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Anti-apoptotic role of Sonic hedgehog protein at the early stages of nervous system organogenesis

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SUMMARY

In vertebrates the neural tube, like most of the embryonic organs, shows discreet areas of programmed cell death at several stages during development. In the chick embryo, cell death is dramatically increased in the developing nervous system and other tissues when the midline cells, notochord and floor plate, are prevented from forming by excision of the axial-paraxial hinge (APH), i.e. caudal Hensen's node and rostral primitive streak, at the 6-somite stage (Charrier, J. B., Teillet, M.-A., Lapointe, F. and Le Douarin, N. M. (1999). *Development* 126, 4771-4783). In this paper we demonstrate that one day after APH excision, when dramatic apoptosis is already present in the neural tube, the latter can be rescued from death by grafting a notochord or a floor plate fragment in its vicinity. The neural tube can also be recovered by transplanting it into a stage-matched chick embryo having one of these structures. In addition, cells engineered to produce Sonic hedgehog protein (SHH) can mimic the effect of the notochord and floor plate cells in in situ grafts and transplantation experiments. SHH can thus counteract a built-in cell death program and thereby contribute to organ morphogenesis, in particular in the central nervous system.

Key words: Apoptosis, Avian embryo, Cell death, Cell survival, Floor plate, Notochord, Quail/chick, *Shh*, Somite, Neural tube, Spinal cord

INTRODUCTION

Tissue interactions play a crucial role in the development of multicellular organisms. They can be mediated either by cell contacts or through secreted factors acting according to distance or concentration. The best documented of the effects of cell to cell signaling are those related to induction of cell proliferation and differentiation. The regulation of cell death has also been recognized to occur in many developing tissues in both vertebrates and invertebrates (reviewed by Glücksmann, 1951; Clarke and Clarke, 1996). Cell death has long been ascribed a role in sculpting and defining the shape and size of organs and organisms. This is seen, for example, in the elimination of the interdigital cells during digit formation and for adjusting the number of motoneurons and sensory neurons along the neural axis in vertebrates.

Although the term programmed cell death (PCD) was initially used to describe cell elimination from certain developing tissues, as part of their developmental program, this term is now used to designate a particular type of cell death (reviewed by Jacobson et al., 1997). Cell death occurs according to two different paradigms in animal cells (Kerr et al., 1972). These authors made a distinction between the type of cell death occurring during tissue homeostasis and development and pathological cell death observed at the center of acute lesions. In the latter case, the cells and their organelles swell and rupture in a process called necrosis that generally induces an inflammatory response. In the former, the cells shrink and condense, but their organelles and plasma membrane retain their integrity. The dead cells are rapidly phagocytosed by neighboring cells without inducing inflammatory responses. Kerr and his colleagues named this process apoptosis and clearly distinguished it from necrosis.

The fact that animal cells possess a built-in suicide program was first demonstrated by genetic studies carried out in *Caenorhabditis elegans* (Ellis and Horvitz, 1986). Execution of this program involves a proteolytic cascade controlled intracellularly by a family of specialized regulatory proteins (for reviews see Martin and Green, 1995; Chinnaiyan and Dixit, 1996; White, 1996; Jacobson et al., 1997). The first known of the proteases involved in this cascade is encoded by a gene called *ced-3* that is required for the 131 PCDs that take place regularly during the development of *C. elegans* (Ellis and Horwitz, 1986; reviewed by Ellis et al., 1991). This gene encodes a cysteine protease that is homologous to the mammalian interleukin-1β-converting enzyme (ICE; Yuan et al., 1993). Mammals possess many members of the *ced*/ICE family involved in PCD (see Chinnaiyan and Dixit, 1996; Kuida et al., 1995; Kuida et al., 1996). They are referred to as caspases (Alnemri et al., 1996). Moreover, there is now evidence that the intracellular death program is constitutively expressed in all nucleated cells (Ishizaki et al., 1995; Weil et al., 1996).

The role of apoptosis in development remained unidentified. *Drosophila* embryos in which the apoptotic program has been genetically altered die early in development (White et al., 1994). Such is also the case for mice in which CPP32 (caspase 3) is deleted by targeted gene disruption. Interestingly, their central nervous system (CNS) shows a large excess of neuroepithelial cells (Kuida et al., 1996).

The cell death program of animal cells is, however, restrained by inhibitors that block PCD. The *C. elegans* gene *ced-9*, or *bcl2* in mammals, is one such example (Hengartner and Horwitz, 1994; review by Korsmeyer, 1995). Evidence from in vitro experiments indicates that, at least in higher vertebrates, developing cells require signals to counteract their intrinsic PCD program (Raff, 1992). These signals come either from themselves, e.g. in lens (Ishizaki et al., 1993) or in cartilage (Ishizaki et al., 1994), or from other cells as an autocrine versus paracrine process. However, the role of the paracrine control of apoptosis in development is not yet fully understood.

We have designed experimental conditions which reveal an unsuspected effect of the floor plate and notochord (here designated as midline structures) on the neural epithelium destined to yield the neurons and glia of the spinal cord. These midline cells are indispensable for the survival of the early neural tube itself, as has been shown previously for the myotomal and sclerotomal lineages of the paraxial mesoderm (Teillet et al., 1998b).

In earlier cell lineage studies, carried out in the avian embryo (Catala et al., 1995; Catala et al., 1996), we demonstrated that the formation of the spinal cord primordium involves the juxtaposition of two populations of cells of distinct embryonic origins. One of them constitutes the neural ectoderm destined to form the lateral walls of the spinal cord (basal and alar plates) and the neural folds (roof plate and neural crest). The other is the presumptive territory of the floor plate, which arises from precursors contained in Hensen's node, the avian organizer. During its caudalward movement involved in the posterior elongation of the embryo, the node leaves in its wake a cord of cells that later splits into dorsal and ventral components that become the floor plate and the notochord respectively. The floor plate, as it becomes isolated from the notochord, is inserted into the neural ectoderm. The precursors contained in the node yield these two midline structures along the entire neural axis without additional cells joining them by convergence from lateral regions, thus functioning as stem cells (Catala et al., 1995; Catala et al., 1996; Teillet et al., 1998a; Charrier et al., 1999).

Inhibition of Hensen's node regression can be obtained by removal of a region that includes the posterior end of the node and the anterior tip of the primitive streak, known as the axial-paraxial hinge (APH) (Charrier et al., 1999). The operation does not prevent the formation of a neural tube posterior to the excised zone. However, the portion of the embryo that develops caudal to the level of the operation (which corresponds to somites 20-21 when the operation is performed at the 5- to 6-somite stage), is devoid of midline cells (i.e. notochord and floor plate). In this region, the neural tube and the somites only possess dorsal molecular markers (Charrier et al., 1999). Subsequently, all the tissues forming the embryo in the midline cell-deprived area undergo apoptosis. After 3 days, embryos are truncated posterior to the forelimbs (see Fig. 7 of Charrier et al., 1999).

In the present work, we demonstrate that midline cells exert a positive effect on the survival and growth of the neural tube. This effect is mimicked by Sonic hedgehog (SHH) a secreted protein produced by the notochord and floor plate among other locations in vertebrates (Echelard et al., 1993; Marti et al., 1995; and see Teillet et al., 1998a).

MATERIALS AND METHODS

Quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus domesticus*) embryos were used throughout this study. The eggs from commercial sources were incubated at 38±1°C in a humidified atmosphere. Embryos were staged according to the number of somites or the number of embryonic days (E). We used the somitic nomenclature of Christ and Ordahl (Christ and Ordahl, 1995) in order to indicate the rostral and caudal limits of the operations. According to this nomenclature, SI indicates the last formed pair of somites, SII the one that is immediately rostral to it. S+n points to the number of presumptive pairs of somites in the non-segmented region behind the last formed somite. All the experiments were performed in ovo and repeated to obtain at least five living embryos.

Microsurgery experiments (Fig. 1)

Two types of experiments (A and B) were performed at E2.5 in order to rescue the caudal neural tube deprived of midline cells. In both experiments, the APH (the caudal end of Hensen's node together with the rostral tip of the primitive streak) was previously excised at the 5- to 6-somite stage (ss) in chick or quail embryos at E1.5, as described previously (Charrier et al., 1999).

Experiments A (Fig. 1C)

Excised embryos were grown further in ovo (1) without graft, as controls; (2) after the graft of a notochord or a floor plate fragment; (3) after the graft of SHH-producing cells. The grafts were inserted between the neural tube and the paraxial mesoderm in the region deprived of midline cells at E2.5. The grafts of notochord or floor plate, about 300 μm-long, were obtained by enzymatic dissociation (GIBCO pancreatine, diluted 1/3 in DMEM) from the non-segmented region of E2 quail embryos (20-25ss). SHH-producing fibroblasts were kindly provided by D. Duprez (Duprez et al., 1998).

Experiments B (Fig. 1D)

Fragments of neural tube deprived of midline cells were dissected out after enzymatic dissociation, as above. These tissues prepared from quail or chick donors were transplanted into chick hosts (20-25ss) that had previously had their own neural tube-notochord complex surgically removed as described previously (Teillet et al. 1998b). Excisions and grafts were performed at the level of the last formed somites and in the non-segmented region over the total length of about 10 somites. The graft was generally separated from the host axial tissues by a gap at least one somite long in order to discriminate host and grafted tissues. Three types of transplantations were performed: (1) floor plate-deprived neural tube alone; (2) floor plate-deprived neural tube above a notochord or a floor plate fragment; (3) floor plate-deprived neural tube above a layer of SHH-producing fibroblast clusters.

Experiment C (Fig. 1E)

A longitudinal slit was made in the neural plate immediately lateral to the notochord and floor plate, through the three germ layers. This operation was performed in 10-12ss chick embryos, at the level of the sinus rhomboidalis, i.e. from SI to rostral Hensen's node level. The

other side of the neural plate, remaining in contact with midline cells, was used as a control.

Experiment D (Fig. 1F)

In order to remove the neural tube from the influence of the paraxial mesoderm, an incision was made lateral to the neural tube, immediately after its closure. This longitudinal incision was at the level of the last formed somites and in the non-segmented region, over a total length of about 10 somites, in 12-15ss chick embryos. The contralateral side served as a control.

Operated embryos were reincubated for periods of time ranging from 1 to 3 days according to the experimental series. Embryos were treated for in situ hybridization either in whole mount (see the following paragraph) or in serial sections. In the latter case, the embryos were immersed in modified Carnoy fluid (absolute ethanol : 37% formaldehyde commercial solution : 100% glacial acetic acid, 6:3:1; v/v/v), dehydrated and embedded in paraplast. Consecutive serial 7 μm sections were mounted on separate slides and treated for in situ hybridization with various RNA probes or for immunohistochemistry.

In situ hybridization

In situ hybridization on whole embryos or on tissue sections were performed as described previously (Charrier et al., 1999). Seven chick riboprobes were used as markers of the different tissues and structures: *HNF3β* (Ruiz i Altaba et al., 1995) and *Shh* (Riddle et al., 1993) for Hensen's node, floor plate and notochord; *Pax6* and *Pax3* (Goulding et al., 1993) and *BMP4* (Francis et al., 1994) as dorsoventral markers of the neural tube; *Pax3* for the paraxial mesoderm and dermomyotome (Williams and Ordahl, 1994); *Pax1* for the sclerotomal part of the somites (Balling et al., 1988); *MyoD* for the myotome (Charles de la Brousse and Emerson, 1990). *Patched* (*Ptc*) was used as an element of SHH signaling pathway (Goodrich et al., 1996).

Immunohistochemistry

Cell death was detected using the TUNEL assay (Gavrieli et al., 1992; Wijsman et al., 1993), using the Roche Kit as previously described (Teillet et al., 1998b).

The QCPN monoclonal antibody (Developmental Studies Hybridoma Bank) was used as a marker of quail cells as previously described (Teillet et al., 1998a).

RESULTS

Notochord or floor plate grafts rescue the neural tube from death after APH excision

Excision of a group of cells from the posterior part of Hensen's node and anterior region of the primitive streak (the APH), stopped the rostrocaudal progression of Hensen's node (Charrier et al., 1999). This did not impair the development of

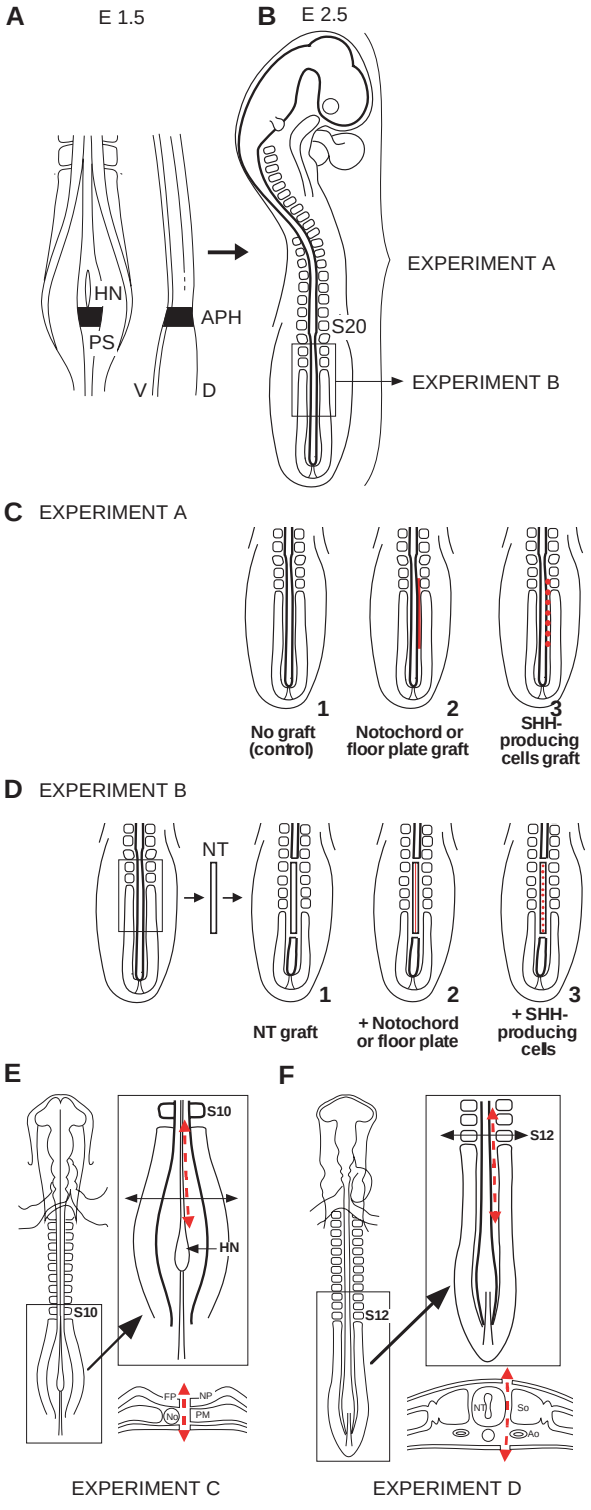

Fig. 1. (A) Dorsal and lateral views of the excision of the axial-paraxial hinge (APH) in chick or quail embryos, at E1.5 (V, ventral; D, dorsal). The APH is the region encompassing the caudal Hensen's node (HN) and the rostral primitive streak (PS). (B) One day after the operation (i.e. E2.5) the neural tube caudal to somite 20 (S20) is deprived of midline structures (floor plate and notochord) and is smaller in diameter than normal. (C) In experiment A, operated embryos are kept in ovo, (1) without a graft, as control; (2) grafted with a fragment of notochord or floor plate; (3) grafted with SHH-producing cells. (D) In experiment B, the neural tube (NT) deprived of midline cells is enzymatically isolated and grafted into a stage-matched chick embryo in place of a segment of its own neural tube-notochord complex, (1) alone; (2) above a notochord or a floor plate fragment; (3) above a layer of SHH-producing cells. (E) In experiment C, the neural plate (NP) and paraxial mesoderm (PM) are separated from the notochord (No) and floor plate (FP) by a slit penetrating the 3 germ layers, from the level of the last formed somites (S) to Hensen's node (HN) level, in chick embryos at the 10- to 12-somite stage. (F) In experiment D, the neural tube (NT) is separated from the paraxial mesoderm or somites (So) by a slit extending from the level of the 2 or 3 last formed somites (S) to the non-segmented region in chick embryos at the 12- to 15-somite stage. Ao, aorta.

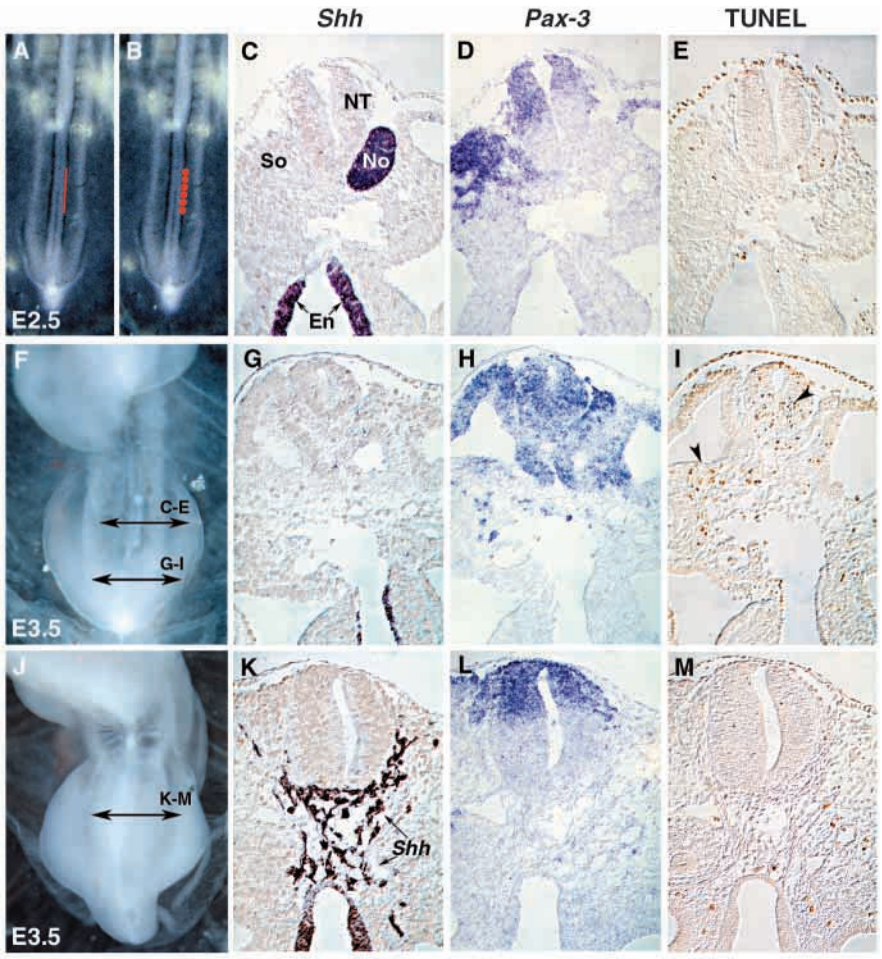

Fig. 2. Experiment A (see Fig. 1C). One day after APH excision (E2.5), (A) a fragment of notochord or (B) SHH-producing cells were grafted between the neural tube deprived of midline cells and the paraxial mesoderm over a length of several somites. (F,J) Dorsal views, at E3.5, of embryos grafted respectively with a notochord or SHH-producing cells. Consecutive sections, at the levels indicated, either in the notochord-grafted region (C-E), or in the non-grafted region of the same embryo (G-I), or in the region grafted with SHH-producing cells (K-M), were hybridized with *Shh* (C,G,K) or *Pax3* (D,H,L) probes or examined for apoptosis using TUNEL (arrowheads) (E,I,M). En, endoderm; No, notochord; NT, neural tube; *Shh*, SHH-producing cells; So, somite.

the truncal and lumbo-sacro-caudal domain of the operated embryos. However, the neural tube formed posteriorly to the excision was deprived of a floor plate and the notochord was absent in this area. Moreover, although it was vascularized, survival of the whole posterior region devoid of midline cells was transient. Massive cell death occurred in the neural tube and other tissues of this region, except the endoderm. Tissues were affected to such an extent that, 48 hours after the operation, the embryos were truncated posteriorly to the forelimbs (see Fig. 7J in Charrier et al., 1999).

With the aim of preventing cell death in a region deprived of midline cells, we inserted a fragment of notochord or floor plate between the neural tube and the paraxial mesoderm, one day after APH excision (Fig. 1C2). At that time, apoptosis had already affected most of the caudal tissues. The posterior neural tube was elongated normally but smaller in diameter than normal (Fig. 2A). One day after the graft of a notochord (i.e. at E3.5; Fig. 2F), the TUNEL technique and *Shh* in situ hybridization applied on consecutive serial sections revealed that cell death in the neural tube and paraxial mesoderm was reduced at the level of the graft (Fig. 2C,E) compared to a control region of the same embryo where no notochord was implanted (Fig. 2G,I). Moreover, dorsoventral polarity of the neural tube was restored in the grafted region as revealed by the expression pattern of the *Pax3* gene. *Pax3* transcripts were restricted to the dorsal half of the neural tube (Fig. 2D) as in normal embryos, while it was distributed over the entire neural tissue in the region deprived of midline cells (Fig. 2H). Lateral grafts of a fragment of floor plate had the same result and restored the development of the corresponding caudal portion of the embryos (not shown).

The notochord and floor plate act on neural tube survival through the SHH pathway

SHH protein has previously been shown to rescue the somites from death after excision of the neural tube-notochord complex (Teillet et al., 1998b). In order to test its capacity to also rescue the neuroepithelium, we grafted SHH-producing cells (Duprez et al., 1998) under a neural tube deprived of midline cells, one day after APH excision (i.e. at E2.5) as represented on Fig. 1C3. Strikingly, at E3.5 (Fig. 2J), not only was cell death significantly reduced in the neural tube in the region of the graft, but, as with notochord grafts, dorsoventral polarity of this structure was restored as confirmed by *Pax3* expression: *Pax3* was no longer expressed in the entire neural tube but was limited to its dorsal region as in normal embryos (Fig. 2K-M).

Another experiment represented in Fig. 1D confirmed the essential role of SHH-producing midline cells on neural tube survival. Fragments of neural tubes devoid of a floor plate, removed from E2.5 embryos that had been subjected to APH excision at E1.5, were grafted into stage-matched chick hosts from which the neural tube and notochord had been excised over a length of about 10 somites. One day later (Fig. 3A-D), the grafted neural tube was still the site of cell death and *Pax3* was expressed uniformly in the neural tube. At E4, the continuity of the embryo was maintained. However, the region containing the grafted neural tube deprived of midline cells appeared indented and foreshortened (Fig. 4A). The host neural tube was normal rostral and caudal to the excision. In contrast, the grafted neural tube, although present, was reduced in diameter and only expressed dorsal markers (Fig. 4B-E).

When a midline cell-deprived neural tube was grafted together with a notochord (Fig. 3E-H), a floor plate fragment (not shown), or SHH-producing cells (Fig. 3I-L), cell death appeared reduced in the grafted neural tube and *Pax3*

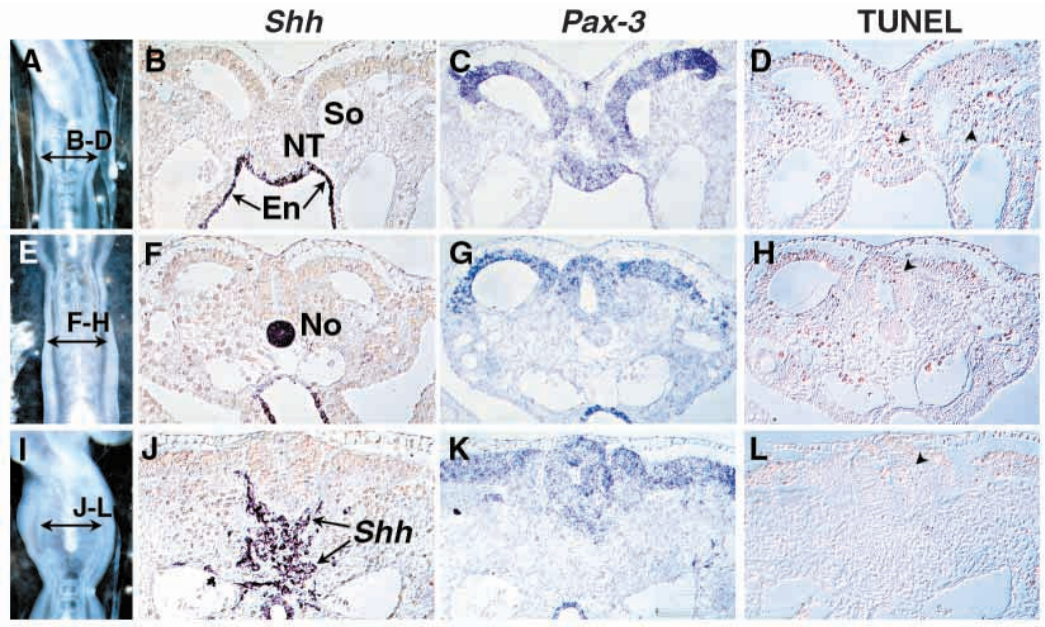

Fig. 3. Experiment B (see Fig. 1D). (A,E,I) Dorsal views of E3 chick embryos grafted at E2 with a fragment of neural tube deprived of midline cells (A) alone (experiment B1); (E) with a notochord (experiment B2); (I) on a layer of SHH-producing cells (experiment B3). (B-D, F-H and J-L) Consecutive sections of each embryo at the level of the graft, hybridized with *Shh* (B,F,J) and *Pax3* probes (C,G,K) or examined for cell death using the TUNEL assay (arrowheads) (D,H,L). En: endoderm; No, notochord; NT, neural tube; *Shh*, SHH-producing cells; So, somite.

transcripts were distributed normally. This result agrees with that obtained when a notochord, a floor plate or SHH-producing cells were grafted in APH-excised embryos, as described above. This suggests that SHH secreted by the notochord and floor plate counteracts PCD in neuroepithelium in avian embryo. Thus, a prominent and precocious role of midline cells (notochord and floor plate) is to ensure survival of the neural ectoderm.

Various studies in flies and vertebrates show that Hedgehog and Patched are linked during development (Marigo and Tabin, 1996; Goodrich and Scott, 1998 for a review). We examined concomitantly the expression patterns of *Ptc* and *Shh* in several of our experiments as compared to their normal expression in the neural tube at E3. As already described (Goodrich et al., 1996; Marigo and Tabin, 1996) *Ptc* was expressed in a dorsoventral gradient, except in the floor plate (Fig. 5E). We observed a low level *Ptc* expression over the entire surface of midline cell-deprived neural tube in APH-excised embryos (Fig. 5F). This expression was considerably upregulated after the graft of SHH-producing cells, on one side of the neural tube (experiment A3; Fig. 5C,G), or after the transplantation of the tube onto a layer of SHH-producing cells, into a stage-matched host embryo (experiment B3; Fig. 5G,H).

Neural tube and somites undergo cell death when separated from the midline cells after their formation

In the above experiments, the neural tube was deprived of midline cells since its origin, and a source of SHH was added the day after the formation of the deprived neural tube. One could not then distinguish whether the survival effect of SHH on the neural tube and somites is indispensable only over a short period of time during midline cell formation, or over a long period of organogenesis. In order to answer this question, we withdrew some neural tube and somites from midline cell influence, by performing a surgical slit through the three germ layers, laterally to the floor plate territory after its formation (see Fig. 1E).

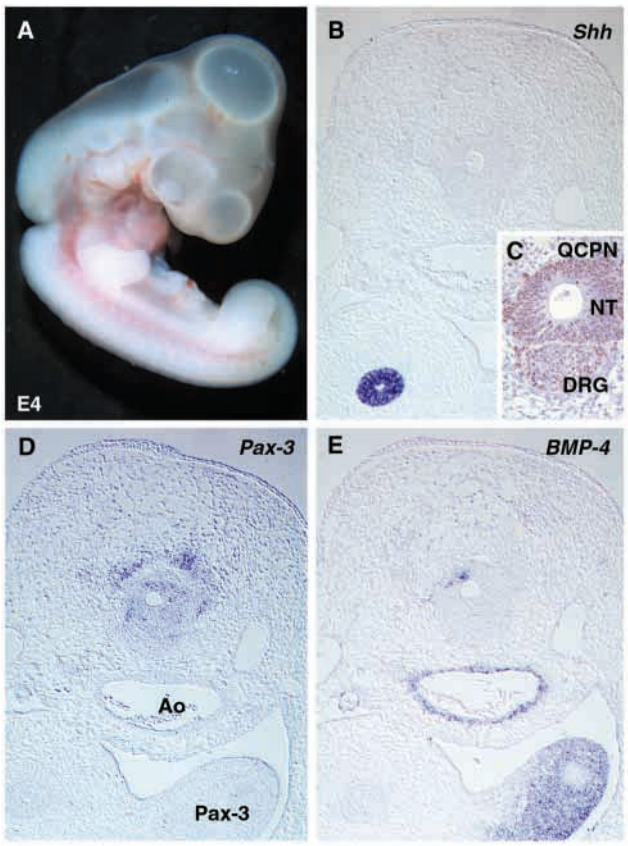

Fig. 4. Results at E4 of experiment B1 (see Fig. 1D1 and Fig. 3A-D). (A) Lateral view of a host chick embryo 2 days after the operation. The position of the graft is apparent from a notch at the cervical level. (B-E) Consecutive cross sections at the level of the graft, hybridized with *Shh* (B), *Pax3* (D) or *BMP4* (E) probes or treated with the QCPN antibody for detection of quail cells (C). In C, the neural tube (NT) and dorsal root ganglia (DRG) are indicated. Ao, aorta.

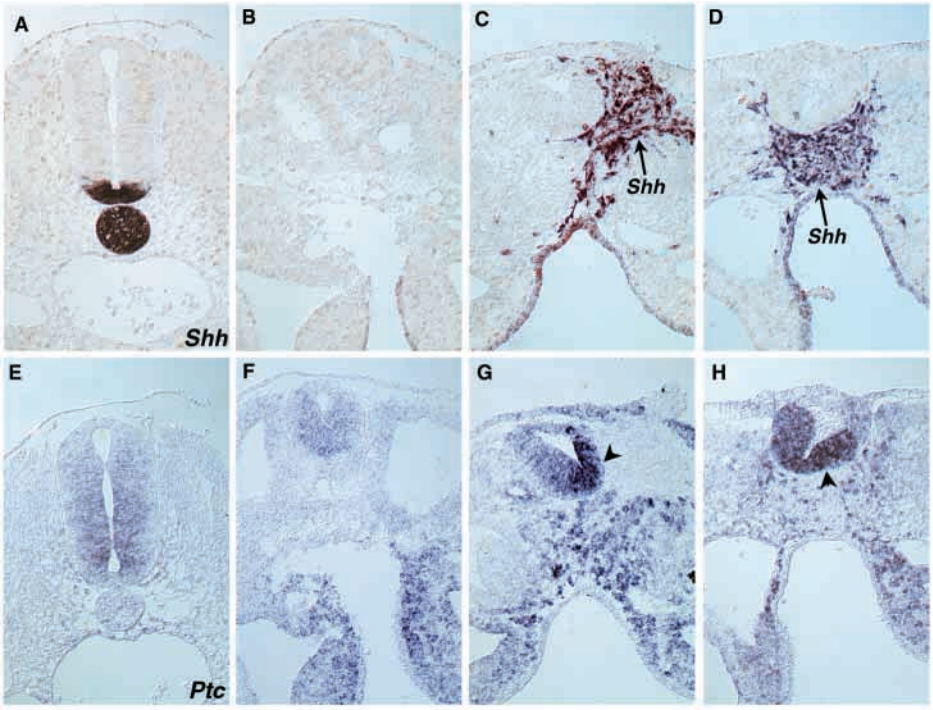

Fig. 5. Expression patterns of *Shh* (A-D) and *Ptc* (E-H) on serial sections at E3. (A,E) control level of expression; (B,F) midline cell-deprived neural tube level; (C,G) SHH-producing cell grafted level (experiment A3); (D,H) midline cell-deprived neural tube grafted on a layer of SHH-producing cells in a host (experiment B3). *Ptc* is upregulated (arrowheads) in the presence of SHH; *Shh*, SHH-producing cells.

One day following the operation (at E2.5), a significant growth defect was observed on the operated side (Fig. 6A-C). This defect became progressively more pronounced so that, at E5, the control side became U-shaped at the operated level (Fig. 6D). As early as E2.5, after whole-mount in situ hybridization, the somites isolated from midline cells, were devoid of *Pax1* and *MyoD* expression (Fig. 6A,C,F), while *Pax3* was expressed homogeneously in what remained of the somites (Fig. 6B). On serial sections (Fig. 6E-G) the control side, that had remained associated with the notochord and floor plate, showed the normal pattern of *Pax6* expression in the ventrolateral neural tube. The other side, separated from the midline cells by the operation, expressed *Pax6* in all the remaining neuroepithelium. It was striking to see that, on the operated side, the neural epithelium expressing *Pax6* was in continuity with the endoderm in which high levels of *Shh* transcripts were detected. In spite of the proximity of this tissue, many apoptotic figures were present in the neuroepithelium as compared to the control side (Fig. 6E-G).

The results obtained following a separation of the neural epithelium and adjacent paraxial mesoderm from midline cells by a slit show that the latter continue to provide the neural tube with indispensable factor(s) over a prolonged period of time.

Evaluation of the role of the somites on neural tube survival

To see if the paraxial mesoderm and somites exert a complementary effect on the survival and growth of the neural tube, a slit was made between these tissues at E2 (15-20ss), from SII, over the length of about 10 somites (Fig. 1F). Embryos were examined one day after the operation (at E3; Fig. 7A-D). Cell death, revealed by the TUNEL assay, appeared increased not only in the medial somites on the operated side, as expected (Teillet et al., 1998b), but also to a lesser extent in the neural tube that remained in contact with the floor plate and notochord (Fig. 7D). On the operated side, the ventrodorsal distribution of *Shh*, *Pax3* and *Pax6* transcripts in the neural tube was the same as in the side still in contact with the somites (Fig. 7A-C). As shown on sections of embryos

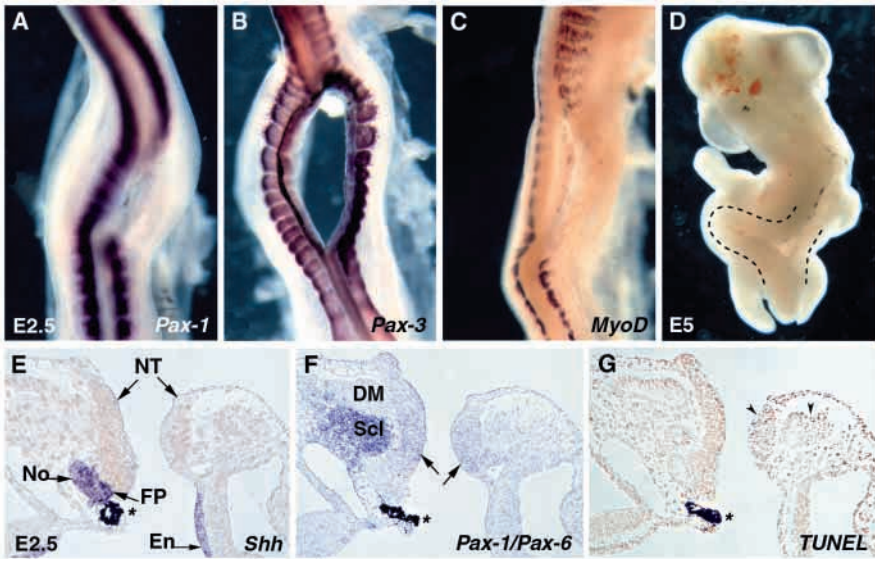

Fig. 6. Results of experiment C (see Fig. 1E). (A-C) Dorsal views of embryos hybridized in whole mount with *Pax1* (A), *Pax3* (B) and *MyoD* (C) probes, one day after the surgery (E2.5). (D) 2.5 days later (i.e. E5), the side deprived of midline cells is very short compared to the contralateral side, which developed normally and became U-shaped. (E-G) Serial sections at E2.5 hybridized with *Shh* (E), *Pax1/Pax6* (F) probes or examined for apoptosis using TUNEL (arrowheads) (G). DM, dermomyotome; En, endoderm; FP, floor plate; NT, neural tube; No, notochord; Scl, sclerotome; *, ink.

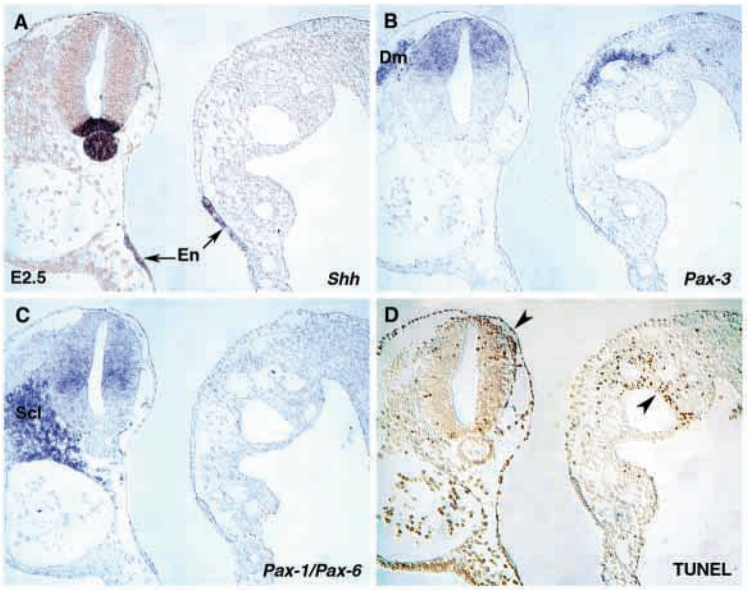

Fig. 7. Results of experiment D (see Fig. 1F). Cross sections 1 day after operation (E2.5) hybridized with *Shh* (A), *Pax3* (B), *Pax1*/*Pax6* (C) probes. (D) TUNEL assay for detection of apoptosis (arrowheads). Dm, dermomyotome; En, endoderm, Scl, sclerotome.

sacrificed 4 days after the operation (E5.5; Fig. 8A), on the operated side, the medial somitic derivatives, vertebral cartilage (Fig. 8B) and vertebral muscles (Fig. 8C) were absent. Moreover, the thickness of the mantle of the spinal cord was considerably reduced and haemorrhagic areas invaded the neural tissue. However *Pax6* and *Pax3* appeared normally distributed at the ventricular level (Fig. 8B,D).

These observations reveal that the paraxial mesoderm plays a prominent role in neural tube development that may be related to the fact that motoneurons may not project to the periphery owing to the absence of their targets, the dorsal muscles, which do not differentiate when the somites are deprived of SHH (Teillet et al., 1998b).

DISCUSSION

The experiments reported above demonstrate that an essential role of the midline structures issued from Hensen's node, notochord and floor plate (Catala et al., 1996), is to ensure the survival of the neuroepithelial cells. As already shown for somitic cells (Teillet et al., 1998b; and see Olivera-Martinez et al., 2001), this activity can be mimicked by SHH.

Midline cells are necessary for neural tube survival

In this work, two experimental designs have been used to isolate the developing neural epithelium from the midline structures. One consisted of removing the APH in 5-6ss embryos (see Charrier et al., 1999). The notochord and floor plate complex was then absent posterior to somite 20 (caudal to the forelimbs). As a consequence, much of the neuraxis developed without any contact, at any time, with the midline cells. In the other paradigm, a slit was made through the three germ layers extending over the length of several somites. In both cases, the deprivation of the influence of the notochord and floor plate from the neural ectoderm resulted in extensive cell death within a few hours and in the complete disappearance of all the embryonic tissues of the region within 48 hours. Insertion of a notochord or a floor plate between the paraxial mesoderm and the neural tube that forms after APH removal rescues these tissues from death and allows their growth and differentiation.

These results suggest that the notochord and floor plate exert an anti-apoptotic effect on the neural ectoderm as it was previously demonstrated for the paraxial mesoderm (Teillet et al., 1998b). This action occurs as soon as the midline structures appear by rostrocaudal displacement of Hensen's node and continues for at least 1 day after the formation of the neural tube. Interestingly, either one of these two midline structures is sufficient, in the avian embryo, to restore both survival and early patterning of the neural tube, suggesting that the factor(s) involved is(are) produced by both of them and able to exert a long-range action.

SHH is an anti-apoptotic factor for the neural tube

One candidate for such a factor was SHH, whose role in patterning the neural tube has been amply documented (reviewed by Tanabe and Jessell, 1996; Goodrich and Scott, 1998). Cells engineered to secrete the SHH glycoprotein were grafted in the same way as for the notochord or floor plate grafts, producing identical survival, growth and early patterning effects on the neural tube and paraxial mesoderm. *Pax3* and *Pax6*, which were uniformly expressed in neural tubes deprived of midline cells, were re-established in their normal dorsoventral patterns (Goulding et al., 1993). *Ptc* transcripts were strongly and homogeneously upregulated in the ventral neural tube close to the SHH-producing cells,

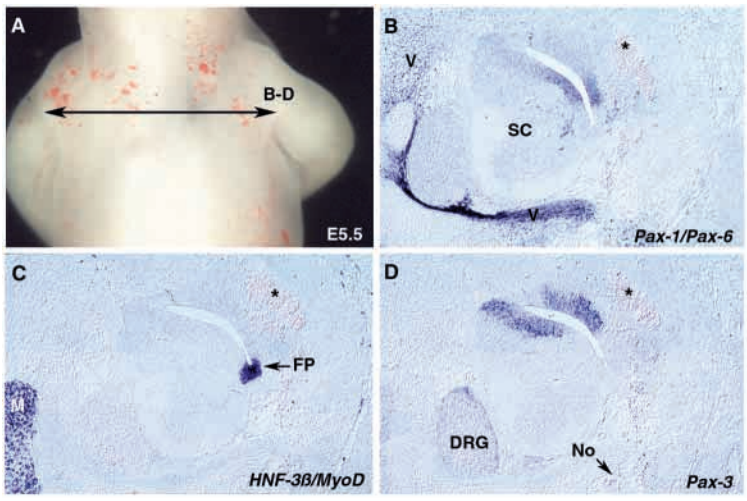

Fig. 8. Results of experiment D (see Fig. 1F) 3 days after operation (E5.5). (A) Dorsal view of an embryo. (B-D) Serial cross sections hybridized with *Pax1*/*Pax6* (B), *HNF3β*/*MyoD* (C) and *Pax3* probes (D). DRG, dorsal root ganglion; FP, floor plate; M, dorsal muscle; No, notochord; SC, spinal cord; V, vertebra; *, haemorrhagic area.

indicating the positive signaling activity of SHH protein on the neural tube. However, it is noteworthy that no floor plate markers (*HNF3β* or *Shh*) were ever detected in this region in the first day of exposure, which is in accordance with the absence of *Ptc* expression in the normal floor plate (see Goodrich and Scott, 1998).

Apoptosis shapes the central nervous system

Several periods of cell death occur during normal nervous system development (reviewed by Oppenheim et al., 1999). One of them concerns supernumerary neurons competing to establish synaptic contacts with their peripheral targets. Limited amounts of survival factors such as NGF and related neurotrophins are produced in the target fields and ensure a match between the size of the neuronal populations and the territory they have to innervate (reviewed by Purves, 1988; Oppenheim, 1991; Raff, 1992; Bibel and Barde, 2000).

PCD is also involved in morphogenesis of the neural epithelium at stages of development preceding the neurotrophic loop between axon-projecting neurons and their targets. Observations in the chick embryo revealed the apoptotic nature of this early cell death (Homma et al., 1994). Mice over-expressing the anti-apoptotic gene *bcl-2* (Martinou et al., 1994) or lacking the pro-apoptotic gene *Bax* (White et al., 1998) increased certain neuronal subpopulations, but they did not show large-scale malformations of the nervous system. By contrast, inactivation of the pro-apoptotic genes *Casp3*, *Casp9* and *Apaf1* have led to severe malformations due to the reduction of cell death in developing neural tissues (Kuida et al., 1996; Kuida et al., 1998; Hakem et al., 1998; Cecconi et al., 1998; Yoshida et al., 1998).

Therefore, as in other parts of the body, cell death is an important mechanism involved in sculpting nervous tissue by adjusting the initial progenitor pool needed for its proper morphogenesis and functional development.

SHH is a regulator of cell death

PCD is necessarily a highly regulated mechanism both at the intra- and inter-cellular levels. The experimental results reported here support the contention that the extent of the naturally occurring cell death in the neural epithelium is controlled by SHH produced by the notochord and floor plate. This implies that embryonic cells need to receive adequate death-inhibiting signals to avoid the activation of their own ready-to-run death program. It seems that such a signal is needed during a temporal window that goes from the time the ectoderm and mesoderm, already specified as neural epithelium and presomitic primordium respectively, begin their organogenesis and extends at least for 48 hours in the avian embryo. This period corresponds to a phase of active proliferation and patterning of the embryonic tissues.

Whether, in the absence of SHH, the PCD is spontaneously activated in the ectodermal and mesodermal cells or is induced by an extrinsic pro-apoptotic signal remains to be elucidated. One extrinsic pro-apoptotic influence could originate from the dorsal ectoderm or the lateral plate mesoderm that, at this stage, produce BMP4, a secreted protein already implicated in triggering PCD in embryonic cells (Graham et al., 1994). The apoptotic action of BMP4 in hindbrain neural crest is supposed to be antagonized by Noggin (Smith and Graham, 2001). Observation of an increased PCD in the dorsal neural tube (see Fig. 7D) after separation from the somites, which are known to produce Noggin at this stage (Hirsinger et al., 1997), can be explained by a deprivation of Noggin of somitic origin.

SHH has already been considered to be involved in the regulation of cell number in the developing CNS. For example, in transgenic mouse embryos, in which *Shh* was ectopically expressed in the dorsal neural tube from 10 days post-coitum until birth, an increase in cell number by an elevation in proliferation rates of spinal cord precursors was observed together with an inhibition in differentiation of CNS precursor cells (Rowitch et al., 1999). In contrast, inhibition of SHH signalling in the cephalic region of chick embryos by injecting a blocking anti-SHH antibody induced massive cell death in the cranial neural tube and in the cephalic mesenchyme of neural crest origin, thus reducing significantly the volume of the head (Ahlgren and Bronner-Fraser, 1999). Moreover, increased cell death was observed both in somites and in the neural tube of *Shh* null mutants (Chiang et al., 1996; Borycki et al., 1999).

SHH may act as a morphogen

A paradoxical effect of SHH has recently been shown in the limb bud of the chick embryo. Apart from its patterning role in this organ, SHH is generally considered to be a proliferation and survival factor for muscle cells (Duprez et al., 1998; Krüger et al., 2001). However, this protein has also been demonstrated to be responsible for cell death in the posterior necrotic zone which thus prevents the zone of polarising activity expanding (Sanz-Ezquerro and Tickle, 2000). In the neural tube of the chick embryo two opposite sites of naturally occurring cell death have been described: one dorsal, in the fusing neural folds, the other ventral, on each side of the floor plate (Homma et al., 1994; our own unpublished observations). Moreover, cells of the early chick spinal cord respond, either by cell death or by cell survival, to SHH in vivo and in vitro (Oppenheim et al., 1999). One can thus hypothesize that, in the neural tube, a high level of SHH could limit the lateral extension of the floor plate by inducing cell death in the neighboring tissues, while lower levels of the protein would have an anti-apoptotic effect on the cells of the lateral neural tube. In the neural folds, away from the SHH anti-apoptotic influence and submitted to high levels of BMP4, cell death could be naturally increased. Thus, SHH might act as a morphogen by promoting either pro-apoptotic or anti-apoptotic activity in a distance/concentration-dependent manner.

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