

Early neurogenesis in Amniote vertebrates

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ABSTRACT Labelling of Hensen's node in a 6-somite stage chick embryo by the quail/chick chimera method has revealed that, while moving caudalwards as the embryo elongates, the node leaves in its wake not only the notochord but also the floor plate and a longitudinal strand of dorsal endoderm. The node itself contains cells endowed with the capacity to yield midline cells (i.e. notochord and floor plate) along the whole length of the neural axis. Caudal node cells function as stem cells. They are responsible for the apical growth of the cord of cells that are at the origin of the midline structures since, if removed, neither the notochord nor the floor plate, are formed caudally to the ablation. The embryo extends however in the absence of midline cells and a neural tube develops posterior to the excision. Only dorsal molecular markers are detectable on this neural tube (e.g. *Pax3* and *Slug*). The posterior region of the embryo in which the structures secreting Shh are missing undergo cell death within the 24 to 48 hours following its formation. Unpublished results indicate that rescue of the posterior region of the embryo can be obtained by implantation of Shh secreting cells. One of the critical roles of floor plate and notochord is therefore to inhibit the cell death programme in the axial and paraxial structures of the embryo at gastrulation and neurulation stages.

KEY WORDS: *Quail/chick labelling, notochord, floor plate, Hensen's node, Shh apoptosis inhibitor.*

Introduction

Two successive steps can be distinguished in the process of neurulation in the vertebrate embryo. The first consists in the determination of two domains in the presumptive ectoderm; a ventral domain fated to become the epidermis and a dorsal domain from which the primordium of the nervous system will develop. The second step is characterised by morphogenetic transformations through which the ectoderm committed to a neural fate (the neural epithelium) will form a neural tube within which neuronal and glial differentiation will take place. The seminal studies of Spemann and his followers on the Amphibian embryo have led to the long standing notion that the critical event through which neurogenesis is initiated is the involution of the notochordal material through the dorsal blastoporal lip during gastrulation. The notochord thus formed, from the organizer region, induces the overlying ectoderm to become neural by the vertical diffusion of a neural inducing substance. According to this view, neural induction is transmitted from the mesodermal to the ectodermal germ layer rather than through a planar diffusion (within the ectoderm) of the neuralizing factor(s) produced by the organizer. Modern studies have revealed that the planar model of neuralization is valid (e.g. Doniach *et al.*, 1992; Sasai and De Robertis, 1997). But this does not mean that neural induction in "trans" does not occur. In fact, it does, as demonstrated many times for example by the Einsteck

Method. In birds, Hensen's node is the equivalent of the Amphibian organizer from which the notochord extends underneath the future neural plate. In line with the model derived from the classical work mentioned above, the notochord has been credited with the role of inducing the overlying ectoderm to become the neural plate in Amniotes as it was in Amphibians. It was also claimed that the notochord is responsible for the induction of the *floor plate (FP)*, a midline transient structure of the neural primordium that plays a key role in patterning the neural epithelium in the spinal cord and brain stem (van Straaten and Hekking, 1991; van Straaten *et al.*, 1988; Placzek *et al.*, 1990; Yamada *et al.*, 1991). This induction was proposed to be mediated by the N-terminal domain of the secreted protein Sonic hedgehog (Shh) produced by the notochord: Shh induces the midline cells of the neural plate to activate the gene encoding the transcription factor HNF3 β and subsequently the one encoding the Shh protein (Roelink *et al.*, 1994; Ericson *et al.*, 1996). This model was supported by several experimental results.

1. If the notochord or the floor plate, that both secrete Shh, are implanted in contact with the neural epithelium of the *sinus rhomboidalis* in a 10 somite-stage (10ss) chick embryo, floor plate properties are induced in the neural epithelium otherwise fated to

Abbreviations used in this paper: CNH, chordo-neural hinge; FP, floor plate; HN, Hensen's node; No, notochord; ss, somite stage.

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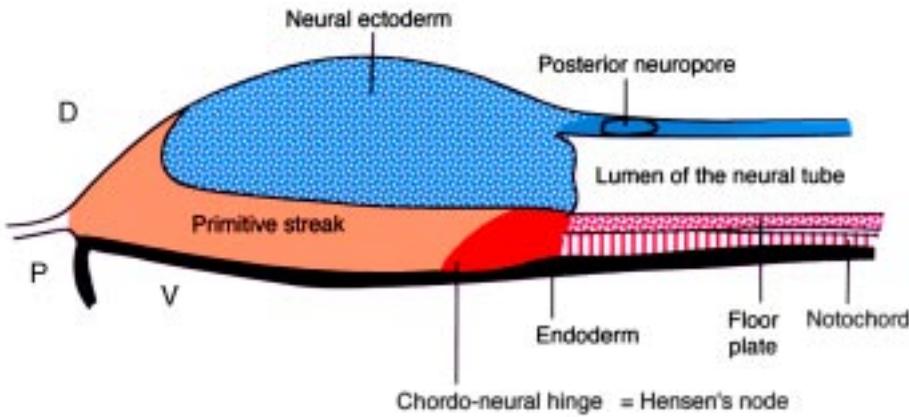


Fig. 1 Fate map of the quail bud represented on a sagittal section of a 25-27ss chick or quail embryo. The superficial cells of the tail bud are fated to form the neural tube by the secondary neurulation process. Ventrally, the chordo-neural-hinge (CNH) prolongs the notochord and floor plate deposited during Hensen's node (HN) regression. CNH can be assimilated to HN. It is located rostral to the remnant of the primitive streak that is at the origin of the paraxial mesoderm of the posterior region of the body.

become the lateral wall of the spinal cord (Placzek *et al.*, 1990, 1991; Yamada *et al.*, 1991; Pourquié *et al.*, 1993). Such a result however can be obtained only during a well-defined time window. At slightly later stages, grafting of the notochord (or floor plate) results in the proliferation of the cells of the neuroepithelium and in the enlargement of the neural tube wall.

2. Removal of the notochord in the region of the chick embryo located rostrally to Hensen's node up to the last formed somites (i.e. at the level of the presomitic mesoderm – PSM) results in the abnormal development of the spinal cord (in a region restricted to the posteriormost part of the domain in which the notochord was removed). At this level (about 4-5 somites in length) the neural tube has a reduced diameter (about half the control, 4 days after the excision). It is devoid of a floor plate and of the ventral horns that normally contain the motoneurons. The neural crest cell emigration is not significantly affected and spinal ganglia are present. In the absence of the two sources of Shh (floor plate and notochord), the sclerotomal part of the somites, normally expressing *Pax1* and *Pax9* and differentiating into vertebral cartilage, fails to develop. In contrast, the myotomes differentiate into two masses of striated muscles that coalesce in a median structure located ventrally to the abnormally developed neural tube.

3. A third experimental result in support of the notochord/floor plate induction model is the fact that, when subjected to Shh *in vitro*, lateral fragments of the posterior neural plate (from 10ss embryo) are induced to express *HNF3 β* , *Shh* gene and are the site of motoneurone differentiation (Roelink *et al.*, 1994, 1995; Ericson *et al.*, 1996).

I will present in this article a series of observations and experiments that address the role of the notochord in neural induction, neural tube morphogenesis and floor plate specification. In addition, I will bring about evidence for a role of Shh in the inhibition of cell death in the neural epithelium and in somitic mesodermal cells

during the early steps of neurulation in Amniote vertebrates.

During its caudalward movement, Hensen's node lays down in its wake the notochord and the floor plate

In the avian embryo, neurulation proceeds according to two different morphogenetic mechanisms in the anterior and posterior parts of the body. In the cephalic, cervical and dorsal regions, the neural epithelium forms a neural plate, whose lateral ridges (designated as the neural folds) fuse in the dorsal midline thus generating the anlage of the central nervous system (CNS): the neural tube and the neural crest which is at the origin of the peripheral nervous system (PNS). This process, called *primary neurulation* takes place from the anterior to the posterior neuropore, located at the level of somites 27 in both chick and quail embryos.

Posteriorly, the neural primordium is formed during the elongation of the tail bud, from a cord of epithelial cells in which the lumen of the neural tube appears by cavitation. This type of neurulation (designated as *secondary neurulation*), which extends on the whole length of the neural primordium in fish embryos, is thus limited to the lumbo-sacro-caudal part of the body in Amniotes (Catala *et al.*, 1995, 1996).

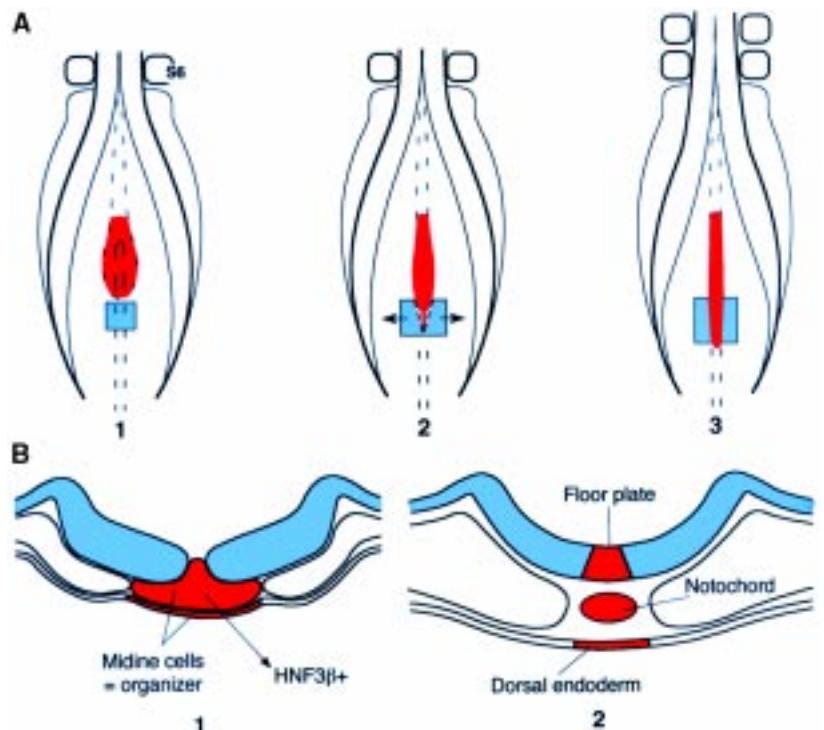


Fig. 2 Schematic representation of the rostro-caudal movement of Hensen's node (HN). (A) Hensen's node cells (red) bisect the superficial layer of the sinus rhomboidalis (blue) which will become the alar plates of the spinal cord. (B) Later on, the bulk of Hensen's node (1) becomes segregated into three layers: the floor plate, the notochord and the dorsal endoderm (2).

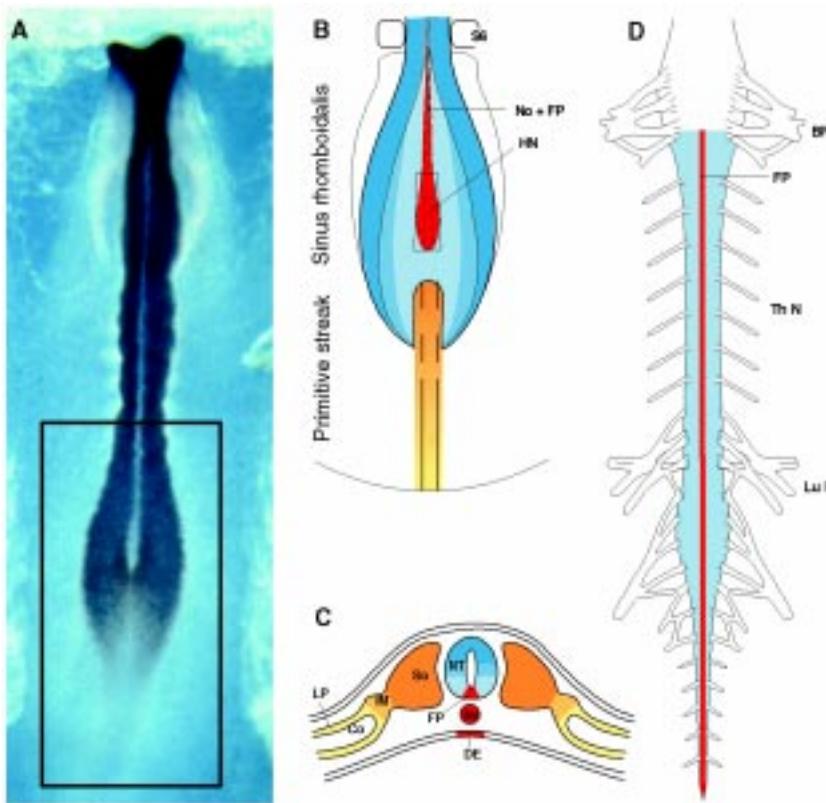


Fig. 3. Fate map of the posterior region of the embryo at 6ss. **(A)** *In situ* hybridization of a 6ss embryo with a *Sox2* probe. At the level of the sinus rhomboidalis, the neural ectoderm, as with the rest of the neural plate, expresses *Sox2*. Note that the floor plate, the node and the primitive streak are *Sox2*-negative. **(B)** The neural ectoderm (blue). Note the similarity between the *Sox2*-positive area in (A) and the region of the sinus rhomboidalis destined to form the neural tube (NT) as revealed by the quail-chick chimera system. In red, the notochord and floor plate (No + FP) and the node (HN). In yellow, the primitive streak. **(C)** Transverse section at the thoracic level in an E3 (embryonic day 3) chick embryo, showing the distribution of the areas labelled in B. The derivatives of Hensen's node are the floor plate, the notochord and the underlying dorsal endoderm (DE). **(D)** Replacement of Hensen's node in a chick embryo by its quail counterpart results in the labelling of midline cells (red) from the level of somite 20 down to the caudal end of the spinal cord. The neural tube itself is derived from the caudal extension of the neural ectoderm of the sinus rhomboidalis. BP, brachial plexus; Lu P, lumbar plexus; s6, somite 6; Th N, thoracic neural tube.

The secondary neurulation is characterised by the caudal growth of the tail bud in which the cells will be progressively segregated into the three germ layers. Labelling of different regions of the tail bud by the quail-chick marker system in 25-27ss chick embryos has shown that the cell movements resulting in the formation of the three germ layers are similar to those going on in the blastoderm during primary neurulation (Catala *et al.*, 1995). The fate map of the tail bud is presented in Fig. 1.

Labelling of Hensen's node region at various stages of neurulation from 5-6ss onward was particularly informative. Thus following the replacement of Hensen's node material (located in the median pit of the *sinus rhomboidalis*) in a 5-6ss chick by its quail counterpart, the notochord, floor plate and dorsal endoderm are made up of quail cells from the level of the node in the 6ss embryo (i.e. the level of somite 20) down to the posterior end of the spinal cord. The node material thus bisects the superficial cell layer of the *sinus rhomboidalis*, caudal to the median pit. During this process the dorsalmost cells of the node become incorporated into the ectoderm and form the floor plate. The

latter is thereafter separated from the future notochord by a basement membrane (Fig. 2) (Teillet *et al.*, 1998).

In these chimeric embryos, the caudal progression of the node can be followed owing to the fact that the node material has been stably labelled by the quail cells. It is then possible to recognize the node material in the tail bud during secondary neurulation. Hensen's node is recognizable at the junction of the floor plate and notochord forming a structure identified by Pasteels (1937) as the chordo-neural hinge (CNH or *charnière cordoneurale*). The capacity to recognize node cells from the 5-6ss stage onward, as they move caudalwards along the AP axis, by the fact that they carry quail type nuclei, has also allowed to selectively identify the genes expressed by those cells during the process of regression: cells of Hensen's node and of their derivatives, floor plate and notochord, express *HNF3 β* and *chordin*. At the early somitic stages (up to 7ss) they also express *Shh*. Later on, *Shh* gene expression is down regulated in the floor plate on the length of about 5 presumptive segments rostrally to Hensen's node (see Fig. 3 of Teillet *et al.*, 1998).

Formation of the floor plate and notochord does not involve convergence of cells located laterally to the midline

The fate map of the *sinus rhomboidalis* and the primitive streak has been completed in the 5-6ss chick embryo by using the same substitution method of definite areas of the chick embryo by their quail counterpart. The results, illustrated in Fig. 3, allow a delineation of the extent of the neural ectoderm in the *sinus rhomboidalis*. It appears that the neural epithelium that will form the spinal cord from the brachial level down to the caudal end of the spinal cord is entirely included in the posterior half of the *sinus rhomboidalis*.

The transcription factor *Sox2* is one of the early genes turned on during the process of neurulation. It is expressed at the primitive streak stage in an area located around Hensen's node (except in the primitive streak itself) (Rex *et al.*, 1997). At 5-6ss, *Sox2* expression encompasses the neural tube of the cephalo-cervical region and the *sinus rhomboidalis* at the exclusion of the midline cells of the floor plate. The neural epithelium as defined by fate mapping studies and by *Sox2* expression are strikingly superimposable (Fig. 3). These observations indicate that neuralisation of the ectoderm is not dependent upon the presence of the notochord. It rather supports a planar diffusion of dorsalizing factors arising from the node region. Moreover, it is striking to see that labelling of Hensen's node at 6ss results in the fact that the notochord and the floor plate are exclusively made up of quail cells from the brachial level down to their caudal end without addition of chick cells during the process of HN regression.

Removal of the notochord in the chick embryo: an experiment revisited

The experiments involving the removal of the notochord are currently performed at a large range of developmental stages depending of the laboratory (e.g. 8 to 22ss) (Placzek *et al.*, 1990,

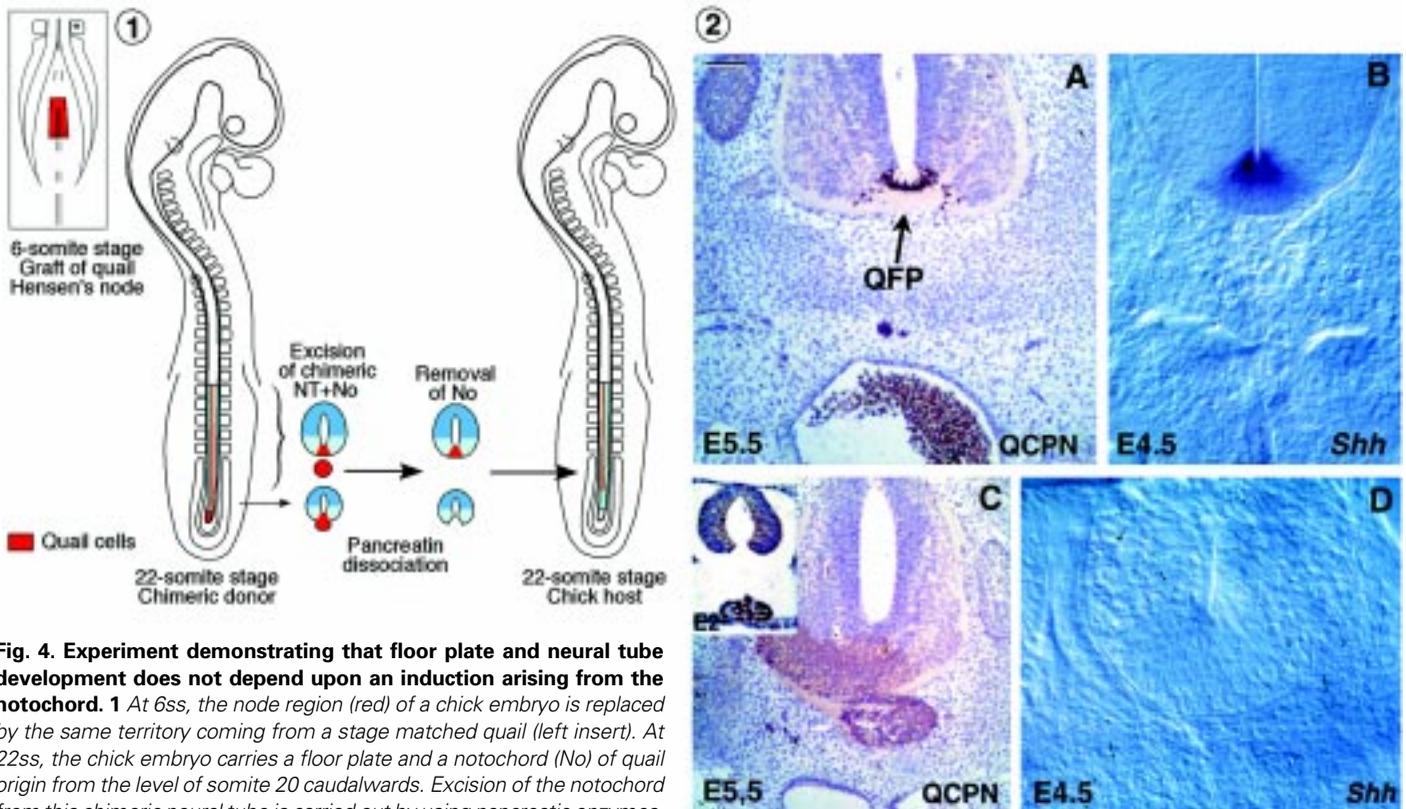


Fig. 4. Experiment demonstrating that floor plate and neural tube development does not depend upon an induction arising from the notochord. **1** At 6ss, the node region (red) of a chick embryo is replaced by the same territory coming from a stage matched quail (left insert). At 22ss, the chick embryo carries a floor plate and a notochord (No) of quail origin from the level of somite 20 caudalwards. Excision of the notochord from this chimeric neural tube is carried out by using pancreatic enzymes.

The notochord is removed while the floor plate remains in place in most of the neural tube subjected to the operation (the floor plate here in red is *HNF3 β* positive, not shown). However, posteriorly, where notochord and floor plate are fused in a single cell cord, the presumptive floor plate material is also removed and the neural tube remains open ventrally (see (2) insert E2). After back grafting, the spinal cord develops normally with a quail floor plate (QFP). **2 (A)** In the absence of the notochord, quail cells are revealed with the QCPN monoclonal antibody (see Teillet *et al.*, 1998). **(B)** The floor plate expresses *Shh*. **(C)** In contrast, in the region of the tube devoid of floor plate, the neural tube develops without the ventral horn and **(D)** does not show *Shh* expression. En, Embryonic day n.

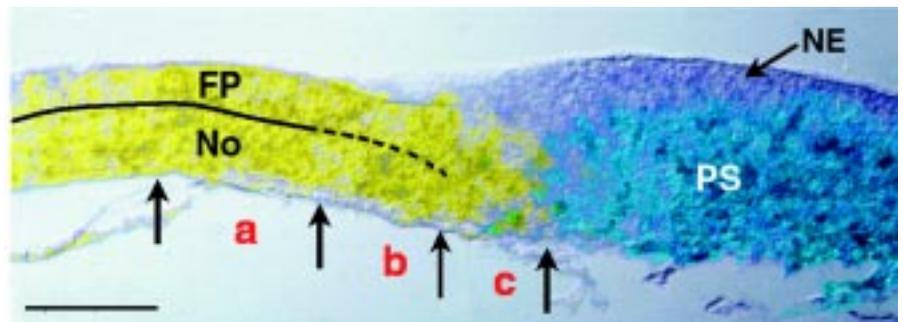
1991; van Straaten and Hekking, 1991; Yamada *et al.*, 1991 and our own experiments). The abnormal development of the spinal cord is obtained only if the excision extended down to the level of the node. Moreover, the absence of floor plate is always restricted to a short length of the AP axis. Knowing that floor plate and notochord are joint in the chordoneural hinge within and rostrally to the node, the notion emerged that during notochord removal, not only the notochord but also the floor plate cells might be removed at this posterior level. This was demonstrated by two experimental procedures described in Teillet *et al.* (1998) and summarized in Fig. 4. In fact, the floor plate remains *in situ* along most of the territory where the excision has been performed as could be evidenced by *HNF3 β* expression. Posteriorly, the neural tube devoid of floor plate was open ventrally. Normal development of the

spinal cord was observed in the area where the floor plate remained *in situ*. In spite of the absence of notochord, the floor plate further developed normally and produced the morphogen *Shh*. In contrast, at the level where the floor plate had been removed, the neural tube was reduced in size and devoid of ventral horns (Fig. 4).

Three distinct A/P regions can be identified in Hensen's node/CNH

Examination of Hensen's node in sagittal section at 5-6ss reveals three regions from rostral to caudal: a rostral one, designated as zone a in which the presumptive floor plate is composed of epithelial cells closely apposed to more ventral cells that are randomly organised and are in continuity with the already indi-

Fig. 5. Heterogeneity in Hensen's node at 6ss. Sagittal section of the node and anterior primitive streak (PS) hybridized with *HNF3 β* (yellow) and *ch-Tbx6L* (blue) (caudal is to the right). Three regions can be distinguished in the node region: zone a where notochord (No) and floor plate (FP) are closely apposed but separated by a basement membrane; zone b where this process is in progress and zone c where the *HNF3 β* + cells are randomly arranged. The PS cells are *ch-Tbx6L*+ and in continuity with the node material. NE, neuroepithelium posterior to the median pit.



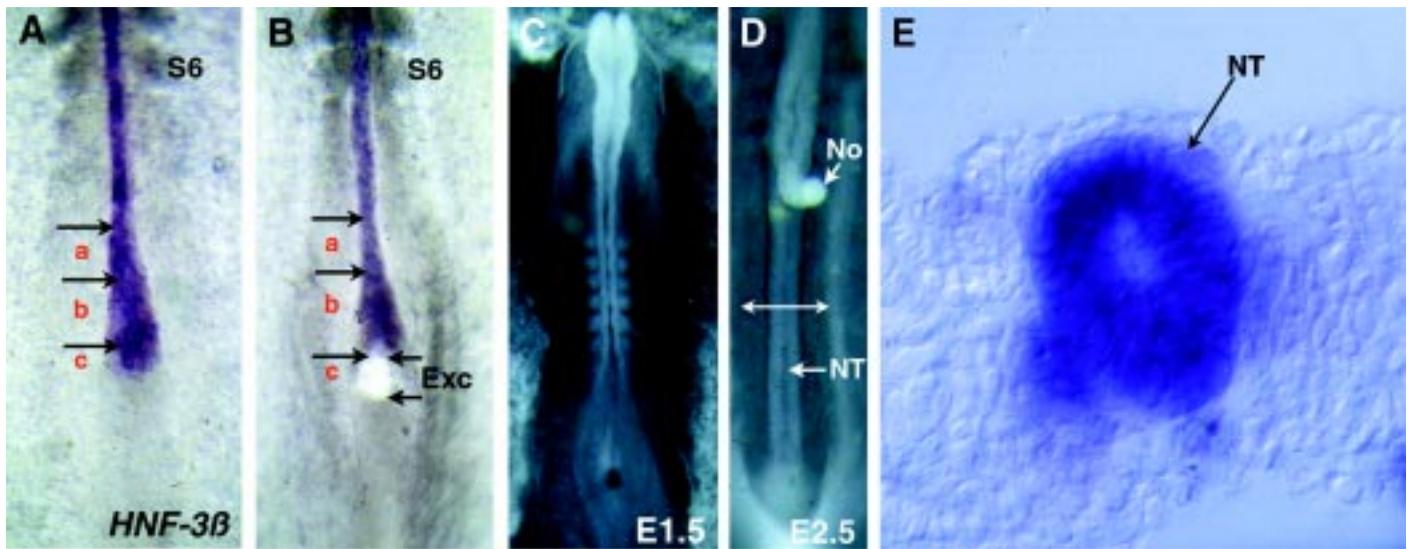


Fig. 6. Consequences of excision of Zone c of Hensen's node. (A) Ventral view of the sinus rhomboidalis following *in situ* hybridization with an *HNF3β* probe. The node is seen with the 3 a, b and c anteroposterior areas. Rostrally, *HNF3β* labels the notochord. (B,C) Zone c has been removed at E1.5. (D) Ventral view of an operated embryo one day after zone c excision. The caudal extension of the notochord (No) is stopped. Notochord forms a mass rostral to the excision site. Posteriorly, the neural tube (NT) forms in the absence of midline cells. (E) Section of the neural tube as shown in D hybridized with a *Pax3* probe.

visualized notochord. These two cellular compartments are separated by a discrete basement membrane. Zone a corresponds to the region removed during neural cord excision (see Fig. 4). In zone b, the future floor plate/notochord domains are recognizable but not yet delaminated. Zone b corresponds to the bulk of the node material. Caudally, zone c corresponds to the posterior end of the midline cells that can be delineated through their expression of *HNF3β*. Zone c is in contact with the primitive streak that expresses the gene *Tbx6L* (Fig. 5) (Charrier *et al.*, 1999).

Removal of Zone a or Zone b results in an interruption of midline cells in a segment of the spinal cord

As described above, removal of zone a can be achieved by pulling out the notochord from the already formed neural tube (or neural groove) rostrally to the node itself. In this case, a segment

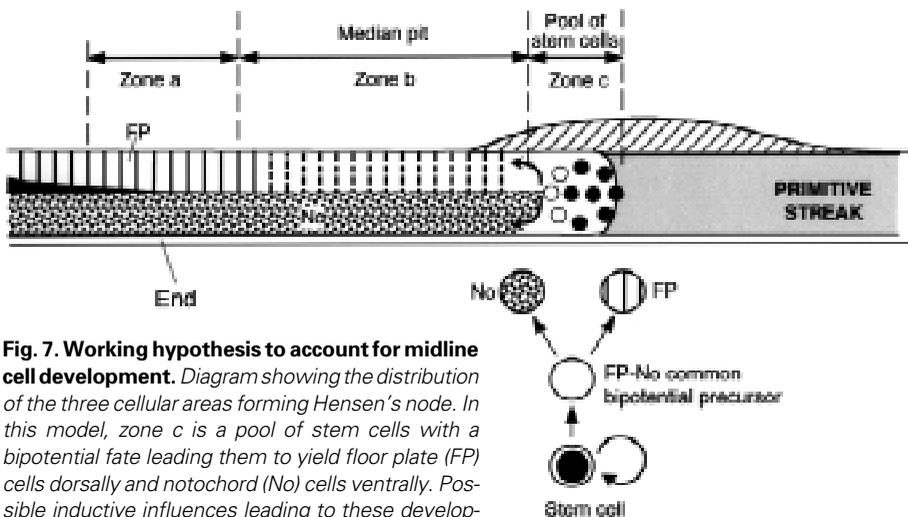


Fig. 7. Working hypothesis to account for midline cell development. Diagram showing the distribution of the three cellular areas forming Hensen's node. In this model, zone c is a pool of stem cells with a bipotential fate leading them to yield floor plate (FP) cells dorsally and notochord (No) cells ventrally. Possible inductive influences leading to these developmental choices have not yet been elucidated. End, Endoderm.

of spinal cord develops in which only dorsal structures, including the neural crest derivatives, survive during a few days following the operation.

Zone b can be extirpated surgically, while zone c remains *in situ*. In this case, as in extirpation of zone a, a segment of neural tube devoid of floor plate and ventral horn develops but the caudal region of the embryo, reached by zone c evolves normally. Therefore, caudal extension of the node can take place, even if most of the node material is extirpated. No significant regulation takes place from either zone a or c to compensate the loss of zone b cells (Charrier *et al.*, 1999).

Extirpation of zone c yields interesting results: midline cells deposition is interrupted from the level of excision down to the caudal end of the embryo. Zone a and b left *in situ* are the site of some growth resulting in the formation of a mass of *Shh*⁺ and *HNF3β*⁺ cells rostrally to the excision. This however did not prevent the extension of the neural primordium and the formation of a neural tube. A neural tube forms in the dorsal region of the body as well as in the tail bud through the process of secondary neurulation. This neural tube, however, is not subjected to the influence of midline cells since neither the floor plate nor the notochord developed posteriorly to zone c excision (Fig. 6). This neural tube expresses *Pax3* on its entire surface and *Slug* in its dorsalmost side corresponding to the neural crest presumptive territory. Already 24 hours after the operation, massive cell death is detectable not only in the neural tube but also in the paraxial mesoderm. At E3.5 the embryonic structures located caudally to the brachial level are undergoing cell death and at E4.5, the embryos are truncated posteriorly to the forelimbs (Charrier *et al.*, 1999).

Discussion and Conclusions

The experimental results described above have led to the reconsideration of the previously accredited model, according to which the notochord is responsible for patterning the neural plate by inducing the midline cells to become floor plate. They prove that floor plate and notochord are derived from a common group of cells present in *zone b* and *c* of Hensen's node. These cells are responsible for the formation of the midline structure along the whole neural axis from the diencephalon down to the tail end. We propose the hypothesis that cells of *zone c* function as stem cells since they form the whole length of midline structures without addition of cells from more lateral region of the embryo during the process of node regression. Whether these stem cells are bipotential or form a heterogeneous group of cells committed either to become notochord or floor plate cannot be deduced from our experiments. The former alternative is the one that I favour since in zebrafish cells with both floor plate and notochord markers have been identified in the organizer region (the shield, considered as Hensen's node or Amphibian organizer equivalent) (Le Douarin and Halpern, 2000). The model presented in figure 7 summarizes the conclusions reached from the above described experiments.

Moreover, various mutations described in zebrafish support the notion that the floor plate and the notochord develop independently from one another. Such is the case of the mutant *Cyclops* in which the floor plate is lacking (more or less completely) whereas the notochord is present.

Another important result that can be deduced from the experiments reported above is that the role of midline structures, floor plate and notochord, is to prevent the neural epithelium as well as mesodermal cells from undergoing cell death. Results recently obtained in our laboratory have revealed that these structures can be rescued from death by back grafting either a notochord, a floor plate or cells engineered to secrete Shh (Charrier *et al.*, in preparation).

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