Anat Embryol (2004) 209:107–117
DOI 10.1007/s00429-004-0421-2

ORIGINAL ARTICLE

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Development of the posterior neural tube in human embryos

Accepted: 3 August 2004 / Published online: 14 October 2004

Abstract Development of the posterior neural tube (PNT) in human embryos is a complicated process that involves both primary and secondary neurulation. Because normal development of the PNT is not fully understood, pathogenesis of spinal neural tube defects remains elusive. To clarify the mechanism of PNT development, we histologically examined 20 human embryos around the stage of posterior neuropore closure and found that the developing PNT can be divided into three parts: 1) the most rostral region, which corresponds to the posterior part of the primary neural tube, 2) the junctional region of the primary and secondary neural tubes, and 3) the caudal region, which emerges from the neural cord. In the junctional region, the axially-condensed mesenchyme (AM) intervened between the neural plate/tube and the notochord at the stage of posterior neuropore closure, while the notochord was directly attached to the neural plate/tube in the most rostral region. A single cavity was found to be formed in the AM as the presumptive luminal surface cells were radially aligned in the junctional region prior to the formation of the neural cord. The single cavity was continuous with the central cavity of the primary neural tube. In contrast, multiple or isolated cavities were frequently observed in the caudal region of the PNT. Our observation suggests that the junctional region of the PNT is distinct from other regions in terms of the relationship with the notochord and the mode of cavitation during secondary neurulation.

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Keywords Posterior neural tube · Primary neurulation · Secondary neurulation · Tail bud · Human embryo

Introduction

In avian and mammalian embryos, the neural tube forms through two distinct phases, primary and secondary neurulation. During primary neurulation, the lateral ends of the neural plate elevate and fuse with each other to form the primary neural tube. Subsequent to closure of the posterior neuropore (the last part of the primary neural tube to be fused), the secondary neural tube develops by elongation and cavitation of the tail bud. This process is called secondary neurulation (Griffith et al. 1992, O'Rahilly and Müller 1994, Colas and Schoenwolf 2001). Development of the posterior neural tube (PNT), which develops into the future lumbar, sacral, coccygeal, and equinal cord, is a rather complicated process because it involves both primary and secondary neurulation. In human embryos, closure of the posterior neuropore occurs at the upper sacral level during Carnegie stage 12 (CS12) (Müller and O'Rahilly 1987, Nakatsu et al. 2000). Because of slow growth of the neural tube relative to that of vertebrae, the junction of the primary and secondary neural tubes is apposed at the lumbosacral level of the vertebral column in neonates (O'Rahilly and Müller 2003). The tail bud is an aggregate of undifferentiated mesodermal cells at the caudal end of the embryo, and it gradually replaces the primitive streak between CS12 and 13. It has a potential to give rise to a variety of tissues, including the caudal portions of the digestive tube, coelom, blood vessels, notochord, somites, and spinal cord in human embryos (O'Rahilly and Müller 1994).

Disorders of primary and secondary neurulation can lead to various forms of neural tube defects (NTD), which are among the most common human congenital malformations, affecting 0.5–8/1,000 live births (Little and Elwood 1992). Failure of the anterior and posterior neuropores to close results in exencephaly/anencephaly and

myeloschisis, respectively. Myeloschisis occurs most frequently at the lumbosacral level of the vertebral column (Dryden 1980), which corresponds to the junctional region of the primary and secondary neural tubes in neonates (O'Rahilly and Müller 2003). Moreover, it has been suggested that skin-covered NTD such as diplomyelia are caused by aberrant cavitation below the lower lumbar level of the spinal cord, which corresponds to the PNT (Dryden 1980, Lemire 1988). In addition, it has been recently reported that decreased occurrence of NTD by folate supplementation to pregnant women can be explained primarily by the decrease in spina bifida (spinal NTD), which results from defects of PNT (Stevenson et al. 2000, Honein et al. 2001, Williams et al. 2002). Therefore, normal development of the PNT needs to be examined to understand the pathogenesis of spinal NTD.

Development of the PNT has been most extensively studied in chick embryos. Multiple cavities are formed at the beginning of cavitation in the medullary cord, which differentiates from the tail bud (Criley 1969, Schoenwolf 1979, Schoenwolf and Delongo 1980). Enlargement and subsequent coalescence of the cavities result in a single cavity, which eventually becomes continuous with the central cavity of the primary neural tube. At the level of the posterior neuropore, there is an overlapping zone where primary neurulation occurs dorsally and secondary neurulation ventrally. On the other hand, development of the PNT in mice appears simpler than that in chicks (Hughes and Freeman 1974, Schoenwolf 1984, Nievelstein et al. 1993). Multiple cavities are not formed during the course of its development. Instead, the cavity of the primary neural tube extends continuously into the "medullary rosette," which consists of elongated tail bud cells around the cavity. There is no overlapping zone of the primary and secondary neural tubes. Such difference between species in the PNT development may account for the higher incidence of lumbosacral myeloschisis in chicks compared with mice under various experimental conditions (Hughes and Freeman 1974, Schoenwolf 1979).

Development of the human PNT is not fully understood. Müller and O'Rahilly (1987, 1988) observed that the cavity of the fully formed primary neural tube extends continuously into the tail bud. The cavity in the tail bud was surrounded by radially-arranged cells that are similar to the "medullary rosette" in mice. No isolated cavities were observed during secondary neurulation in human embryos. They also showed that there was no overlapping zone of the primary and secondary neural tubes. These observations suggest that development of the PNT in human embryos is similar to that in mice rather than to that in chicks. On the other hand, some other investigators claimed that multiple cavities were present in the human PNT as observed in the chick PNT (Bolli 1966, Lemire 1969, Hughes and Freeman 1974, Saraga-Babic et al. 1995). The developmental mechanism of the human PNT is controversial partly because human embryo specimens around the stage of posterior neuropore closure have only rarely been available to date.

The Kyoto Collection of Human Embryos (Nishimura 1975, Shiota 1991) has provided us with a unique opportunity to observe in detail a large number of human embryos at the stage of neurulation. In the present study, we histologically examined 20 cases of externally normal human embryos at CS12 and 13 with special reference to development of the PNT. We propose that the developing PNT can be divided into three parts in terms of its relationship with the notochord and the mode of cavitation in the secondary neural tube.

Materials and methods

The human embryos examined in this study were from the Kyoto Collection of Human Embryos held in the Congenital Anomaly Research Center of Kyoto University. The embryo collection consists of approximately 44,000 embryos, most of which were procured after termination of pregnancy in healthy women for social reasons (Maternity Protection Law of Japan). Most embryos in the collection were within 8 weeks of fertilization. Because the obstetricians did not examine the aborted embryos in detail and sent

Table 1 Studied embryos of stages 12 and 13 (*PNT* posterior neural tube, *ND* not determined)

CS12			CS13			
Embryo no.	Embryonic length (mm)	Plane of PNT	Embryo no.	Embryonic length (mm)	Plane of PNT	Multiple cavities in PNT
Posterior neuropore			560	5	Oblique	◯
Closing			584	6.6	Transverse	◯
589	3.5	Transverse	622	ND	Transverse	-
741	3.1	Transverse	632	4.6	Transverse	-
2694	2.8	Oblique	1224	5.9	Oblique	◯
Closed			1259	ND	Oblique	-
2431	3.6	Transverse	1313	5.8	Oblique	-
3005	ND	Transverse	1542	4.3	Transverse	-
13087	4.2	Sagittal	1586	5.5	Transverse	-
			3300	4.9	Oblique	◯
			3740	5	Transverse	◯
			3918	5.1	Transverse	◯
			14049	4.6	Transverse	-
			14205	5.7	Transverse	◯

them to our laboratory without any prior selection, the collection of specimens was not biased by the outcome of the embryos. Further details of the embryo collection and its demographic characteristics have been previously described elsewhere (Nishimura 1975, Matsunaga and Shiota 1977, Shiota 1991). The embryos were fixed in 10% formalin or Bouin's fluid soon after procurement, and after being sent to the laboratory in Kyoto University, they were staged (O'Rahilly and Müller 1987), measured, and examined for structural abnormalities and signs of intrauterine death under a dissection microscope. Some of the well-preserved embryos were photographed and serially sectioned at 10-μm thickness for histological examination. In the present study, we examined 20 externally normal embryos around the stage of posterior neuropore closure (CS12 and 13; Table 1). All the embryos except one (No. 1542) were procured after induced abortion for social reasons. Case No. 1542 was obtained from ectopic pregnancy. All the embryos appeared healthy and had no sign of intrauterine death. To examine the relationship between neural plate/tube and notochord, and cavity formation in secondary neurulaton, transverse or oblique sections of the caudal region were examined in detail. A sagittally sectioned embryo (No. 13087) is also described to illustrate the junction between the primary and secondary neural tubes.

Results

The axially-condensed mesenchyme intervenes between the neural plate/tube and the notochord in the junctional region of the primary and secondary neural tubes

In a CS12 embryo with its posterior neuropore widely open (No. 741), the notochord was attached to the floor plate of the neural plate (Fig. 1a). At the posterior end of the neural plate, the notochord was not formed and instead, axially-condensed mesenchyme (AM) derived from the tail bud (Müller and O'Rahilly 1986) was located beneath the neural plate (Fig. 1b). Similar findings were observed in a CS11 embryo by Müller and O'Rahilly (1986).

In more advanced embryos with the posterior neuropore closing (CS12; No. 589, 2694), AM intervened between the notochord and the neural plate for a short distance (Fig. 2b). In No. 589 embryo, the depth of the neural groove gradually decreased posteriorly at the level of the posterior neuropore, as observed by Nakatsu et al. (2000), while the thickness of AM progressively increased posteriorly (Fig. 2b–g). The notochord was closely attached to the neural plate at the rostral part of the posterior neuropore (Fig. 2a).

In slightly advanced embryos in which the posterior neuropore had closed (No. 2431, 3005, and 13087 at CS12 and No. 1586 at early CS13), AM was still present between the notochord and the primary neural tube. Sagittal sections of a CS12 embryo (No. 13087) showed that the notochord was in close contact with the neuroepithelium at most parts of the primary neural tube. As the primary neural tube became tapered caudally, AM appeared to intervene between the notochord and the primary neural tube in the junctional region (Fig. 3a, b). This feature was also observed in transverse sections of No.2431, 3005 and 1586 embryos (Fig. 3f, g). A small cavity in the AM seemed to be a cavity of the secondary neural tube (Fig. 3c–e, *white arrows*). The size of the cavity became enlarged rostrally, and AM intervened between the notochord and the primary neural tube (Fig. 3f, g), while the primary neural tube was closely attached to the notochord in the most rostral region of the PNT (Fig. 3h). Because AM appeared to be incorporated into the most ventral part of the neural tube, it is likely that both the primary neural tube and AM participate in the formation of the PNT in the junctional region. AM was still present between the notochord and the newly-formed secondary neural tube (Fig. 3a, b). At CS13, there was no cell population between the notochord and the neural cord, which is a mass of the neuroepithelial cells derived from the tail bud (Fig. 4, Fig. 5, Fig. 6, Fig. 7).

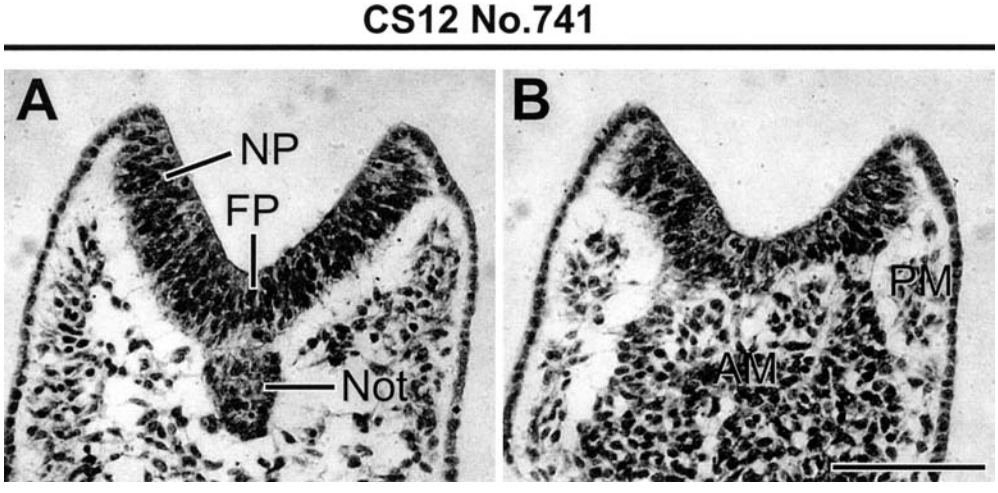

Fig. 1 Transverse sections through the posterior part of an early CS12 embryo (No. 741). **a** At the level of the posterior neuropore, the notochord (*Not*) is attached to the floor plate (*FP*) of the neural plate (*NP*), which is widely open. **b** In a section approximately 80 μm more posterior to **a**, the notochord is not formed, and axially-condensed mesenchyme (*AM*) is located beneath the neural plate. (*PM* paraxial mesenchyme, bar=100 μm)

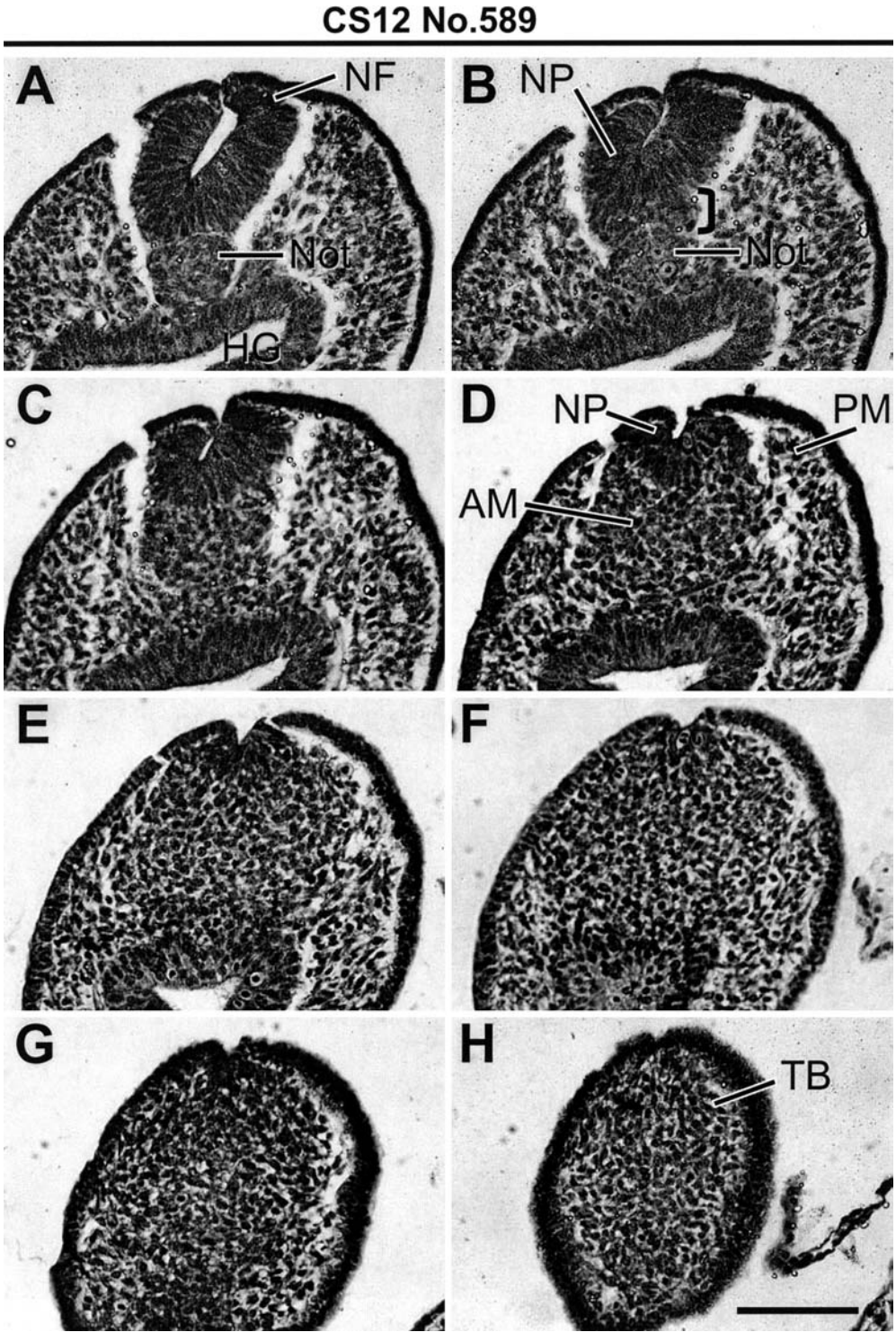

Fig. 2 Serial transverse sections of a CS12 embryo (No. 589) at approximately 20-μm intervals through the closing posterior neuropore in a rostrocaudal sequence (**a–h**). Neural folds (*NF*) come in contact with each other in **a–c**, but the neuropore is still open in **d–g**. Toward the caudal end of the posterior neuropore, the neural groove becomes gradually shallower while the dorsoventral thickness of the AM progressively increases (**b–g**). As the notochord (*Not*) is formed from the AM, the AM intervenes between the notochord and the neural plate (*NP*) (**b**, *bracket*), although the notochord is closely attached to the neural plate in section **a**. The boundary between the neuroepithelium and the surrounding mesenchyme also becomes less distinct toward the caudal end of the neural groove (**e–g**). No cavities are observed in the AM and the tail bud (*TB*) at this stage (**b–h**). (*HG* hind gut, bar=100 μm)

The cavity of the early secondary neural tube is continuous with that of the primary neural tube in the junctional region

In embryos with the posterior neuropore closing, no cavity was observed in the AM and the tail bud (Fig. 2b–h). The early stage of secondary neurulation was observed after posterior neuropore closure, as previously reported by Müller and O'Rahilly (1987). A single cavity of the newly-formed secondary neural tube was continuous with that of the primary neural tube in the junctional region (Fig. 3). The rapid enlargement of the cavity was observed at the transition of the primary and secondary neural tubes (Fig. 3e, f). No cavity was observed under the cavity of the primary neural tube as observed in chicks (Fig. 3a, b, f, g). It seemed that cavitation of the sec-

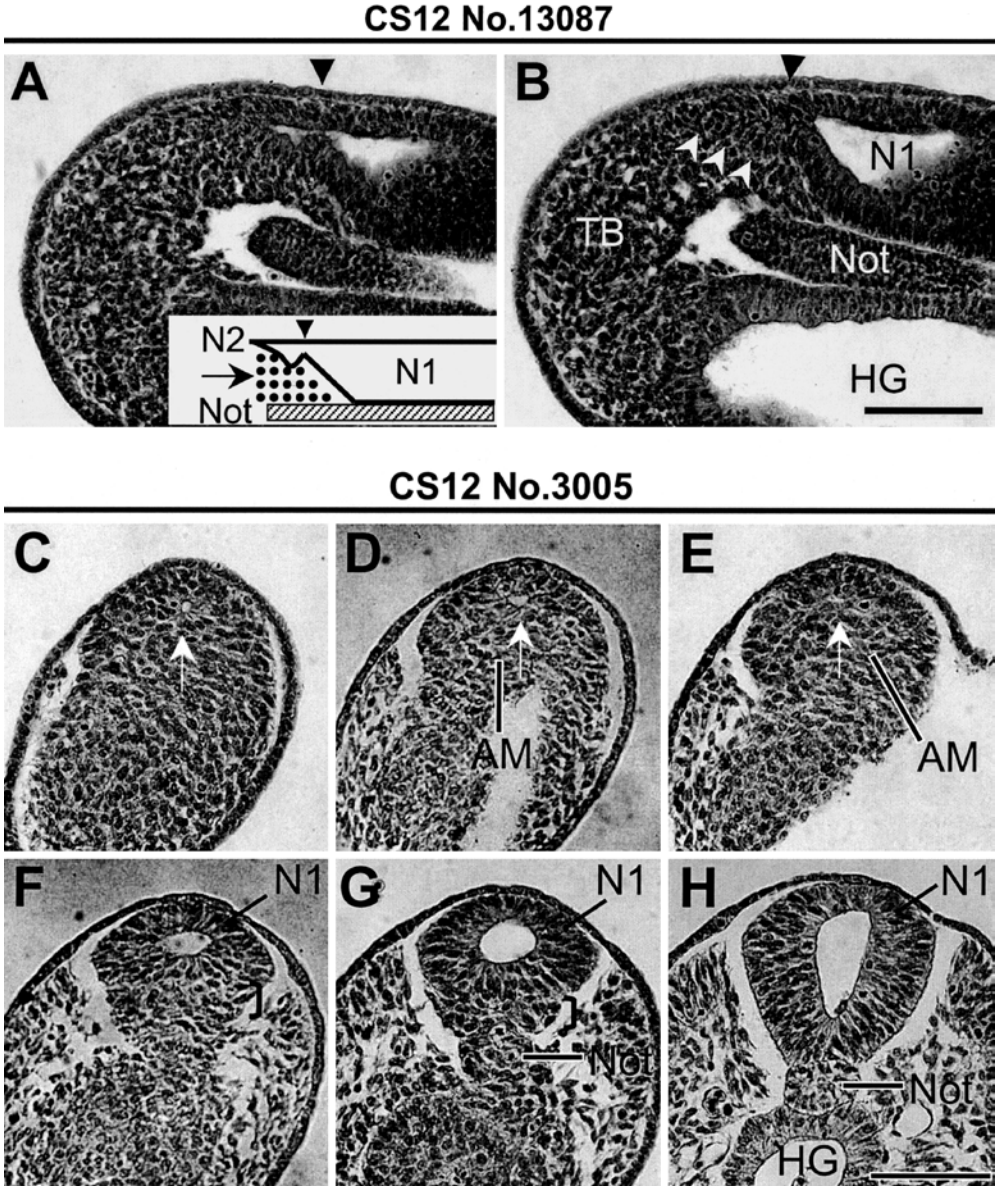

Fig. 3 Sections of the junctional region of the primary and secondary neural tubes in late CS12 embryos. **a, b** Serial sagittal sections of No. 13087 embryo. Diagrammatic representation is shown in inlet of **a**. The notochord (*Not*) is attached to the primary neural tube (*N1*) almost along its entire length. As the primary neural tube tapers caudally, the AM intervenes between the notochord and the primary neural tube (*arrow* in inlet). The cavity of the secondary neural tube (*N2*) is single and continuous with that of the primary neural tube. The junction between the primary and secondary neural tubes is indicated by a *black arrowhead*. (*White arrowheads* neural tissue of the secondary neural tube, *TB* tail bud, *HG* hind gut, bar=100 μm) **c–g** Serial transverse sections of No. 3005 embryo in a caudorostral sequence. **c–e** A small cavity of the secondary neural tube is surrounded by the radially-arranged cells in AM (*white arrows*). **f, g** In more rostral sections, the size of the cavity is enlarged, and AM intervenes between the notochord and the primary neural tube (*brackets*). Note that the rapid enlargement of the cavity is observed at the transitional zone between the primary and secondary neural tubes (**e, f**). **h** A section approximately 110 μm rostral to **g**. The notochord is attached to the primary neural tube in the most rostral section. (bar=100 μm)

ondary neural tube in the junctional region occurred as the AM cells around the presumptive cavity were rearranged radially, prior to the formation of the neural cord (compare Fig. 3a–e with Fig. 4–Fig. 7). Radial rearrangement of the AM cells, in addition to the cavitation, seemed to begin at the caudal tip of the primary neural tube (Fig. 3a, b).

Cavities of the secondary neural tube in the caudal region are frequently formed at multiple or isolated sites in the neural cord

The caudal region of the secondary neural tube predominantly differentiated from the neural cord, which is derived from the tail bud (Müller and O'Rahilly 1988) (Fig. 4–Fig. 7). In the neural cord, it appeared that cavi-

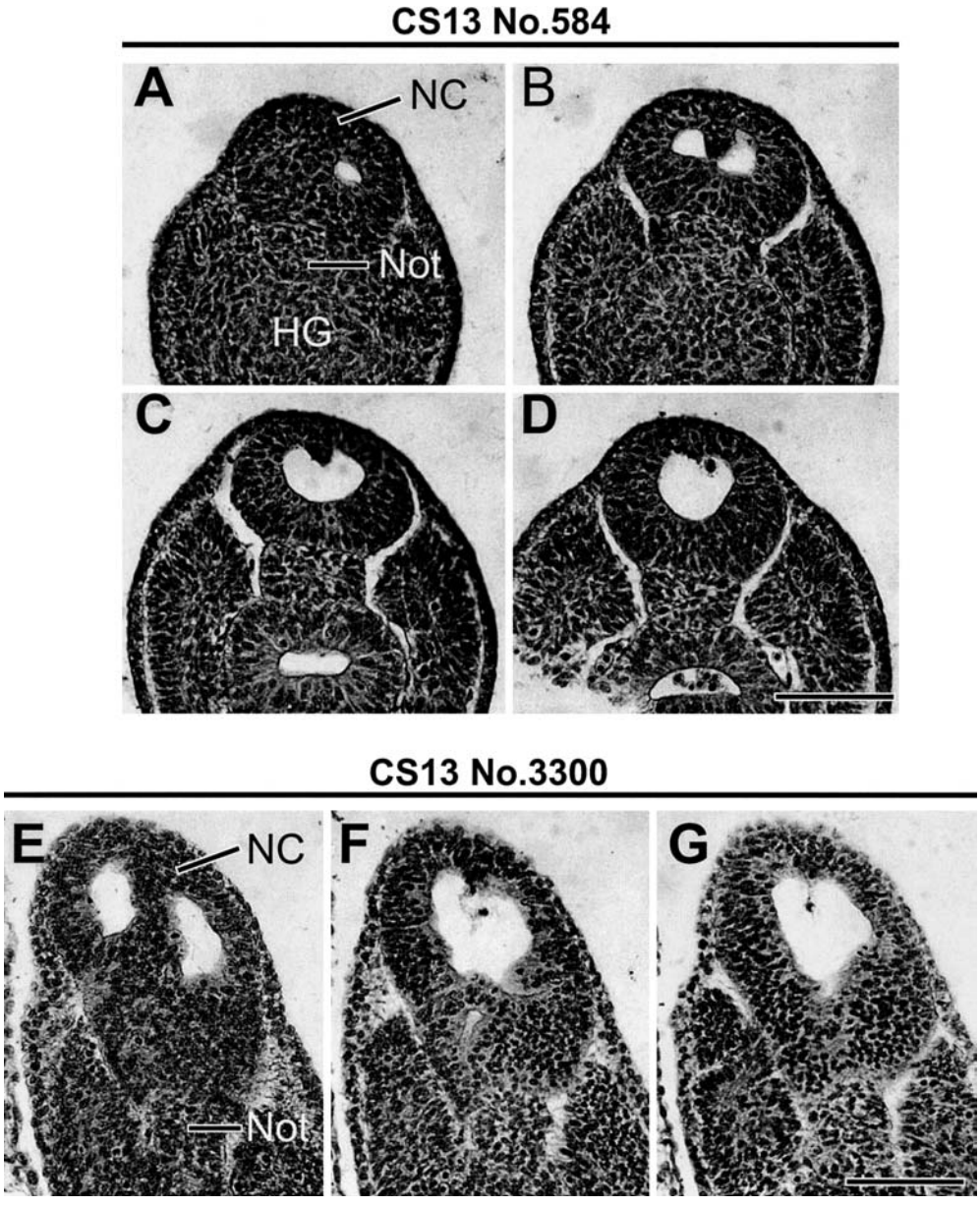

Fig. 4 Sections of the caudal region of CS13 embryos. Multiple cavities are formed in the neural cord (*NC*), which is attached to the notochord (*Not*). **a–d** Serial transverse sections of No. 584 embryo at approximately 20-μm intervals in a caudorostral sequence. The septum between the laterally-located two cavities (**b**) disappears in more rostral sections, forming in a single central cavity (**c, d**). Note that a small mass of cells protrudes from the dorsal wall of the neural tube into the central cavity (**c**). **e–g** Serial oblique sections of No. 3300 embryo at approximately 10-μm intervals in a caudorostral sequence. Mediolaterally (**e**)- and dorsoventrally (**f**)-located two cavities coalesce to form a central cavity (**g**). (*HG* hind gut, bar=100 μm)

tation was not a simple extension of the cavity of the primary neural tube as was the case in the junctional region (compare Fig. 4–Fig. 7 with Fig. 3). Contrary to the previous report by Müller and O'Rahilly (1988), multiple cavities were frequently observed in CS13 embryos (7/14; Table 1) (Fig. 4–Fig. 6). Out of seven embryos without multiple cavities, five embryos had a single continuous cavity (Fig. 7a–d) and two embryos (No. 1313, 14049) had discontinuous cavities that were aligned straight along the long axis (Fig. 7e–j). Cavitation seemed to occur off the center, not at the position of the presumptive central cavity. As the centrally-located cells disappeared, the cavities seemed to enlarge and/or coalesce with each other to form a single central cavity that became continuous with the cavity of the primary neural tube (Fig. 4–Fig. 7). Because cavitation occurs between peripheral and central cells in the chick medullary cord (Schoenwolf and Delongo 1980), our findings suggest that cavitation in the neural cord that occurs at a later stage of secondary neurulation resembles, at least in part, that in the chick medullary cord.

Other findings

A small mass of cells was found to protrude from the dorsal wall into the central cavity in the PNT of embryos with the posterior neuropore closed (No. 2431 at CS12, and No. 622, 632, and 1586 at CS13) (Fig. 7d). Although the origin and fate of these cells are not clear, they may possibly be some PNT cells that disappear as cavitation proceeds.

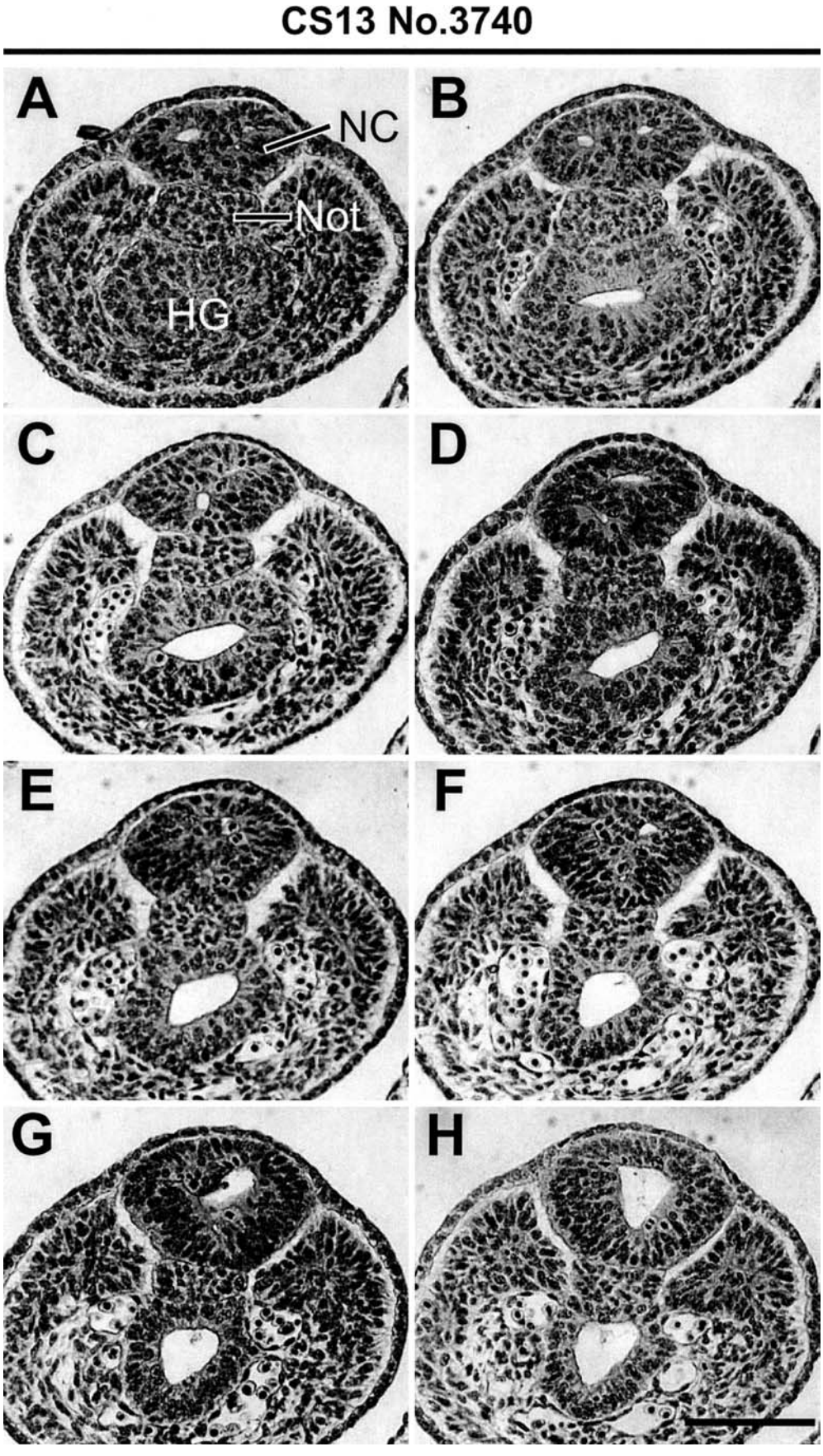

Fig. 5 Serial transverse sections of the caudal region of a CS13 embryo (No. 3740) at approximately 10-μm intervals in a caudorostral sequence (**a–h**). Mediolaterally (**a, b**)- and dorsoventrally (**c, d**)-located two cavities are observed in the neural cord (*NC*). Note that the cavities are formed off the center, which is not the position of the presumptive central cavity. The cavities seem to enlarge and coalesce with each other as the centrally-located cells disappear (**e–h**). (*Not* notochord, *HG* hind gut, bar=100 μm)

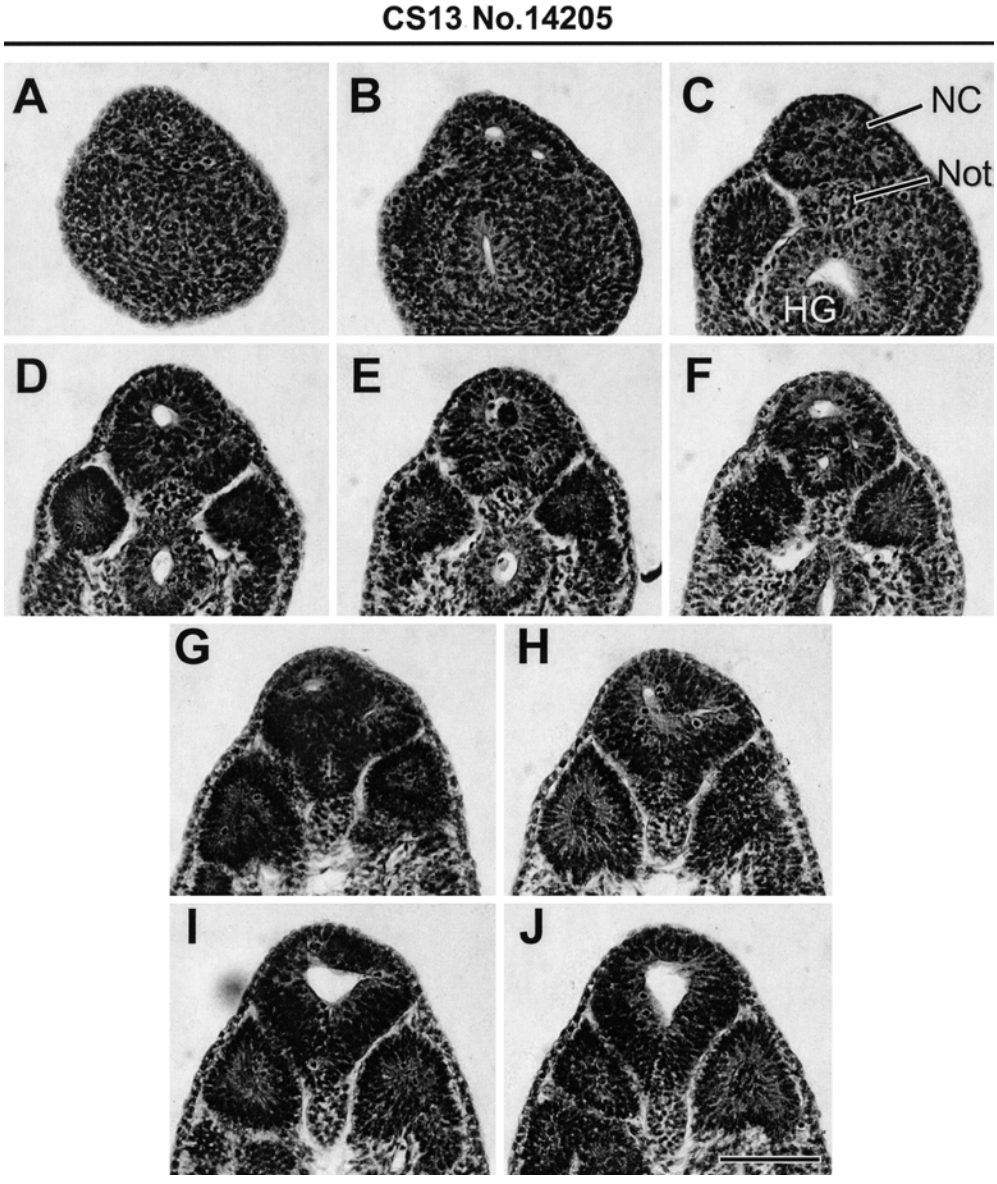

Fig. 6 Serial transverse sections of the caudal region of a CS13 embryo (No. 14205) in a caudorostral sequence (**a–j**). **a–f** Sections at approximately 30-μm intervals. Multiple cavities in the neural cord are formed intermittently along the rostrocaudal axis, resulting in apparently isolated cavities. **g–j** Serial sections from approximately 20 μm rostral to **f**. The cavities appear to coalesce with each other to form a central cavity. (*NC* neural cord, *Not* notochord, *HG* hind gut, bar=100 μm)

Discussion

PNT development in human embryos is summarized in Fig. 8. One noticeable observation is that in human embryos, the AM intervened between the notochord and the neural plate/tube in the junctional region of the primary and secondary neural tubes. The neural plate in this junctional region was in close contact with AM but not in the more rostral region of the developing PNT. This feature is similar to that in chick embryos in which the neural plate/tube is attached to the ventrally-located medullary cord in their overlapping zone. In chick embryos, lumbosacral myeloschisis can be easily induced by various experimental conditions (Hughes and Freeman 1974, Schoenwolf 1979), and they have been used as an experimental model for human myeloschisis (Rosenquist et al. 1996, Epeldegui et al. 2002). Schoenwolf (1979) suggested that the high incidence of lumbosacral myeloschisis in chicks may be related to the existence of the overlapping zone of the primary and secondary neural tubes described above. Although no cavity was observed in AM beneath the primary neural tube in human embryos, a similar relationship between the neural plate/tube and AM (medullary cord in chicks) in the junctional region supports the suitability of chick embryos for studying the pathogenetic mechanism of human myeloschisis.

As shown in the present study, the primary neural tube shifts to the secondary neural tube in the junctional region. Early cavitation was found to start at the caudal tip of the primary neural tube with radial rearrangement of AM cells, and the newly-formed cavity of the secondary neural tube was continuous with the cavity of the primary neural tube. This observation is consistent with the previous report by Müller and O'Rahilly (1987) in which 24 embryos at CS12 were examined. They demonstrated that the cavity of the secondary neural tube was contin-

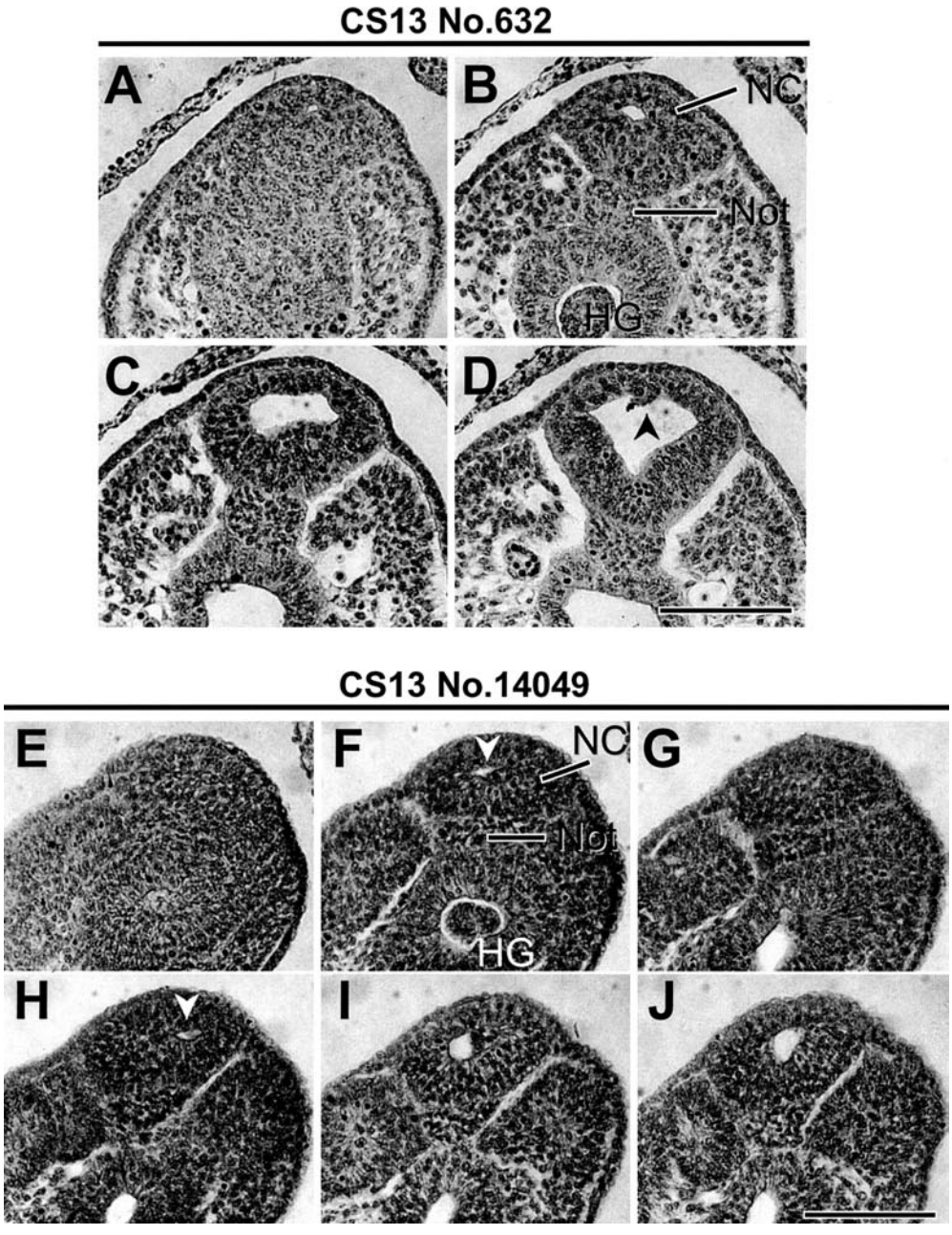

Fig. 7 Serial transverse sections of the caudal region in CS13 embryos in a caudorostral sequence. **a–d** Sections at approximately 50-μm intervals of No. 632. From caudal to rostral, a small cavity gradually enlarges and forms a central cavity. Note that a small mass of cells protrudes from the dorsal wall of the neural tube into the central cavity (**d,** *black arrowhead*). **e–j** Sections at approximately 10-μm intervals of No. 14049. An isolated cavity is present in **f, h** (*white arrowheads*). In **i**, some cells are located in the central cavity, which can be distinguished from the neuroepithelial cells of the neural tube. The cavity may eventually enlarge as the centrally-located cells disappear (**i, j**). (*NC* neural cord, *Not* notochord, *HG* hind gut, bar=100 μm)

uous with that of the primary neural tube and was surrounded by radially-arranged cells as observed in mice. Therefore, in terms of cavity formation, the transition from the primary to the secondary neural tubes and the early developmental features of the secondary neural tube in the junctional region are similar to those in mice, whereas cavitation in the neural cord in the caudal region resembles, at least in part, that in the chick as mentioned above. Thus, mouse embryos may be suitable for studying the mechanism of how the cavity of the primary neural tube connects with that of secondary neural tube.

Accumulating evidence indicates that when tail bud mesenchymal cells fail to differentiate into the paraxial mesoderm, they follow the process that leads to the formation of the secondary neural tube. Much of the evidence has been obtained by mutation studies of transcriptional factors or signaling molecules that are active in paraxial mesoderm formation, such as *Fgfr1* (Deng et al. 1997), *Wnt3a* (Yoshikawa et al. 1997), *Tbx6* (Chapman and Papaioannou 1998), and compound mutants of *Lef1* and *Tcf1* (Galceran et al. 1999). In these mutants, ectopic neural tubes were present where the paraxial mesoderm normally develops, which suggests transformation of paraxial mesoderm progenitors into neural cells. Recently, ectopic expression of *Gcm1* in the developing tail bud was shown to downregulate *Tbx6* expression and produce multiple neural tubes in the lumbosacral region (Nait-Oumesmar et al. 2002). In our present study, early cavitation, a sign of neural differentiation, was found to start at the caudal tip of the primary

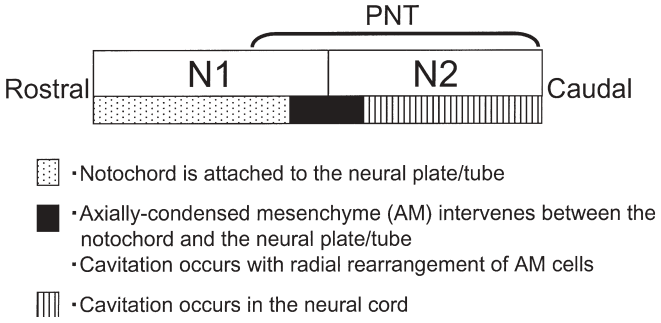

Fig. 8 A model of the PNT development in the human embryo. The posterior neural tube can be divided into three parts. In the most rostral region, the notochord is attached to the neural plate/tube. In the junctional region, AM intervenes between the notochord and the neural plate/tube, while the notochord is directly attached to the neural plate/tube in the most rostral region. Cavitation in the junctional region occurs with radial rearrangement of AM cells. A single cavity is formed in the junctional region. In contrast, the cavitation of the secondary neural tube occurs in the neural cord in the caudal region. Multiple or isolated cavities are frequently observed in the neural cord. (*PNT* posterior neural tube, *N1* primary neural tube, *N2* secondary neural tube)

neural tube in human embryos. These data suggest the possibility that some signals secreted from the caudal tip of the primary neural tube induce tail bud mesenchymal cells to differentiate into neural cells, possibly by antagonizing mesoderm-inducing signals.

While the cavitation occurs with radial rearrangement of AM cells in the junctional region, cavitation of the secondary neural tube in the more caudal region was predominantly observed in the neural cord. Multiple or isolated cavities were frequently formed, and they were not continuous with the cavity of the junctional region. Lemire (1969) found accessory cavities in the caudal neural tube in seven out of eight human embryos between CS14 and 21. Hughes and Freeman (1974) also reported multiple cavities in the same region at approximately CS14 and 16. Moreover, Bolli (1966) reported that accessory cavities were present in the caudal neural tube of human embryos of 4–31 mm in length, with a peak frequency in 8–9-mm embryos (approximately CS16). All these previous studies together with ours suggest that multiple cavities are first formed in the neural cord of human embryos. On the other hand, Müller and O'Rahilly (1987, 1988) did not find multiple cavities in most of their specimens at CS13, and argued that cavitation of the secondary neural tube in human embryos proceeds as a simple extension of the cavity of the primary neural tube. The discrepancy between their observation and ours may be partly due to different orientations of the sections examined. It seems that Müller and O'Rahilly (1988) included some tangentially-sectioned embryos, in which it is difficult to identify accessory cavities, particularly small ones. In the present study, we analyzed only transverse and oblique sections to identify cavities in the secondary neural tube. We also observed a single and continuous cavity in the early secondary neural tube, which is consistent with the findings by Müller and O'Rahilly (1987). So it is likely that their specimens represented the early stage of secondary neurulation.

The high frequency of multiple cavities in the caudal region of the PNT appears to be consistent with the following observation in children: It has been reported that even in children with normal appearance, the incidence of the canal forking of the equinal cord (including conus medullaris, ventriculus terminalis, and filum terminalis) is as high as 45% (Lendon and Emery 1970). Although multiple cavities in the caudal region of the PNT seemed to normally coalesce with one another to form a single cavity, multiple cavities may persist in some cases as separate neural cavities, leading to canal duplication.

Acknowledgements The authors wish to thank the many cooperating obstetricians and acknowledge the contribution of past and present staff of the Congenital Anomaly Research Center and the Department of Anatomy, Kyoto University, in establishing the Kyoto Collection of Human Embryos. This work was supported by grants from the Japanese Ministries of Health, Labor and Welfare, and of Education, Culture, Sports, Science and Technology.

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