) and assembly of a complex of regulatory proteins (Hua et al., 1996), respectively. Although a role in Shh signal transduction for the sterol-sensing domain of Patched is suggested by our results, the nature of such a role is unclear. Processes possibly affected by steroidal alkaloids or abnormal membrane sterols include presentation of the Patched/Smoothed complex on the cell surface, internalization of receptor/ligand complexes, or conformational changes upon ligand binding that are necessary for Smoothed activity. *Drosophila* hedgehog protein has been observed to be internalized by target cells in the embryo (Tabata and Kornberg, 1994). Caveolae and other cholesterol-rich membrane microdomains that are enriched with proteins involved in signal transduction have been isolated from many terminally-differentiated cell types (Lisanti et al., 1994; Stefanova et al., 1991), but a potential role for such structures in Shh signal transduction is unknown. Cyclopamine will be a useful tool for the pharmacologic manipulation of Shh-mediated processes and the further dissection of mechanisms involved in Shh signal transduction.

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