Chapter 4

Establishment of the Embryonic Body

When the primitive streak is fully formed (stage 4, *Plate 9*), the first stages of gastrulation are complete and the tissues that will develop into the body are arranged around its anterior end. During the next 12 h they will be transformed into a basic embryonic axis consisting of ectoderm, including neural plate and neural crest, notochord, somites, lateral plate and intermediate mesoderm, and endoderm. Steps in these changes are illustrated in:

- Normal Tables in Appendix II.
- selected stages of whole-mounted embryos (e.g. *Plates 9, 13, 14*).
- selected stages of whole-mounted embryos seen by scanning electron microscopy (*Plates 15–59*).
- histological sections (*Plate 60 et seq.*).

The key structure in the primitive streak stages is **Hensen's node** (alternative names are primitive node or primitive knot and, more recently, chordoneural hinge by Charrier *et al.,* 1999), the anterior tip of the primitive streak, where the primitive groove ends in the primitive pit and is enclosed anteriorly and laterally by the swollen primitive folds (*Plates 9, 10*). It corresponds in many ways to the dorsal lip of the blastopore in amphibians. One of the most famous and influential experiments ever performed on an embryo was that of Spemann (1938), who grafted a piece of tissue from the dorsal lip of an amphibian blastopore into the flank of a similar embryo of the same stage of development, which resulted in a second axis forming in the host embryo. It became apparent that the graft had influenced the host tissues adjacent to it to form an embryo, and Spemann concluded that the dorsal lip of the blastopore possessed special properties which enabled it to 'organize' the other tissues around it to form an embryonic axis. The dorsal lip of the gastrula therefore became known as the 'organizer'. Subsequently, it was shown by Waddington (1932 and later, summarized by Waddington, 1952) that the same sort of activity occurred in birds. He demonstrated that if Hensen's node was extirpated, stripped free of any adherent lower layer and implanted beneath the ectoderm at the edge of the area pellucida of another embryo, it was able to induce a secondary axis consisting of neural tube, gut and somites. It soon became apparent, moreover, that the ability to induce a secondary axis was not restricted to Hensen's node, but was present also in at least the anterior third of the primitive streak (Waddington and Schmidt, 1933), although the power to bring about an induction of neural tube was found to be greater in Hensen's node than in any other part of the primitive streak (see below for neural induction). A major characteristic of Hensen's node is that it expresses the chick homeobox-containing gene, *goosecoid* (*gsc*) (Izpisúa-Belmonte *et al.,* 1993). This gene was initially derived from a *Xenopus* blastopore dorsal lip cDNA library, and is therefore considered as a marker for the organizer region. Its presence in Hensen's node emphasizes the analogous organizer roles of the amphibian dorsal lip and chick Hensen's node. An important finding of Izpisúa-Belmonte *et al.* was that *goosecoid* is first expressed in a restricted region of middle layer cells in Koller's sickle, implying that these cells are the precursors of Hensen's node.

By the time the primitive streak is fully formed, the main presumptive areas lie clustered around its anterior end and it now expresses a range of markers. For example, in addition to *goosecoid,* it expresses HNF3β, *Otx2,* ADMP, FGF8 and *chordin*, though some (e.g. *goosecoid, Otx2,* ADMP and FGF8) are no longer expressed after stage 4. At later stages Hensen's node expresses other genes, such as *sonic hedgehog*, *noggin* and *nodal.*

Detailed fate maps for stages 3–4 have been published by Selleck and Stern (1991) and Garcia-Martinez *et al.* (1993). Selleck and Stern's (1991) fate maps of Hensen's node were based on DiI labelling of

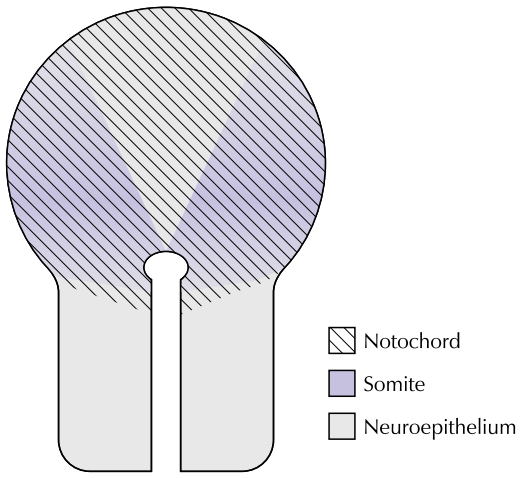
The Atlas of Chick Development. http://dx.doi.org/10.1016/B978-0-12-384951-9.00004-6

small groups of cells and lysine–rhodamine–dextran labelling of single cells. They concluded that the node consists of several clearly defined regions (*Text-Figure 17*), each possessing its own presumptive fate. By stage 4 all the definitive endoderm has ingressed (*Text-Figure 11*) and the node contains only the mesoderm and that part of the presumptive neural plate that will form the floor plate. By stage 5 the presumptive notochord region has become narrower in the midline, since many cells have ingressed to form the head process and the notochord.

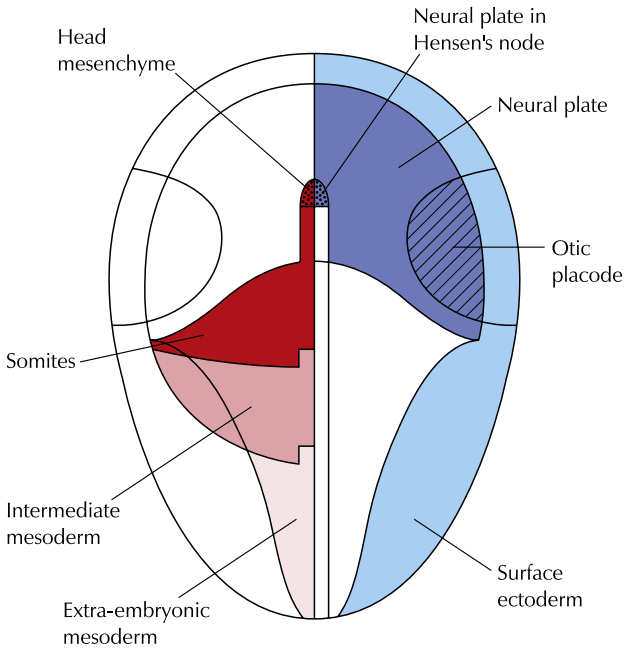
The lateral folds of Hensen's node give rise to the medial halves of the somites, whilst a separate region of primitive folds posterior to the node forms the lateral half of each somite. The fate map of Selleck and Stern was limited to the primitive streak, but Garcia-Martinez *et al.* (1993) published a map of the entire area pellucida ectoderm at stage 4, based on the results of chick–quail transplantation experiments (*Text-Figure 18*).

Other maps include:

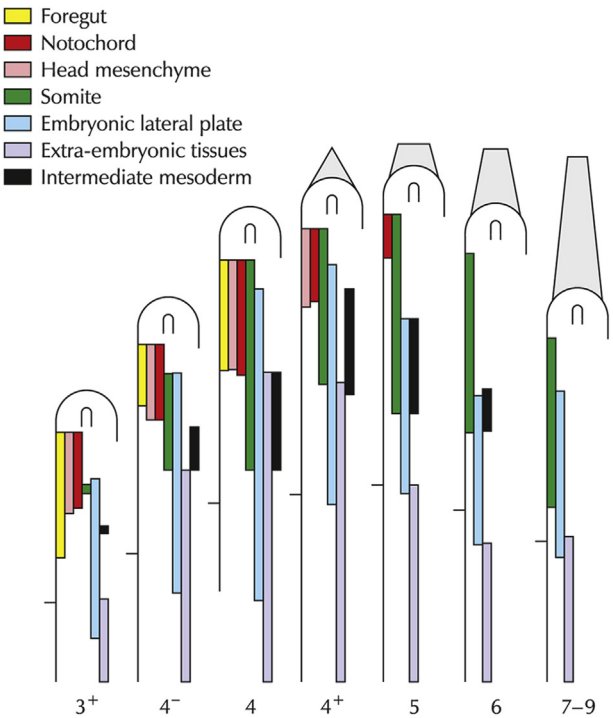
- the location of the presumptive heart rudiments in the primitive streak at stages 3–4 (Garcia-Martinez and Schoenwolf, 1993);
- the early neural plate (Bortier and Vakaet, 1992);
- the primitive streak (Psychoyos and Stern, 1996) (*Text-Figure 19*) and the mesoderm (Sawada and Aoyama, 1999 (*Text-Figure 20*); Lopez-Sanchez *et al.* (2001);
- a spinal cord fate map at regression stages (Catala *et al.*, 1996);
- tail bud (Le Douarin, 2001) (*Text-Figure 21*).



Text-Figure 17. *Fate map of the tissues in Hensen's node at the fully grown primitive streak stage (stage 4-). (After Selleck and Stern (1991), with permission of The Company of Biologists Ltd.)*

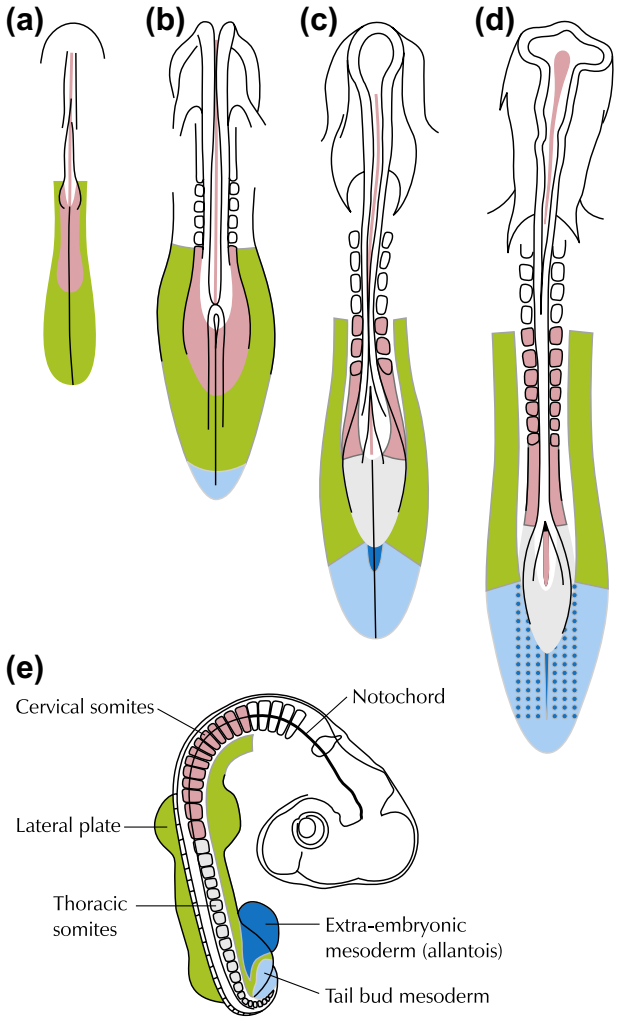


Text-Figure 18. *Fate map of the ectoderm and primitive streak at about stage 4 to show the layout of the presumptive areas. (After Garcia-Martinez et al., 1993; reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.).*



Text-Figure 19. *Fate maps of the primitive streak, summarising the relative antero-posterior positions of the precursors of the main tissue types along the axis. The prospective cell types are shown side-by-side for clarity, but this should not be taken to imply any defined medio-lateral position in the diagram. (After Psychoyos and Stern (1996), which should be consulted for further details. Reprinted with permission of The Company of Biologists Ltd.)*

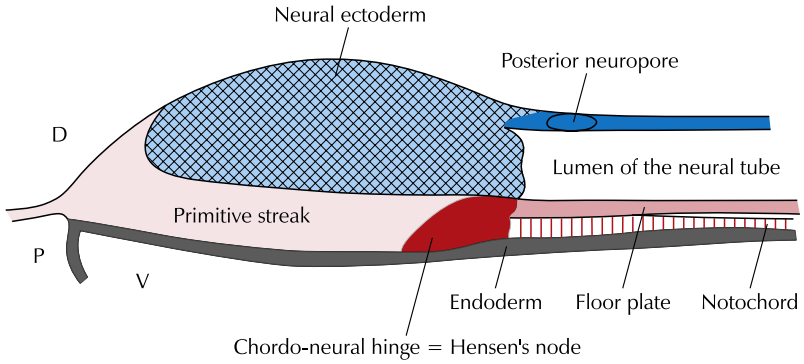
Until about stage 4– most of the mesoderm cells are as yet uncommitted to their individual fates, but the presumptive notochord cells, situated in Hensen's node and the anterior end of the primitive streak, appear to be totally committed to form notochord. The evidence comes from a wealth of experiments in which tissues are transplanted from one region to another of the embryo (e.g. Selleck and Stern, 1992; Garcia-Martinez and Schoenwolf, 1993). The cells in most of the grafts were able to ignore their presumptive fate and develop according to their new position, but the presumptive notochord cells appeared to be able to form only notochord.



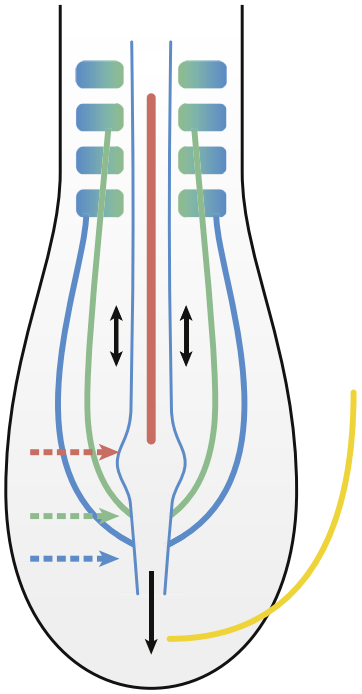
Text-Figure 20. *Fate maps of the mesodermal cells of the primitive streak between stages 6 and 20. (a) Stage 6; (b) Stage 8; (c) Stage 9; (d) Stage 10; (e) Stage 20. (After Sawada and Aoyama (1999), with permission of UBC Press.)*

HEAD PROCESS AND REGRESSION

In the fully formed primitive streak (stage 4) ingression has ceased and the presumptive areas are grouped around its cranial end (see above). As regression occurs, they become rearranged to lie along the future body axis (*Text-Figure 22*). The presumptive notochord cells in Hensen's node and the anterior primitive streak give rise to the **notochord** proper, a rod of cells running along the length of the embryo. The most anterior part of this rod is the **head process** (*Plate 13*); it is formed by the anteriorward migration of cells from Hensen's node, whereas the trunk notochord is formed by the caudalward movement of cells during the process of regression. The first appearance of the head process is as a small dark triangle anterior to Hensen's node at stage 4 (Appendix II). The onset of regression initiates the start of the disappearance of the primitive streak. Differentiation of the tissues takes place gradually from anterior to posterior as regression occurs, so that the development of the anterior tissues is always in advance of the posterior ones. Wnt5a appears to promote the migration of cells from the posterior end of the primitive streak, which will form lateral plate mesoderm, whilst Wnt3a opposes this action at the anterior end of the primitive streak in the cells that will become somites (Sweetman *et al.*, 2008).



Text-Figure 21. *Fate map of the tail bud of the quail at a stage of about 26 somites (corresponding to about stages 15–16 of the chick). D = dorsal; P = posterior; V = ventral. (From Le Douarin (2001), with permission of UBC Press.)*



Text-Figure 22. *Cell migration patterns during regression. Anterior primitive streak cells migrate to form the medial part of each somite (pale green), whilst posterior streak cells migrate out to become the lateral part of each somite (blue). The yellow line indicates the origin of the cells that migrate into the extra-embryonic area to become blood islands. The regression of Hensen's node is indicated by the black arrow, the trail of notochord that has been left by this process is shown in red. (After Chuai and Weijer (2009), with permission of Elsevier.)*

The **head process** is more clearly visible at stage 5 (*Plates 13, 14* and Appendix II). It must be distinguished from the **prechordal mesoderm**, which lies anterior to it and does not form notochord. Like the head process it is derived from Hensen's node, but there is evidence to suggest that TGFPs from the underlying endoderm repress the notochord characteristics (Vesque *et al.,* 2000). The loose mesenchyme which later appears on either side of the head process is known as the **head mesenchyme**, and is critical in the formation of the head muscles and skull (see Chapter 10).

A major event in development is the conversion of the flat, almost 2-dimensional, embryo into a rounded, 3-dimensional one. The first visible event is the appearance of the **headfold**. This is a tuck in the ectoderm which pushes beneath the head process and prechordal mesoderm and lifts the head above the area pellucida. It begins to form at about stage 6 and has become clearly visible by stage 7 (see *Text-Figures 50 and 52*). Varner *et al.* (2010) provided experimental and computational evidence that shape changes in the neural plate, active cell shape changes at the anterior border of the neural plate, as well as shape changes in the non-neural plate ectoderm all played a role in the formation of the headfold.

There has been much confusion over the term **regression**. Essentially, it describes the gradual disappearance of the primitive streak, which shortens from anterior to posterior as the presumptive mesodermal tissues ingress and leave it, but it is also used to describe a series of morphogenetic movements that take place at this time. The main feature is that most of the presumptive notochord cells leave Hensen's node and become deposited as a trail of notochord cells down the body (*Text-Figure 19*). There is recent evidence to indicate that the floor plate of the neural tube and a longitudinal strip of endoderm are deposited at the same time (Catala *et al.,* 1996; Le Douarin, 2001) (*Text-Figure 21*). The individual cells of the notochord become greatly elongated by the stretching and extension of the area pellucida, which occurs at this time. It is, however, not only the presumptive notochord and floor plate tissues that are affected by the regression movements. Other presumptive tissues that are still within the primitive streak (*Text-Figures 19 and 20*) also become extended. The morphogenetic movements involved in node regression also take place to a lesser extent in the regions lateral to the primitive streak, so that all the presumptive areas become elongated along the body axis.

THE TAILBUD

By about stage 11 the remnants of Hensen's node and the primitive streak lie at the posterior end of the area pellucida, and by stages 13–14 have become a clump of cells, the *tailbud,* which lengthens until about stage 25 (4–5 days) (Griffith *et al.*, 1992). The tailbud becomes undercut by the *tail fold* (*Text-Figure 51*). Examination of the tail bud at these stages show a mass of mesoderm enveloped by ectoderm (*Plates 31b, 72*). This mesoderm is not a homogeneous collection of cells. Using grafts from transgenic chick embryos, McGrew *et al.* (2008) showed that a population of mesodermal progenitor cells is present in the tailbud, the descendants of which contribute to the somitic mesoderm, the notochord, and the neural tube. Their destination seems to depend on their position within the tailbud.

The tailgut, which is continuous with the hindgut, also forms from the tailbud mesenchyme (Griffith *et al.*, 1992).

With the formation of the tailfold the remnant of the primitive streak and Hensen's node becomes shifted to the ventral side of the tailbud where it forms a thickened ridge, the *ventral ectodermal ridge.* It is thought that the cells probably continue to ingress (Ohta *et al.*, 2007) since there is no basal lamina beneath the ventral epidermal ridge to enable it to remain as an epithelium, and this may be due, at least in part, to the inhibition of BMP signaling, which is normally associated with epithelial/mesenchymal transformations as the cells pass through the primitive streak (Ohta *et al.*, 2010). Ingression ceases at about stages 21 to 24 (Ohta *et al.*, 2007).

There has recently been much consideration whether the tailbud is a secondary organizer (i.e. a signaling centre) comparable with the primary organizer (i.e. Hensen's node) or the apical epidermal ridge of the limb bud (Griffith and Sanders, 1991). Knezevic *et al.* (1998) demonstrated by means of tail grafting experiments that the tip of the tailbud has residual organizer-like properties, being able to recruit cells to overcome their presumptive fate and become neural. Ohta *et al.* (2007, 2010) proposed that the ventral ectodermal ridge is the signaling centre for the tailbud and that it regulates that part of the ectoderm that is not, at least initially, underlain by a basal lamina, and so regulates the tail length. They suggested that the expression of *Nog* genes adjacent to the ventral ectodermal ridge inhibits Bmp signaling in the tailbud, which in turn suppresses the ridge and leaves to a formation of basal lamina, and so regulates the tail length. They concluded that the expression of *Nog* genes adjacent to the ventral ectodermal ridge inhibits Bmp signaling in the tailbud, which in turn suppresses the ridge and leads to formation of the basal lamina.

The structures that develop from the tailbud are continuous with those that form in the main part of the body, though some of the developmental processes differ. The tail neural tube does not form by a rolling up of the ectoderm into a tube, but by cavitation of a solid mass of mesenchyme (secondary neurulation). Similarly, although the foregut and hindgut both form by folding, the tail gut develops by cavitation of the mesenchyme. By the time the tailbud starts to regress the neural tube and notochord have reached the tip of the tail, but the somites never do so; the terminal tailbud mesoderm never becoming segmented (Sanders *et al.*, 1986; Bellairs and Sanders, 1986). Eventually, the tail gut and the tail somites are overcome by cell death and disappear. According to Le Douarin (2001) cell death in the tail is due to the absence of adjacent structures secreting *sonic hedgehog* protein, and the tissues can be rescued if *sonic hedgehog-secreting* tissues are implanted. Fate maps of the tail bud are illustrated in *Text-Figures 21 and 24*.

NEURAL INDUCTION

The presumptive neural plate at stage 3+ to 4− is an extensive area of epiblast lying over and around Hensen's node (*Text-Figures 17 and 18*), anteriorly, posteriorly and laterally. The evidence comes from experiments of many authors in which small areas of the ectoderm have been labelled and traced to their destinations (e.g. Pasteels, 1937; Rosenquist, 1966; Garcia-Martinez *et el.* 1993). According to Garcia-Martinez *et al.* it extends 0.5 mm posterior to Hensen's node. Before the primitive streak has formed the presumptive neural plate region lies at the posterior end of the area pellucida either within Koller's sickle (*Text-Figure 9*).

By the time the primitive streak is fully formed the ability of the ectoderm to form neural plate extends beyond the presumptive neural plate region. There is ample evidence that if a small piece of ectoderm, together with its underlying mesoderm, is taken from almost anywhere in the anterior half of the area pellucida at stage 3 and isolated (even on the chorioallantoic membrane; see page 129), it can give rise to neural tissue.The underlying cells are necessary for this to happen, so we say that they induce the neural tissue.

For many years after the organizer region was discovered in amphibians, attempts were made to identify the supposed chemical nature of a signalling molecule, but it was not until about 70 years later that molecular biological techniques began to make possible an understanding of a very complex process involving a cascade of interacting molecular events. The earliest signals appear to originate in the hypoblast (reviewed by Streit and Stern, 1999; Wilson *et al.*, 2009). A body of information began to be acquired about the interacting genes and their products in early vertebrate embryos. The sequencing of the entire chick genome opened up a new range of molecular markers (especially the definitive neural markers, *SOX3,* and *ERI*) in stage 3 embryos. It had long been suspected that the hypoblast was the source of neural induction, but Albazerchi and Stern (2007) and Bertocchini and Stern (2008) found that although the hypoblast could indeed induce a group of markers (especially the definitive neural markers, *SOX* and ER1) in stage 3 embryos they were transitory in nature, and that there was no real evidence that they were responsible

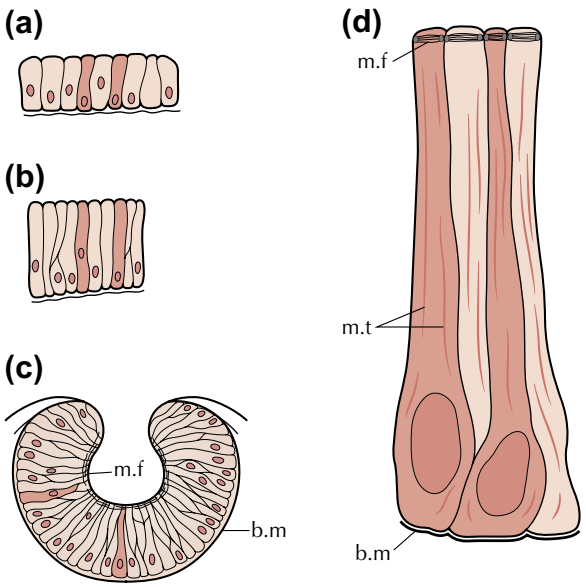
for neurulation. By contrast, there is considerable evidence that FGF signalling plays a major role in neural induction (Albazerchi and Stern, 2007; Karabagli *et al.*, 2002). The problem has proved to be a highly complex one, not least because of the difficulty of identifying the first critical steps in the process and deciphering the important reactions occurring both within the inducing tissue and the induced. For further clear reviews see Wittler and Kessel (2004) and Stern (2005). The epiblast loses its ability to respond to induction between stages 4 and 4+ (Darnell *et al.,* 1995), the three main signalling molecules, FGF, Wnt, BMP apparently playing significant roles. FGF does not act directly but has an essential role (as an antagonist for BMP), and can induce the transcription factor SOX2, which is expressed throughout the neural plate. The region where BMP4 and two of its antagonists, *chordin* and *noggin*, are active appears to be the boundary between neural and non-neural ectoderm. Streit and Stern suggested that the main functions of *noggin* at this stage may be in somite formation, by protecting the prospective somite cells from the inhibitory actions of BMPs, whilst *chordin* is primarily involved in axis formation. Two markers, *ERNI* (early response gene for neural induction) and *Sox3* (a similar early response gene), are induced by Hensen's node (as well as other neural inducters such as head process mesoderm), and each may, therefore, be used as an early marker for neuralising activity. Streit and Stern (1999) found that when cells from the middle layer of Koller's sickle were grafted into the area opaca of a primitive streak stage embryo, which is a region that would not normally form neural tissue, both *ERNI* and *Sox3* were induced. Markers for later development of the neural tissue were, however, not induced unless epiblast from the centre of the area pellucida was also included. These authors, therefore, suggested that neural induction is initiated before the start of gastrulation by signals (FGF signals) from a small population of organizer precursor cells in Koller's sickle, and that when these cells spread anteriorly and make contact with a second precursor population centre (in the middle of the area pellucida epiblast), the two groups of cells interact with one another to produce a functional organizer; this then leads to a cascade of reactions, including the sensitisation of the epiblast to the BMP antagonists. Interestingly, transplantation experiments indicate that the inducing ability is restricted to the medial region of Hensen's node, and is not present in the deep portion of the posterior-lateral part (Storey *et al.,* 1995).

By the time that the neural tube has formed it already possesses a dorso-ventral polarity (see below).

FORMATION OF THE NEURAL PLATE AND NEURAL TUBE

At the end of neural induction the embryo possesses a thickened region, the neural plate, which subsequently rolls up to form the neural tube. The individual cells of the neural plate become narrower at the apical surface; evidence from transmission electron microscopy suggests that this is due, at least in part, to the contraction of microfilaments (see *Text-Figure 23*) which appear to act like purse strings drawing the apices together. At about the same time the neural plate cells elongate, an event which is associated with a reorientation of the microtubules.

Initially there is no sharp morphological border between the cells of the neural plate and the adjacent ectoderm, the height of the epithelium changing gradually in the transitional zone. If this transitional zone is removed, the neural plate is unable to form neural folds (*Plates 10 and 13)* and remains flat (Moury and Schoenwolf, 1995). The traditional view is that the neural plate is induced from Hensen's node, even though some neural plate formation can take place in its absence. The inducing ability of the various subpopulations of the node was investigated by Storey *et al.* (1995). After grafting carefully selected small populations of cells into an extra-embryonic



Text-Figure 23. *A: Transverse section through ectoderm prior to neurulation. B: Cells have become elongated. C: Microfilaments have contracted at the apical surfaces of the cells resulting in the rolling up of the neural plate. D: Enlargement of part of C. To show the nuclei at the basal side of the neural tube, the microtubules which play a role in the elongation of the cells, and the microfilaments. bm: basal membrane; mf: microfilaments; mt: microtubules.*

site beneath ectoderm that would not normally form neural plate, they concluded that the neural inducing ability was associated not only with specific regions of Hensen's node, but also with certain types of cells.

After the neural plate has formed, the morphogenetic movements of **regression** lead to a narrowing and posterior extension of the trunk region, and about this time there is a widening of the brain area. These regression movements are accompanied by dramatic changes in the shapes and arrangements of the cells, the epidermal cells becoming flatter and the neural cells deeper. The notochord is attached to the floor plate (the floor of the neural plate) (*Plate 71)* and acts as a hinge about which the neural plate folds and bends to form a tube (Schoenwolf and Smith, 1990). The floor plate expresses HNF3β and is probably induced by the notochord, since grafts of notochord can induce an ectopic floor plate which expresses HNF3β (Ruiz I Altaba *et al.,* 1995). According to Catala *et al.* (1995), the notochord and floor plate express so many genes in common that they should be considered as the same tissue.

Theories about the possible mechanisms involved in the process of neural folding are discussed by Moury and Schoenwolf (1995) and by Lawson and Schoenwolf (2001), who emphasise that neurulation, especially neural plate formation, is a multifactorial process resulting from forces both intrinsic and extrinsic to the neural plate. Changes in the tenascin component of the cells are associated with changes in cell shape, whereas changes in the cell membranes are involved in the increasing adhesion of the cells to one another.

Fusion of the neural folds begins at about stage 8 at the level of the midbrain, and is rapidly followed by fusion throughout the entire brain and the anterior regions of the trunk. The final point of fusion in the brain is the anterior neuropore (*Plate 14),* which is obliterated at about stage 12. The neural tube closes progressively down the trunk until by about stage 9 or 10 the process is almost complete and an opening remains only at the posterior end, the sinus rhomboidalis or the neural crest, which by stage 13 has become the posterior neuropore. As the neural folds fuse, the cells of the neural crest (see below) come to lie at its dorsal side. The ectoderm that is not enclosed within the neural tube subsequently becomes the epidermis.

The dorsoventral patterning of the neural tube is brought about by the interaction of at least two major gene products, ventralization by *sonic hedgehog* protein from the notochord, and the dorsalizing influence results from BMPs probably secreted by the ectoderm (Patten and Placzek, 2002).

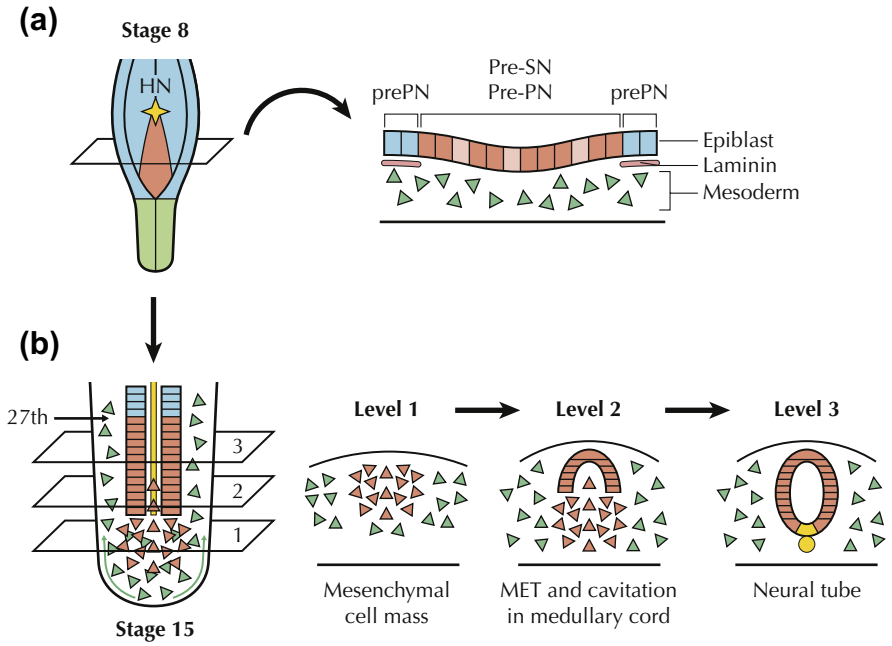
The way that the neural tube forms in the tail bud (see page 33) is designated **secondary neurulation** to distinguish it from the rolling up of the neural plate described above, which is sometimes called **primary neurulation.** In secondary neurulation, mesenchyme cells on the dorsal side of the tail bud become condensed into an epithelial-like rod, which then separates off from the remaining tissues and becomes hollowed out. This mesenchyme lies in the epiblast caudo-medially to Hensen's node at stage 8, but becomes drawn further posteriorly into the tailbud during further development) (*Text-Figure 24*). A region of overlap occurs where the so-called **primary neural tube** of the trunk gives way to the secondary neural tube of the tail (*Plate 87*). The secondary neural tube forms the lumbo-sacral and coccygeal region of the spinal cord (Griffith *et al.*, 1992).

Transverse sections through the early neural tube of the trunk region show that it possesses thick walls (*Text-Figure 25; Plates 17, 19*); these will give rise to both the neurones and glia. The dorsal and ventral regions of the tube, the roof plate and floor plate, respectively, become narrower than the lateral walls with wedge-shaped cells, and form neuroglia only.

REGIONALIZATION OF THE NEURAL TISSUE

We have seen that in addition to the cells forming the notochord and head process, several other types of cells originate in Hensen's node. These are the **definitive endoderm,** which gives rise to the gut, the **mesendoderm** cells, which pass anteriorly to lie beneath the anterior-most end of the node and contribute to the gut and prechordal plate mesoderm, and the lateral cells, which contribute to the somites. Initially, it is difficult to identify the types of cells as they leave the node since, morphologically, they are indistinguishable from one another. For this reason they are often collectively, but confusingly, called mesendoderm cells. The ability of Hensen's node to induce neural tissue is lost when the mesendoderm cells have left it (Storey *et al.,* 1992).

Markers for the forebrain are *GANF*, *CNOT1* or *Nkx2.1*, which are normally expressed in the neural plate as it forms (Boettger *et al.,* 2001), though they can be induced experimentally by grafts of the mesendoderm. Grafts taken from the region of



Text-Figure 24. *Fate maps of precursors of secondary neurulation.* ***Stage 8:*** *The ectoderm (red) posterior to Hensen's node gives rise to secondary neural tissue (SN); the primary neural tissue (PN) is shown in light blue. The primitive streak (green) gives rise exclusively to mesoderm.* ***Stage 15:*** *Cells that have ingressed from the pre-SN region form a mesenchymal cell mass at the posterior tip of the neural tube. These cells progressively undergo epithelialisation. (From Shimokita and Takahashi (2011); reproduced with permission of John Wiley and Sons.)*

the primitive streak just posterior to Hensen's node are still able to induce neural plate, though not forebrain. It appears therefore that the forebrain is not established at the time of neural induction, but is probably induced at the anterior end of the neural plate when it becomes underlain by the underlying mesendoderm.

Foley *et al.* (2000) put forward a model to explain forebrain patterning. They found that the migrating hypoblast induced the expression of early forebrain markers (*Sox3* and *Otx2*) but was unable to induce forebrain. They suggested that a role of the hypoblast was to direct the cell movements in the overlying epiblast so that the prospective forebrain was removed from the caudalizing effects of the node.

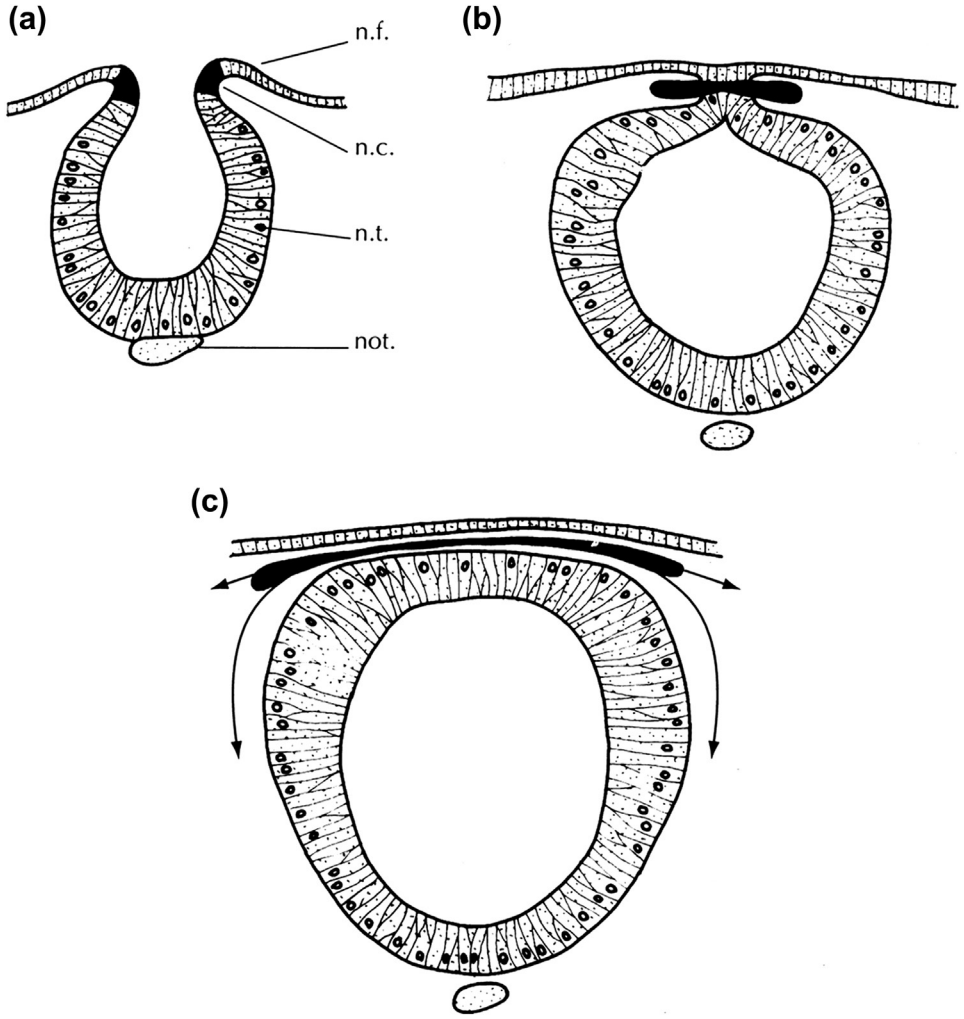
There are a large number of other genes involved in regionalization of the neural tube. For example, the homeobox gene *cDix* is restricted to the presumptive ventral forebrain region of the neural plate that ultimately gives rise to the hypothalamus and adenohypophysis (Borghjid and Siddiqui, 2000). By the time that the neural tube has formed it already possesses a dorso-ventral polarity. *Sonic hedgehog* secreted by the notochord and floor plate is important in specifying ventral telencephalic cells (Gunhaga *et al.,* 2000). The response of the neural cells to *sonic hedgehog* can be altered *in vitro* by BMP signals causing cells that would have become ventral to change into dorsal-type cells (Liem *et al.,* 2000).

FORMATION OF THE NOTOCHORD AND SOMITES

The notochord and somites are structures that are found only in the early embryo and are not present at later stages.

The **notochord** (*Plate 19, 20*) plays a critical role in development. As we have seen above, it is derived from Hensen's node and is formed as regression takes place. It occupies a central position running down the length of the body and during its early stages is closely adherent to both the floor plate of the neural tube and to the underlying endoderm. The notochord is important in the dorso-ventral patterning of the neural tube. We have seen above that the notochord expresses *sonic hedgehog* protein, which has a ventralizing effect on the neural tube. But it also has a ventralizing effect on the somites. Subsequently, the neural tube and notochord become encased in the vertebrae (see Chapter 10), and the remnants of the notochord survive as the nucleus pulposus in the intervertebral discs.

The *somites* (*Plates 21, 22*) are paired blocks of mesoderm that form in a row on either side of the



Text-Figure 25. *Sections through the neural tube at three consecutive stages to illustrate the origin of the neural crest. Arrows show the paths taken by the migrating neural crest cells. n.c.: neural crest; n.f.: neural fold; n.t.neural tube; not: notochord.*

notochord. They are the first segmented structures to develop in the embryo, and their layout influences that of all the other segmental structures that form subsequently; these are the vertebrae and ribs, the cranial and spinal nerves, the vertebral arteries and the skeletal muscles and ligaments.

The mesoderm that will give rise to the somites lies around the anterior end of the primitive streak at about stages 3–4 (*Text-Figures 18, 19*), including the lateral edges of Hensen's node, and probably starts to ingress soon afterwards. Cells pass into and through the primitive streak and then migrate laterally, passing between the ectoderm and the endoderm, usually keeping to the same side of the embryo, although occasionally individual cells may enter the primitive streak on one side and then leave on the contra-lateral side. Some of the ingressing cells become lateral plate mesoderm, others intermediate mesoderm and the remainder (nearest the neural plate) give rise to the segmental plate (sometimes called pre-segmental plate or even unsegmented paraxial mesoderm) mesoderm (*Plates 8, 17, 62*) from which the somites will form. The term paraxial mesoderm is sometimes applied as a joint description of the somites and segmental plate. The fate of any cell is determined (committed) soon after leaving the primitive streak and appears to be brought about by the relative concentration of several signals. There is a high concentration of TGFβ-like molecules, especially BMP4, in the lateral plate. (This region corresponds to the ventral region of amphibian embryos and so the effect of BMP4 is said to be a ventralizing one.) The node, meanwhile, produces *noggin* protein, which is an antagonist of BMP4 and so plays a role in preventing the pre-somitic mesoderm from developing into lateral plate (Streit and Stern, 1999). The interplay of these and other signals is discussed by Pourquié (2001). Aulehla and Pourquié (2008) suggest that oscillations may also be important in the development of limb outgrowth and neural progenitor maintenance, though they are

careful to point out that no underlying core oscillator has yet been identified.

As the cells leave the primitive streak, two major morphogenetic movements are occurring in the mesoderm. These are the medio-lateral migration of the newly ingressed cells and the antero-posterior movements associated with regression, and it seems likely that these two streams of migrating cells interact with one another (discussed by Ooi *et al.,* 1986).

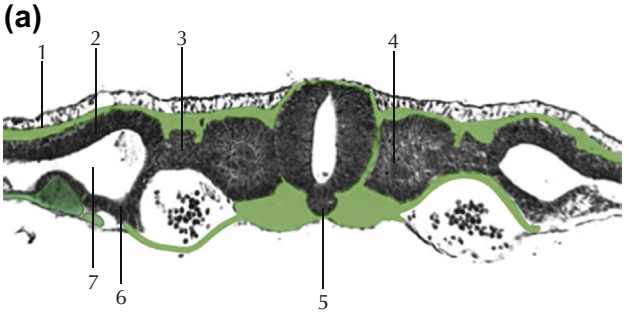
The first pair of somites appears at about stage 7, and succeeding pairs are laid down sequentially further and further posteriorly (*Plate 60*). The cells that will become somitic mesoderm enter the segmental plate as they leave the primitive streak. The left and right segmental plates lie on either side of the neural tube (*Plate 72*), and each consists of a strip of condensed mesoderm. They are easy to dissect from other tissues, especially if the embryo is treated for about 15 min with 1% trypsin in calcium- and magnesium-free saline. The cells at the posterior end of the segmental plate are mesenchymal, whereas those at its anterior end are arranged as an epithelial ball. Groups of cells at the anterior end of the two segmental plates become separated off simultaneously to form the left and the right of a pair of somites. Each newly formed somite is a ball of columnar epithelium, the walls consisting of a single layer of cells (*Plate 22*) surrounding a cavity, the **somatocoele**, which contains mesenchyme cells. Iimura *et al.* (2007) have suggested that the lateral part of each somite is formed from cells that ingress during the process of regression.

A new pair of somites forms from the anterior end of each segmental plate about every 90–100 min, but meanwhile a steady stream of mesenchyme cells is being added to the posterior end of the segmental plate. As a result the segmental plate remains visible, although there is a continual turnover of the individual cells which constitute its population. Between stages 10 and 18 it possesses the equivalent of about 10 pairs of somites (Packard and Meier, 1983). Once the cells have entered the segmental plate they are committed to becoming part of a somite. Differentiation into somites takes place gradually along the segmental plate as the original mesenchyme cells become arranged into an epithelium and, in appropriate preparations, it is possible to see part-formed somites (named **somitomeres** by Meier (1979)). Although best seen by scanning electron microscopy, the somitomeres are also visible in segmental plates that have been dissected from the embryo and viewed by light microscopy. The order in which the somites form establishes the order in which they lie in the body, the first formed giving rise to anterior structures, such as thoracic vertebrae and head muscles, whilst the somites formed later develop into more posterior structures.

Two genes that have been implicated in initiating epithelialization of the somites are *paraxis* (Sosic *et al.,* 1997) and *Epha-4* (Schmidt *et al.,* 2001), the latter gene, at least, being probably induced by signals from the adjacent ectoderm. More recently, mathematical modelling has been applied to the problem of how cells become rearranged during epithelialisation (Kulesa *et al.,* 2007).

As the cells gradually become arranged into epithelia, their cell-to-cell adhesiveness increases, the most anterior somite compacting immediately before segmenting off from the segmental plate. This increased adhesiveness appears to be brought about, at least in part, by the action of the glycoprotein, fibronectin (Lash *et al.,* 1984) (*Text-Figure 26*), which increases in concentration as the somitomeres mature along the segmental plate. This can also be brought about experimentally by the synthetic peptide, GRGDS, which corresponds to the adhesive segment of fibronectin (Lash *et al.,* 1987). Protogenin, an immunoglobulin protein, has been shown to be expressed on the surface of the future somatic mesoderm cells as they ingress (Ito *et al.* 2011); an over-expression of protogenin delayed the mesoderm migration by increasing cell-to-cell adhesion. Each newly segmented somite contains about 2,500 cells (Christ and Ordahl, 1995).

Before molecular biological experiments were possible, one of the greatest problems in embryology was that of explaining the underlying causes of segmentation. What causes a strip of tissue, the segmental plate, to become broken up into a repeating series of somites? Another way of looking at the same problem is to ask, ‘What causes the boundaries between somites to be established?’ And why does



Text-Figure 26. *Transverse section of a 13-somite embryo through somites to show extracellular material, mainly fibronectin (coloured green). 1. Ectoderm; 2. Somatic lateral plate mesoderm; 3.intermediate mesoderm; 4. Somite; 5. Notochord; 6. Splanchnic mesoderm; 7. Coelomic cavity.*

this take place step-by-step from the anterior to the posterior end of the developing embryo? Although a number of alternative theories were proposed in the past, the concept that oscillating genes control the process is now widely accepted. This idea was first put forward in a theoretical paper by Cooke and Zeeman (1976) and is now supported by modern molecular evidence (Pourquié, 2001). Members of the *Notch* signalling cascade, especially *lunatic fringe,* appear to be essential components in setting up the oscillatory clock as well as Wnt and FGF (Gomez and Pourqié, 2009). For recent reviews of other molecules involved in segmentation, see Stockdale *et al.* (2000) and Pourquié (2011).

Traditionally, the somites are numbered sequentially from the anterior to the posterior end of the embryo, which corresponds to the sequence in which they form. An alternative scheme for the numbering of somitomeres and somites was devised by Ordahl (1993), the aim being to provide a standard description of the developmental state of each somitomere and when comparing different stages. For example, the most recently formed somite of a 13-somite embryo (stage 11) and that of a 26-somite embryo may be considered comparable. Although there are certain advantages to this scheme, they are probably overshadowed by the possibilities for confusion generated, especially as it involves the numbering of the somites from posterior to anterior. In general, therefore, it is probably best to use this scheme only under very specialized circumstances.

The number of somites that develop depends on the species. In the chick it is about 52. The most caudal somites form in the tail, but somite formation ceases at about stage 24 before all the available mesoderm is exhausted, a small amount remaining at the tip of the tail arranged in two bands, which correspond topologically and morphologically to the segmental plate in the trunk (Mills *et al.*, 1990). It appears that the somite oscillator ceases about this time, at least partially due to a restriction of *Notch* activity, but according to Tenin *et al.* (2010) may also be due to endogenous retinoic acid in this region suppressing the oscillators Wnt3a and FGF8). This is unlikely to be the whole story, however, since other changes occur at this time; in particular, a patch of cell death appears in the tail bud (Schoenwolf, 1981; Mills and Bellairs, 1989), which may be of significance.

Individual somites appear to be rectangular or globular (*Plates 21, 22*) in shape when seen from the dorsal or the ventral side, but transverse sections show them to be wedge-shaped, the medial wall being curved to the shape of the neural tube. Their shape is largely dependent on the tension exerted by the collagen fibrils in the extracellular matrix that surrounds them and attaches them to the adjacent epithelia, the ectoderm, endoderm, notochord and neural tube (Veini and Bellairs, 1990; Chernoff *et al.,* 2001). A mass of extracellular material, including fibronectin (*Text-Figure 26*), is now conspicuous. The earliest somites are formed before the neural tube has rolled up completely, and have a flattened shape (*Text-Figure 45a*). As the neural tube closes, the somites apparently become drawn upwards and acquire the more familiar shape seen in transverse sections (*Text-Figure 45b*). Longitudinal sections show each somite as an epithelial ball of cells with walls one cell thick and centred around a lumen (*Plate 22*). Mesenchymal cells are present in the lumen.

Each newly formed somite consists of two main regions, the medial and lateral, each divided anterior and posterior (*Text-Figure 72*). The medial portion is derived from a stem cell population in Hensen's node, which is drawn posteriorly during regression, and the most anterior part of the primitive streak, whilst the lateral part originates in cells that ingress through the primitive streak during regression (Chuai and Wejer, 2009; Iimura *et al.*, 2007). Experiments in which regions of the chick segmental plate have been replaced with the corresponding tissue from quail embryos have shown that the fates of the cells, or groups of cells, depend on their position along the segmental plate.

One of the first visible changes in the mature somite is that the basement membrane in the medio-ventral region breaks down and the cells lose their epithelial character and become mesenchymal (*Plate 71*). They are the **sclerotome** cells. The change in the ventral wall of the somite releases the cells within the somitocoele, and these also become part of the sclerotome.

A further change follows as the epithelium at the dorso-lateral side increases in height and is then known as the **derma-myotome** (often spelled dermomyotome). In longitudinal sections it can be seen as a cap with its edges curved inwards (*Plates 23b, 71*); it gives rise to the dermatome and myotome. The sclerotome and the derma-myotome are formed almost exclusively from the cells of the medial half of each new somite, the derma-myotome from the dorsal part, the sclerotome from the ventral region. The cells of the lateral part of the somite migrate away to form the muscles of the limbs and probably of the ventral body wall (see Chapter 10). There is evidence that this medio-lateral patterning of the somites is the result of a cascade of molecular signals. The neural tube

produces *Wntl,* which promotes *noggin* expression in the medial part of each somite, and this antagonizes the BMP4 which is produced by the lateral plate mesoderm (Hirsinger *et al.,* 1998). The patterning of the somites as they segregate into different regions involves many more molecular changes (reviewed by Stockdale *et al.,* 2000). The dorso-ventral patterning appears to be brought about principally by *sonic hedgehog* and *Wnt* signalling, whilst antero-posterior polarity is due to *notch.*

The anterior and posterior regions of the somite are separated from one another by a distinct gap, **von Ebner's fissure** The spinal nerves are able to enter the anterior half of each somite but not the posterior half.

The first myotome cells form in the anterior-medial region of the somite from the edge cells of the derma-myotome. By the time they have extended into the posterior half of the somite they have already begun to produce muscle proteins and are, therefore, probably already determined, though experiments have shown that the sclerotome cells are not (Dockter and Ordahl, 2000). Once the myotome has formed it becomes separated from the dermatomal component of the somite by a basement membrane. Later in development, dermatome cells migrate dorsally to become the dermis.

THE LATERAL PLATE MESODERM AND THE INTERMEDIATE MESODERM

The **lateral plate mesoderm** arises from the posterior region of the area pellucida and ingresses through the primitive streak, eventually spreading out to reach the edge of the area opaca and then continuing into this region as extra-embryonic mesoderm. As the lateral plate mesoderm migrates away from the primitive streak it becomes replaced by the ingressing somitic mesoderm. Similarly, as Hensen's node regresses caudally it comes to lie in the site formerly occupied by the presumptive lateral plate mesoderm, which now shifts laterally. Sweetman *et al.* (2008) suggested thatWnt3a and Wnt5a are involved in promoting and/or inhibiting these movements. The contribution from the primitive streak to the lateral plate ceases at about stage 10/11 (Knezevic *et al.*, 1998). Unlike the somitic mesoderm, the lateral plate is not segmented, but by stage 4 is present as two sheets, the somatic layer, which lies beneath the ectoderm (together forming the bilaminar sheet, the somatopleure) and the splanchnic layer, which lies over the endoderm (together forming the bilaminar layer, the splanchnopleure) (Text- Figure 35 and at a later stage *Plates 65, 72*). They are separated by a space, the future coelom (see Chapter 8). The development of the lateral plate appears to be linked in some ways to the somites. For example, *Epha-4* is expressed both in the lateral edge of the somites and in the lateral plate mesoderm and, as discussed above, the BMP4 produced by the lateral plate affects somite development. The lateral plate mesoderm subsequently forms the mesenteries, the lining of the pleural, cardiac and abdominal cavities, and the major substance of the heart, as well as contributing to the extra-embryonic membranes.

The **intermediate mesoderm** lies between the somites and the lateral plate mesoderm, to both of which it is initially attached (*Plate 65*). It appears at the same time as the somites and develops simultaneously with them from anterior to posterior down the trunk, but it differs from the somites in that it is not overtly segmented and does not extend into the postcloacal tail. It later forms most of the urino-genital system (see Chapter 7).

THE NEURAL CREST

Like the notochord and somites, the neural crest is found only in the early embryo and is not present at later stages. It is, as its name implies, a strip of cells situated along the dorsal side of the early neural tube, forming a 'crest' down its entire length (*Plate 24a,b*). Its former name, ectomesenchyme, is descriptive of its origin and fate rather than of its position, in that it is an ectodermal derivative that gives rise to a wide range of mesenchymal structures. There is extensive literature on the neural crest, including excellent reviews by Gammill and Bronner-Fraser (2002), with emphasis on the migration of specific neural crest cells, and Harris and Erickson (2007) and Kuo and Erickson (2010), with especial attention to gene profiles.

The neural crest arises from a broad crescent-shaped area in the gastrula ectoderm which is characterized by BMP4 expression (Ezin *et al.*, 2009). It originates from the ectoderm at the junction of the non-neural ectoderm and the presumptive neural ectoderm (Selleck and Bronner-Fraser, 1995) and there is evidence that the two tissues interact to induce it. Dickinson *et al.* (1995) found that neural crest could be induced from neural plate in culture either by ectoderm or by two proteins secreted by the ectoderm (BMP4 and 7). These two proteins induce the expression of *slug* protein and *Rhob* protein in the cells which become neural crest, and in the absence

of either one of them the neural crest cells fail to leave the neural tube. Even the extra-embryonic ectoderm can be induced to form neural crest (Ruffins and Bronner-Fraser, 2000). *Rhob* is involved in the production of cytoskeletal elements that are needed for migration (Hall, 1999). The non-neural ectoderm remains able to induce neural crest from the neural ectoderm until about stage 10, though the neural plate loses its ability to respond after about this time (Basch *et al.,* 2000). These authors found that the marker, *slug,* was present in the neural folds even by the end of gastrulation, and suggested, therefore, that neural crest induction involves a series of stages. The gene, *lunatic fringe,* which is expressed in the neural tube but not in the neural crest cells, also plays a role, since excess *lunatic fringe* results in excess proliferation of neural crest cells at least in the cranial region (Nellemann *et al.,* 2001).

The neural crest is first visible as a thickened region just before the neural tube closes (*Text-Figure 25*). Its formation and subsequent development follow the normal anterior to posterior sequence. Three major regions of the neural crest (*Text-Figure 27*) are usually recognized:

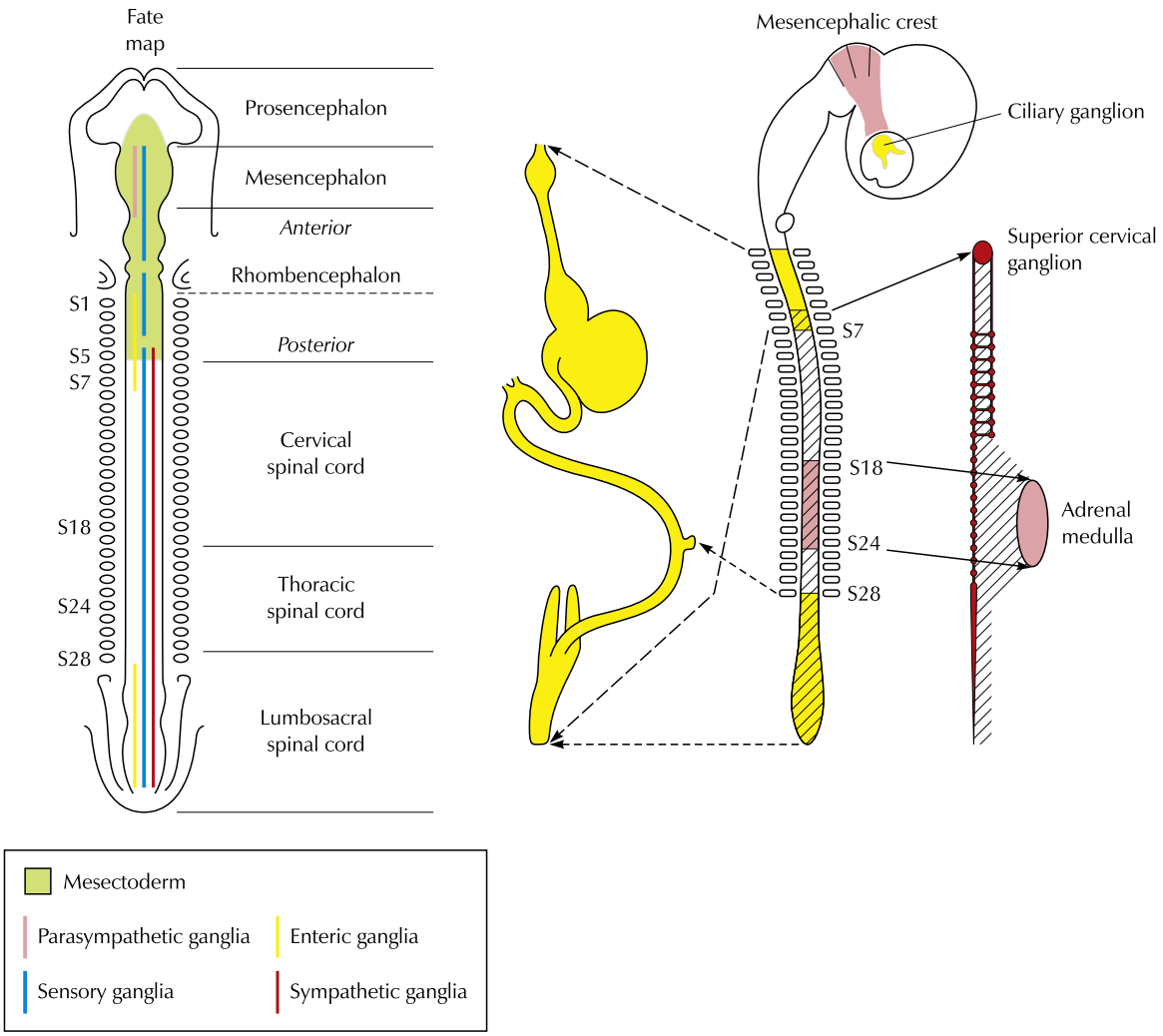
1. **Cranial neural crest**, which lies anterior to the somites and gives rise to skeletal structures of the face, melanocytes, neurons, ganglia and glial cells.
2. **Vagal neural crest**, which is associated with somites 1–7, and includes the **Cardiac neural crest**, at somite levels 1–3, gives rise to enteric neurons of the gut, melanocytes, etc., as well as components of the heart.
3. **Trunk and tail neural crest**, which lies posterior to somite 8 and includes the **Adrenal neural crest** at somite levels 16–24.

There are differences in the behavior of neural crest cells from, and within, these different regions as they migrate, as well as in their eventual differentiation.

As the neural tube closes, the neural crest becomes lodged between it and the overlying ectoderm (*Text-Figure 25*), and as the three tissues separate from one another the neural crest cells settle down onto the dorsal surface of the neural tube (*Plates 24, 25*). Shortly before they leave the neural tube they flatten and reorientate so that the longest axis of each cell is arranged at right angles to that of the embryo, and there is a reduction in the intercellular spaces (Bancroft and Bellairs, 1976). The emigration from the neural tube takes place in a wave from anterior to posterior down the body. An important aspect is that it follows on closely after the wave of somite segmentation, and there is evidence that each pair of somites influences the release of the neural crest in its vicinity by affecting the BMP4 and *noggin* interrelationship. BMP4 appears to play a major role in releasing the neural crest cells from the neural tube. Sela-Donenfeld and Kalcheim (2000) found that in regions of the embryo where *noggin* expression is high in the neural tube, BMP4 is inactive and neural crest cells fail to emigrate, but progressive reduction in *noggin* activity coincides with activation of BMP4 and the emigration of neural crest cells. The authors suggested, as a result of a series of experimental ablations, that an inhibition of *noggin* was produced by the dorso-medial part of the epithelial somites. This would explain why neural crest delamination follows somite segmentation.

Once the neural crest cells have left the neural tube, they follow well-defined pathways through the body until they reach their target tissues. What are the controls that guide the cells as they migrate? And are the cells destined to differentiate into their appropriate tissues when they finally reach their destinations, or are their fates determined at an earlier stage? The answers to these questions seem to depend on the particular crest cells we are considering. For example, those cells in the trunk destined to become melanoblasts become specified (determined) soon after leaving the neural tube (Harris and Erickson, 2007), whereas others remain more flexible, even if not totally pluripotent, until they reach their destination. The major cues that direct their migration are provided by the surrounding environment: e.g. the neural crest cells pass through the anterior half of each somite but never through the posterior half. This rule is followed even in experimental situations where the somites are rotated or inverted so that their relationship to the emigrating neural crest cells is altered. *F-spondin,* which is expressed in the posterior region only of the somites, appears to play a role in inhibiting the crest cells from entering this region (Debby-Brafman *et al.,* 1999). Similarly, *ephrin* proteins inhibit the passage of crest cells through this region (Newgreen *et al.,* 1986). The neural tube also influences the direction in which the neural crest cells migrate, since if it is inverted in its dorso-ventral axis the crest cells that were destined to migrate down the side of the neural tube continue to do so, even though that leads them toward the ectoderm instead of the endoderm.

In the trunk, two major routes are apparent. The first cells migrate down the lateral side of the neural tube and into the anterior part of adjacent somites, following the basal lamina of the dermamyatome (*Text-Figures 28, 29*). This is the **ventral route** and

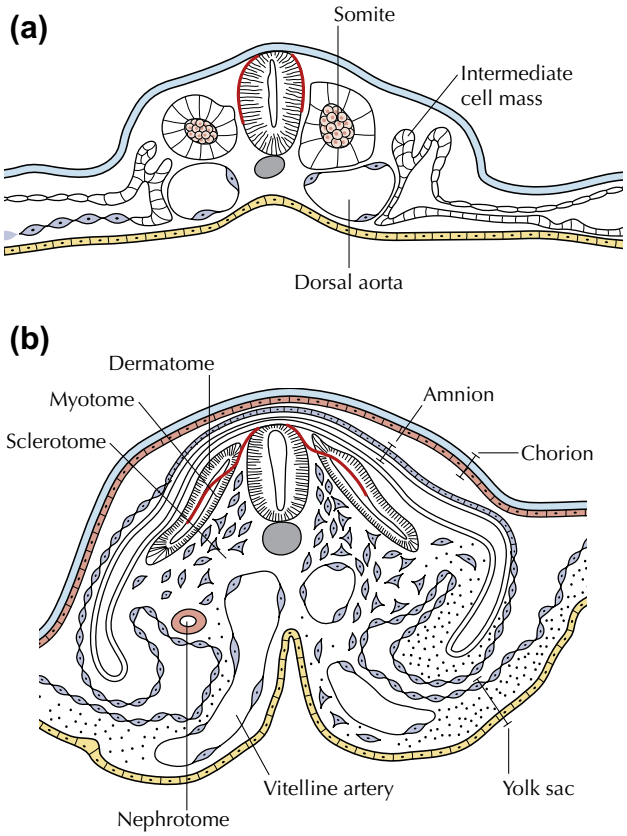


Text-Figure 27. *Regions of the neural crest and their contributions to the autonomic nervous system; the* ***cranial neural crest*** *gives rise to the cranial nerves and ganglia; the* ***vagal neural crest*** *(level somites 1-7) and the* ***sacral neural crest*** *(posterior to somite 28) form the nerves of the gut; the* ***cardiac neural crest*** *(level somites 1–3) contribute to the heart; the* ***trunk neural crest*** *give rise to the sympathetic neurons, those between somite levels 18–24 forming the adrenal medulla. (After Le Douarin and Kalcheim (1999); reproduced with permission of Cambridge University Press.)*

is repeated at one somite level after another down the trunk. It is succeeded by the **dorso-lateral route** (*Text-Figure 29*) where the cells migrate beneath the dermis.

The neighbouring tissues exert much of their influence on the neural crest through the medium of the extracellular matrix which surrounds them, the most important components being fibronectin, laminin, tenascin, collagen and various proteoglycans. Integrins and other adhesion molecules enable the neural crest cells to interact with the extracellular matrix. It is likely that the differing concentrations in which these materials are present in different regions of the body play a role in directing migration. The environment also plays a role in the final differentiation of the neural crest cells. Most neural crest cells appear to be pluripotent at the time they leave the neural tube in that they can often be made to abandon their presumptive fate and differentiate according to a new environment if transplanted. The major exception is that the trunk neural crest, which does not form cartilage or bone in the trunk, appears to be unable to do so even when transplanted into the head.

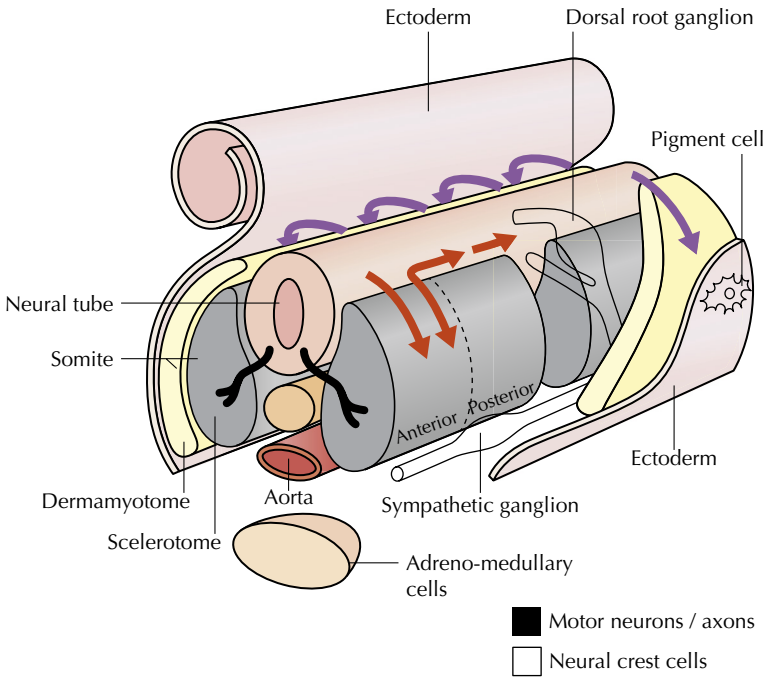
Neural crest which arises in the head region (cephalic neural crest), and forms cartilage and bone of much of the head and face skeleton, also gives rise to connective tissue as well as Schwann cells and cranial sensory ganglia (see Chapter 9). Neural crest in the vagal (just posterior to the head) and sacral regions forms the entire enteric (gut) nervous system. The neural crest in the trunk migrates as two separate streams; the dorsal one passes laterally beneath the ectoderm until it



Text-Figure 28. *The trunk level neural crest cells (red) migrate ventrally between the neural tube and somites, some subsequently passing through the anterior part of each somite, moving along the basal lamina of the sclerotome.*

reaches the mid-ventral body wall. Eventually, it migrates into the substance of the dermis and forms the pigment cells. As the ventro-lateral stream passes through the anterior halves of the somites, some cells remain there and differentiate into dorsal root ganglia, whilst others give rise to sympathetic ganglia, Schwann cells and adrenomedullary cells. The cardiac neural crest extends from the first to the third somites, between the cranial and the trunk neural crests (*Text-Figure 27*). There have been fewer studies on neural crest development during secondary neurulation (i.e. in the tail region), but recent evidence suggests that the molecular aspects are similar to those in the trunk neural crest (Osório *et al.* 2009).

Some interesting parallels exist between the neural crest and the primitive streak (see Bellairs, 1987). In each case cells leave an epithelium and migrate to another part of the body; they move from the outer layer to the inside of the embryo and upon reaching their destination become arranged into new structures. The cells that leave the primitive streak inevitably differ from those of the neural crest in that they give rise to a different range of tissues. There is, however, an additional difference. The cells that arise from the primitive streak all give rise to epithelial tissues on reaching their destination (somites, lateral plate, pronephric duct, endoderm), but in birds the cells that arise from the neural crest seldom become rearranged as epithelia.



Text-Figure 29. *Schematic diagram showing the two pathways taken by the neural crest cells as they pass to their target tissues.* ***Purple:*** *the dorso-ventral pathway between the ectoderm and somites by cells that give rise to pigment cells.* ***Red:*** *the ventro-medial pathway, passing through the anterior, but not the posterior, parts of somites. (After Krull (2010), with permission of Landes Bioscience.)*