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Making a Neural Tube: Neural Induction and Neurulation

Raj Ladher and Gary C. Schoenwolf

INTRODUCTION

As subsequent chapters will describe, the vertebrate nervous system is necessarily complex. However, this belies its humble beginnings, segregating relatively early as a plate of cells in the dorsal ectoderm of the embryo. This process of segregation, termed neural induction, occurs as a result of instructive cues within the embryo and is described in this chapter. Once induced, the neural plate, in most vertebrates, rolls into a tube during a process known as neurulation. This tube is then later elaborated to form the central nervous system. In this chapter, we describe the model for how ectodermal cells become committed to a neural fate, and the studies that have led to this model. We will then review the mechanisms by which the induced neural ectoderm rolls up to form the neural tube.

SETTING THE SCENE

In this section, we describe some of the fundamental events that occur in embryogenesis prior to neural induction. We also introduce the main vertebrate model organisms used to investigate neural induction, and we discuss their strengths and appropriateness for various types of experimental studies.

Neural induction, the process by which a subset of the ectoderm is instructed to follow a pathway leading to the formation of the nervous system, has been studied in model systems comprising four classes of vertebrates. Despite obvious differences in the geometry of the embryos of these classes (e.g., the early frog embryo is spherical, whereas the early chick embryo is a flat disc), by and large their embryogenesis is comparable, and researchers can use the respective strengths of these models to address experimentally very specific research questions. By synthesizing data that have emerged from these studies, a model has been formulated of how neural tissue is induced.

Model Organisms

Four vertebrate model systems have been used extensively to study neural induction (Fig. 1). Two of these are classified as lower vertebrates—zebrafish and *Xenopus*—and two are classified as higher vertebrates—chick and mouse. Two major differences exist between lower and higher vertebrates. First, lower vertebrates lack an extraembryonic membrane called the amnion, which was developed by higher vertebrates as an adaptation to terrestrial life. Thus, lower vertebrates are anamniotes and higher vertebrates are amniotes. Second, true growth (i.e., cell division followed by an increase in cytoplasm in each daughter cell to an amount comparable to the parental cell—in contrast to cleavage, where cells get progressively smaller with division) is minimal during morphogenesis in lower vertebrates, but plays an integral role in morphogenesis of higher vertebrates. In addition to these differences between lower and higher vertebrates, another major difference exists among the four model organisms: the relationship between the formative cells of the embryo and their food source. Namely, *Xenopus* eggs contain a large internal store of yolk. With cleavage of the egg to form the spherical blastula, this yolk is incorporated into the forming blastomeres with the vegetal blastomeres being much larger than, and containing much more yolk than, the animal blastomeres. In both zebrafish and chick, a blastoderm forms as a disc on top of the yolk mass. Finally, in the mouse egg, yolk is sparse; rather the embryo receives its nourishment from the mother, initially by simple diffusion and later through the placenta. These differences in the amount and distribution of yolk in the eggs of the four vertebrate models result in very different geometries in the four organisms. Thus, during the early developmental stages of cleavage, gastrulation, neural induction, and neurulation, the four model organisms appear very different from one another, yet developmental mechanisms at the tissue, cellular, and molecular–genetic levels are highly conserved.

In *Xenopus* and zebrafish, early development is directed by maternal products laid down during oogenesis; at the

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mid-blastula transition, or MBT, zygotic transcription commences (Newport and Kirschner, 1982; Kane and Kimmel, 1993). Maternally provided products are important in axis formation and germ layer identity. In chicks and mice, "MBT," or the onset of zygotic transcription, occurs soon after fertilization; thus, the exact role of maternal products in early development has been difficult to decipher.

The *Xenopus* Embryo

A large body of literature exists on the development of the amphibian embryo. Indeed, two of the most important findings regarding the embryogenesis of the vertebrate nervous system—the discovery of the organizer and the elucidation of its role in neural induction (Spemann and Mangold, 1924, 2001) and the discovery of the molecular mechanisms of neural induction (Sasai and De Robertis, 1997; Nieuwkoop, 1999; Weinstein and Hemmati-Brivanlou, 1999)—were obtained using amphibian embryos. These will be discussed later in this chapter. The class itself can be split into the Anurans (frogs and toads) and the Urodeles (newts and salamanders), and despite some differences in the details of their development, the many similarities make it possible to generalize the results and extend them to other organisms. Although the Anuran, *Xenopus*, is the model most used today, the starting point for most studies was the pivotal work performed in Urodeles by Spemann and Mangold in the course of discovering the organizer (Spemann and Mangold, 2001). For a summary of the differences between Anurans and Urodeles, see the excellent review by Malacinski *et al*. (1997). For a schematic view of key phases of early *Xenopus* development, see Fig. 2.

The amphibian embryo is large, easily obtained, readily accessible, and easily cultured in a simple salt solution. As all cells of the embryo have a store of yolk, pieces of the embryo and even single cells from the early embryo (i.e., blastomeres) can be cultured in simple salt solution. A recent advantage in the use of *Xenopus* is the ability to overexpress molecules of interest. Because early blastomeres are large, it is a simple matter to make RNA corresponding to a gene of interest and inject it into selected cells. The injected RNA is translated at high efficiency

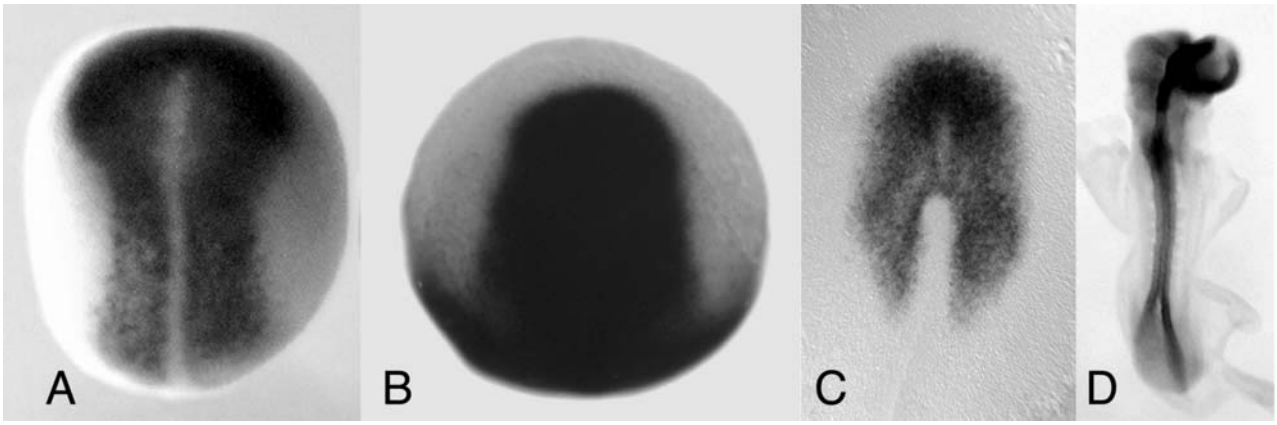

FIGURE 1. Photographs showing the locations of the neuroectoderm at neurula stages in (A) *Xenopus* (dorsal view, immunohistochemistry for N-CAM at stage 15; courtesy of Yoshiki Sasai); (B) zebrafish (dorsal view, *in situ* hybridization for *Sox-31* at tail bud stage; courtesy of Luca Caneparo and Corinne Houart); (C) chick (dorsal view, *in situ* hybridization for *Sox-2* at stage 6; courtesy of Susan Chapman); and (D) mouse (dorsolateral view, *in situ* hybridization for *Sox-2* at 8.5 dpc; courtesy of Ryan Anderson, Shannon Davis, and John Klingensmith).

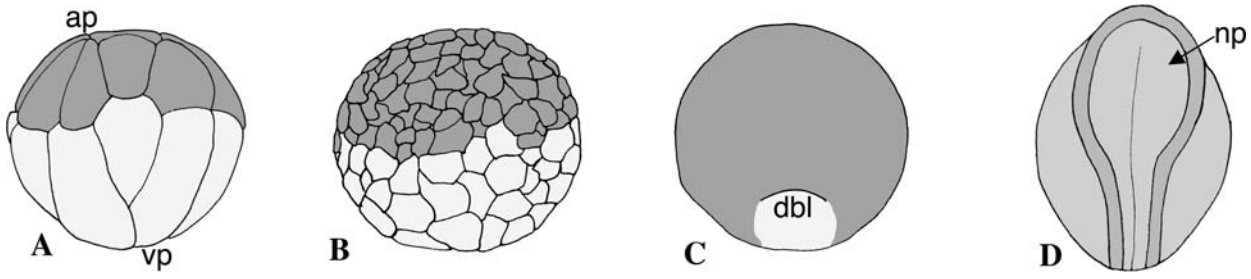

FIGURE 2. *Xenopus* development leading up to neurulation. Diagrams of embryos at the (A) morula, (B) blastula, (C) gastrula, and (D) neurula stages of development. Once the egg is fertilized, cleavage occurs, with the cells of the animal hemisphere darker and smaller than cells of the vegetal hemisphere. At blastula stages, mesoderm is induced. In particular, dorsal mesoderm is specified and at gastrula stages, this mesoderm starts to involute, forming the dorsal blastoporal lip and marking the site of the organizer. The organizer induces neural tissue in the overlying animal hemisphere. ap, animal pole; dbl, dorsal blastoporal lip; np, neural plate; vp, vegetal pole. Modified from Nieuwkoop and Faber (1967).

and is active. Indeed this technique has been used not only to assay a whole molecule, but also modified (i.e., systematically and selectively mutated) versions of the gene.

As most developmental biology research in amphibians is performed on the *Xenopus* embryo, we will consider its development. Smith (1989) provides an excellent synthesis of the early embryological events that occur prior to neural induction.

The *Xenopus* egg has an animal–vegetal polarity, with the darker (i.e., more heavily pigmented) animal hemisphere forming the ectoderm and mesoderm, and the lighter vegetal, yolk-rich hemisphere forming the endoderm. Fertilization imparts an additional asymmetry on the egg, with the sperm entering the animal hemisphere. The sperm entry point also determines the direction of rotation of the cortex of the egg in relation to the core cytoplasm, and this activates a specific pathway leading ultimately to the establishment of the dorsal pole of the embryo (Vincent and Gerhart, 1987; Moon and Kimelman, 1998). Specifically, the region of the vegetal hemisphere, the Nieuwkoop center, which is diametrically opposite the sperm entry point, is now conferred with the ability to induce the Spemann organizer in the adjacent animal hemisphere (Boterenbrood and Nieuwkoop, 1973). The Spemann organizer has the ability to induce dorsal mesoderm and pattern the rest of the mesoderm, as well as to direct the formation of the neuroectoderm (Gimlich and Cooke, 1983; Jacobson, 1984; see below and Box 1).

Following fertilization, mesoderm is induced in the equatorial region of the embryo, at the junction between the animal and vegetal poles (Nieuwkoop, 1969). Amazingly, this induction has been experimentally recreated to great effect in later assays for both mesoderm-inducing signals and neural-inducing signals. When challenged with the appropriate signal, an isolated piece of *Xenopus* animal tissue, which would normally form epidermal structures, will change its fate accordingly. This animal cap assay has, for years, provided researchers with a powerful assay for induction. One important caveat must be noted here though. Barth (1941) found that the animal cap of the amphibians *Ambystoma mexicanum* and *Rana pipiens*, amongst others, autoneuralizes; that is, the removal of the presumptive epidermis from its normal environment actually changes its fate to neural, a result supported and extended by Holtfreter (1944), who among other things showed that neural induction could occur even after the inducer had been killed (Holtfreter, 1947). This result could only be contextualized years later when the pathway for neural induction was worked out (see below). It should be noted here, however, that the animal cap of *Xenopus* does not show such auto-neuralization; indeed as we discuss below, the *Xenopus* animal cap is resistant to nonspecific neural induction by diverse agents (Kintner and Melton, 1987). This resistance to nonspecific neural induction strengthened the role of *Xenopus* embryos in the search for inducing signals.

Neural induction occurs during the process of gastrulation when the mesoderm and endoderm invaginate through the blastopore and, via a set of complex morphological movements (see Keller and Winklbauer, 1992, for details of this process), are internalized. This results in the ectoderm remaining on the surface and forming the crust, and the mesoderm and endoderm coming to lie deep to the ectoderm, forming the core. A fuller description of neural induction is given below.

The Zebrafish Embryo

Two large-scale mutagenesis screens propelled the zebrafish embryo to the forefront of developmental biology (Mullins and Nusslein-Volhard, 1993; Driever, 1995). The combination of

BOX 1. The Organizer

The discovery of the organizer in 1924 is one of the major milestones in developmental biology. This discovery has had a major influence on our thinking about the mechanisms underlying neural induction (Spemann and Mangold, 1924). The German scientists, Hans Spemann and Hilda Mangold, discovered that a region of the amphibian gastrula, the dorsal lip of the blastopore, had the ability to direct formation of the neural plate (Fig. 3A). By transplanting the dorsal lip from a donor embryo to the ventral side of a host embryo, they found that a second axis can be initiated. The experiment was performed using salamander embryos, not *Xenopus*, the current favorite amphibian model. By using two species of salamander, one pigmented and the other unpigmented, Spemann and Mangold could identify which structures in the duplicated axis were derived from the donor and which were derived from the host. Careful analysis showed that whereas the secondary notochord and parts of the somites were derived from the donor dorsal lip, the neural plate and other regions of the somites within the secondary axis were derived from the host. As host tissues should have been fated to form ventral derivatives, such as lateral mesoderm and epidermal ectoderm, Spemann and Mangold reasoned that the action of the donor dorsal tissue was not autonomous, and that a nonautonomous action induced the surrounding tissues to take on a dorsal fate. By using a classical definition of the word "induction"—the action of one tissue on another to change the latter's fate, Spemann and Mangold defined neural induction in vertebrate embryos and localized its center of activity.

As mentioned above, the action of an organizer is not just limited to amphibian embryos. A large number of studies have extended the findings of Spemann and Mangold to embryos of the fish, bird, and mammal (Waddington, 1934; Oppenheimer, 1936; Beddington, 1994; Fig. 3B). All of these studies have found that the organizer can induce the formation of a secondary axis. However, in the mouse, there is an important difference. Whereas in the fish, frog, and chick, transplantation of the organizer can induce a secondary axis with all rostrocaudal levels (i.e., from the forebrain to the caudal spinal cord), transplantation of the node in the mouse can induce only a supernumerary axis that begins rostrally at the level of the hindbrain (Beddington, 1994; Tam and Steiner, 1999). This has led to the identification of a second organizing center, the anterior visceral endoderm (Thomas and Beddington, 1996; Tam and Steiner, 1999). Using a series of transplants, it has been found that the anterior visceral endoderm, unlike the node of the mouse, cannot induce neural tissue. Instead, it provides a patterning activity, imparting rostral identity upon already induced neuroectoderm. As this is beyond the scope of this chapter, the anterior visceral endoderm will be more appropriately covered in greater detail in Chapter 3 on neural patterning.

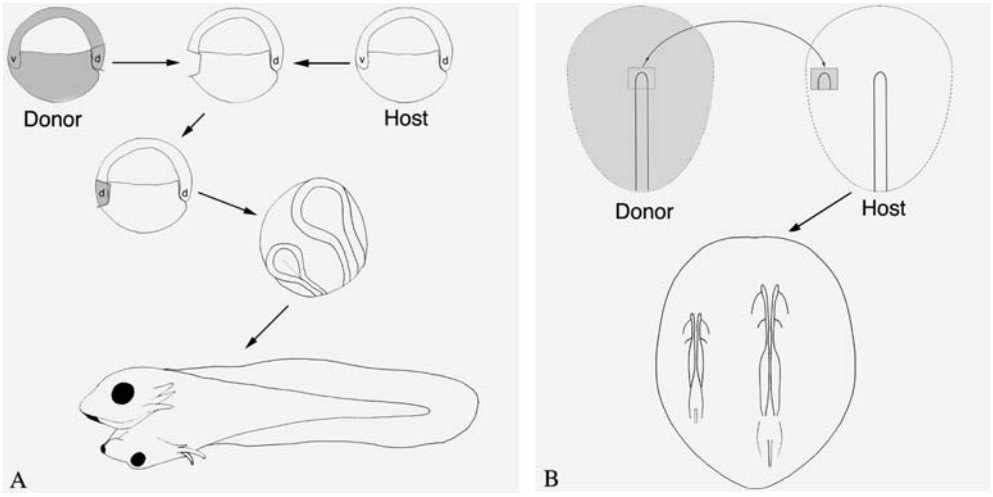

FIGURE 3. Axis duplication in (A) amphibians and (B) the chick after transplantation of the organizer regions of these embryos to ectopic locations. Details of the experiments are given in the main text. Transplantation of the dorsal lip (in amphibians) or Hensen's node (in chick) gives rise to a duplicated neuroaxis, derived from host tissue. This experiment mapped the site of neural induction to the organizer. d, dorsal; v, ventral. (A), modified from Spemann and Mangold (1924); (B), modified from Waddington (1932).

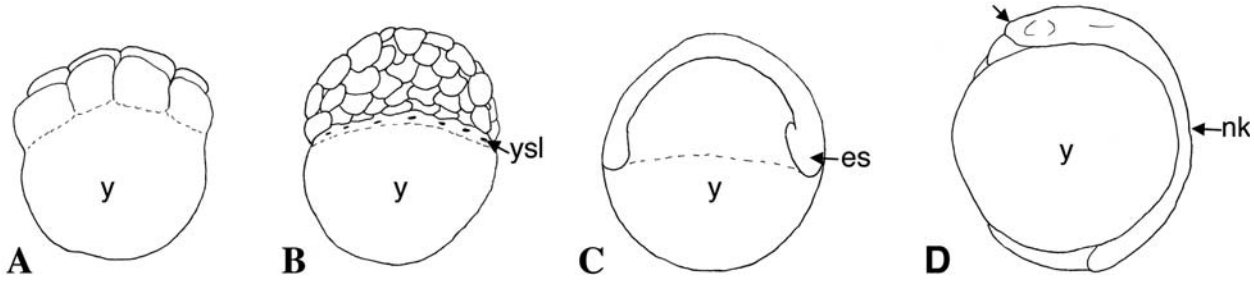

FIGURE 4. Zebrafish development leading up to neurulation. Diagrams of embryos at (A) morula, (B) blastula, (C) gastrula, and (D) neurula stages. The zebrafish embryo floats on top of the yolk (y), a situation that is not changed until gastrulation. At blastula stages, a belt of cells is formed at the junction between the embryo and the yolk; it is known as the yolk syncytial layer (ysl). This induces the formation of the mesoderm and also directs the formation of the embryonic shield (es), the organizer of the fish embryo. The embryo shield also induces the formation of neural ectoderm (i.e., the neural keel, nk). Arrow indicates the head end of the embryo. Modified from Langeland and Kimmel (1997).

generating mutants, cloning the affected genes and using traditional embryological techniques has made the zebrafish embryo especially attractive to researchers. For a schematic view of key phases of early zebrafish development, see Fig. 4.

Fertilization causes the segregation of the cytoplasm from the yolky matter in the egg, resulting in a polarity manifested by the presence of a transparent blastodisc on top of an opaque yolky, vegetal hemisphere (Langeland and Kimmel, 1997). Cell division increases the number of cells, forming the blastoderm, and at the 256-cell stage, the first overt specialization occurs within the blastoderm. The most superficial cells of the blastoderm form an epithelial monolayer, known as the enveloping layer, confining the deeper cells of the blastoderm. At around the tenth cell division, the cells at the vegetal edge of the enveloping layer of the blastoderm fuse with the underlying yolk cell. Interestingly, the tenth cell cycle marks the MBT for the zebrafish embryo. A belt of nuclei, the yolk syncytial layer (YSL), resides within the yolk cell cytoplasm just under the blastoderm. It provides a motive force for gastrulation, and it has been postulated also to function in establishing the dorsal–ventral axis of the zebrafish (Feldman *et al*., 1998).

The initial phase of gastrulation is marked by the blastoderm flattening on top of the yolk. This causes the embryo to change from dome-shaped to spherical, and it results from the process of epiboly: the spreading of the blastoderm over the yolk hemisphere. The YSL drives epiboly, pulling the enveloping layer with it. The process has been likened to "pulling a knitted ski hat over one's head" (Warga and Kimmel, 1990). At about 50% epiboly, that is, when the blastoderm has covered half of the yolk hemisphere, the germ ring forms. This is a bilayered belt of cells: The upper layer is the "epiblast," whereas the lower layer is the "hypoblast." The lower layer forms by involution; that is, as the deeper cells of the blastoderm are driven superficially toward the vegetal margin, they fold back under and migrate toward the

animal pole. At the same time, there is a movement of deep blastoderm cells toward the future dorsal side of the embryo. This creates a thicker region in the germ ring, marking the organizer of the zebrafish, a structure known as the embryonic shield. Similar to the situation in amphibia, this structure can be transplanted to the ventral side of a host fish embryo, where it induces the formation of a secondary axis (Oppenheimer, 1936; Box 1). As gastrulation proceeds and the body plan becomes clearer, the neural primordium becomes apparent as a thickened monolayer of cells. The mechanisms by which this happens will be discussed in detail later in this chapter.

The Chick Embryo

Chick eggs are readily available and embryos are easily accessible throughout embryogenesis. Embryos readily tolerate manipulation such as microsurgery. As a result of these attributes, the chick embryo has long been a favorite organism for experimental embryology. For a schematic view of key phases of early chick development, see Fig. 5.

After the egg is fertilized, which occurs within the oviduct of the hen, shell components are added during the day-long journey through the oviduct prior to laying. Cleavage begins immediately after fertilization, and by the time the egg is laid, it contains a bilaminar blastoderm floating on the surface of the yolk (Schoenwolf, 1997). The upper layer of the bilaminar blastoderm is termed the epiblast, whereas the lower layer (i.e., the one closest to the yolk) is termed the hypoblast. The epiblast gives rise to all of the tissue of the embryo proper, that is, the ectodermal, mesodermal, and endodermal derivatives. The hypoblast is displaced during embryogenesis and will contribute to extraembryonic tissue.

Like the fish embryo, the region of the chick egg that gives rise to the embryo proper floats on top of a yolky mass. During cleavage, the blastoderm becomes 5–6 cells thick and is separated from the yolk by the subgerminal cavity. The deep cells in the central portion of the disc are shed, leaving the monolaminar area pellucida. This region of the blastoderm will give rise to the definitive embryo. The peripheral ring of cells, where the deeper cells have not been shed, is the area opaca. This region, in conjunction with the peripheral part of the area pellucida, will give rise to the extraembryonic tissues. Many of the extraembryonic tissues will eventually cover the entire yolk, providing the embryo with nourishment during development. At the border between the area opaqua and area pellucida at the time of formation of these two regions is a specialized ring of cells, the marginal zone. This zone plays an important role in establishing the body axis of the embryo (Khaner and Eyal-Giladi, 1986; Khaner, 1998; Lawson and Schoenwolf, 2001).

Shortly after the formation of the area pellucida, some of the cells in this region delaminate and form small polyinvagination islands beneath the outer layer (the epiblast). These cells flatten and join to form a structure known as the primary hypoblast. Within the caudal marginal zone, a sickle-shaped structure appears called Koller's sickle; it gives rise to a sheet of cells, called the secondary hypoblast, which migrates rostrally, joining the primary hypoblast. This results in an embryo with two layers—the uppermost layer epiblast and the lowermost hypoblast. These layers are separated from the yolk by a fluid-filled space called the blastocoel.

Once the egg is laid, further development requires incubation at about 38°C. After about 4 hr of incubation, the first signs of gastrulation become apparent. The cells of the hypoblast begin to reorganize in a swirl-like fashion, termed a Polinase movement. Viewed ventrally, that is, looking down on the surface of the hypoblast, the cells of the left side of the hypoblast move counterclockwise, whereas those on the right side move clockwise. Concomitantly, epiblast cells as they extend rostromedially

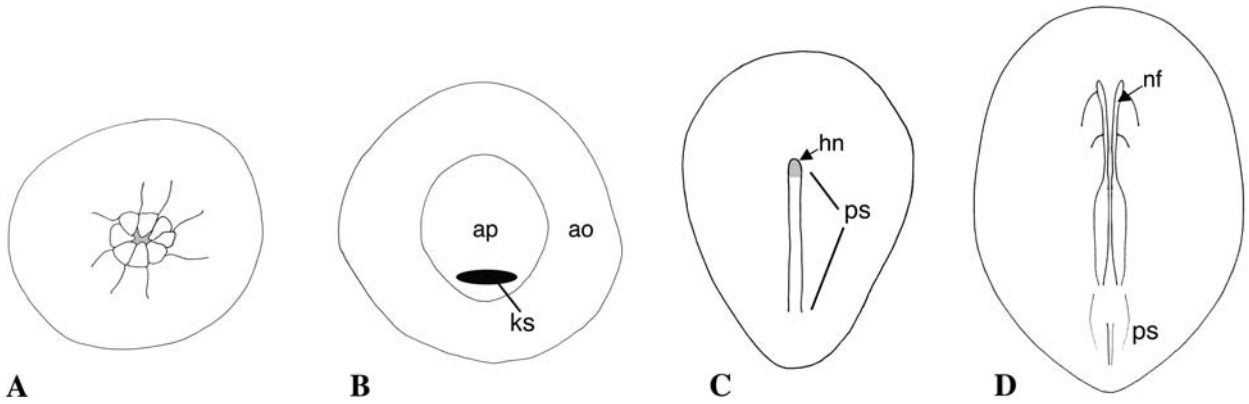

FIGURE 5. Chick development leading up to neurulation. Diagrams of embryos at (A) morula, (B) blastula, (C) gastrula, and (D) neurula stages; the blastoderm is shown removed from the yolk and viewed from its dorsal surface. At the time that the chick egg is laid, a multicellular blastoderm floats upon the yolk. The blastoderm is subdivided into an inner area pellucida (ap) and an outer area opaca (ao), with Koller's sickle (ks) marking the caudal end of the blastoderm. The ao forms the extraembryonic vasculature, providing nutrition for the growing embryo. By blastula stages, the central portion of the embryo is two cell layers thick: the upper epiblast will form all of the structures of the adult; the lower hypoblast will contribute to extraembryonic tissues. The primitive streak (ps) forms in the epiblast of the embryo, and the mesoderm and definitive endoderm ingress through it and into the interior. The primitive streak extends rostrally and once it has reached its maximal length, it forms a knot of cells known as Hensen's node (hn; shaded). This is the organizer of the chick embryo; it is responsible for neural induction. Shortly after neural induction, the embryo undergoes neurulation. nf, neural folds. Modified from Schoenwolf (1997).

from Koller's sickle begin to pile up at the caudal of the midline of the area pellucida. These cells accumulate as a wedge, with the base of the wedge at the caudal end and the apex pointing along the midline rostrally. This wedge-like structure is the initial primitive streak, the equivalent to the blastopore lip in the frog and the embryonic shield in fish, that is, the structure through which cells of the epiblast will ingress to give rise to mesoderm and definitive endoderm. It forms just rostral to Koller's sickle, and this has led to the belief that Koller's sickle acts in much the same way as the Nieuwkoop center in *Xenopus* (Callebaut and Van Nueten, 1994). As development progresses, the streak elongates reaching a maximal length at about 18 hr of incubation. As the streak reaches its maximal length, its rostral end forms a knot of cells called Hensen's node. Hensen's node is the embryological equivalent of the dorsal lip in *Xenopus* and the embryonic shield in zebrafish; that is, Hensen's node is the organizer of the avian embryo (Waddington and Schmidt, 1933; Waddington, 1934). The role of Hensen's node in neural induction is discussed further in Box 1.

The Mouse Embryo

The mouse, being a mammal, has an embryo that should be highly relevant for understanding development of the human embryo. Nevertheless, there are some caveats that make this model less than ideal. The fact that mouse development occurs within the maternal uterus and that the embryo is highly dependent upon its mother for respiration, nutrition, and the removal of its waste products makes the embryo relatively unsuitable for the kinds of embryological experimentation that have characterized research on the other three model systems discussed above. Early development of the mouse embryo also is peculiar in that unlike the other three model organisms, the gastrula stage of the mouse develops "inside-out"; that is, with its ectoderm on the "inside" and its endoderm on the "outside." For a schematic view of key phases of early mouse development, see Fig. 6.

Recent advances in whole-embryo culture have substantially increased the value of the mouse embryo for experimental embryology. Consequently, cutting- and pasting-type experiments in the mouse embryo are becoming increasingly common. However, it is in the realm of genetic analysis that the mouse embryo has excelled as a model organism. The ability to remove genes, to place genes into an unnatural context and to elucidate the genetic controls that genes are subject to, has advanced developmental biology considerably. These molecular genetic techniques are introduced in this chapter where necessary; for further information, the reader is directed to several excellent reviews (Capecchi, 1989; Rossant *et al*., 1993; Soriano, 1995; St-Jacques and McMahon, 1996; Beddington, 1998; Osada and Maeda, 1998; Stanford *et al*., 2001). In the subsequent section, we discuss development of the mouse up to the stage when neural induction occurs.

The mouse oocyte is released into the oviduct from the ovary and it is in the ampulla of the oviduct that fertilization occurs (Cruz, 1997). Cleavage begins as the oocyte passes down the oviduct toward the uterus. It should be noted that cleavage occurs within the confines of the zona pellucida, the covering of the oocyte. The zona plays an important role in regulating the site (and time of) implantation in that until the embryo hatches from the zona pellucida, the embryo cannot implant. If the embryo hatches too early, then implantation can occur in the oviduct, resulting in an ectopic pregnancy.

After the third cleavage, that is, after the eight-cell stage, the conceptus transforms from a group of loosely arranged blastomeres called a morula (Latin for mulberry) to a mass of flattened and tightly interconnected cells. This change is referred to as compaction. As a result of compaction, the blastomeres flatten against each other at the surface of the morula, maximizing their contact with one another, and a blastocoel appears within the morula. As the blastocoel is forming, a small group of internal cells appears, known as the inner cell mass, surrounded by external cells, known as the trophoblast. With formation of the inner cell mass and trophoblast, the morula is converted into the blastocyst. Formation of these two cell types constitutes the first lineage restriction that occurs in mouse development, with cells of the trophoblast eventually forming the chorion—the embryonic portion of the placenta—and those of the inner cell mass forming the embryo proper and some associated extraembryonic tissue.

By the 64-cell stage, a large blastocoel has formed and the inner cell mass is displaced to one side of the blastocyst. There is now polarity to both the inner cell mass (a blastocoel-facing side and a trophoblast-facing side) and the trophoblast (the polar trophoectoderm in contact with the inner cell mass and the opposite side, not in contact with the inner cell mass, the mural trophoectoderm). This polarity plays an important role in subsequent development. The cells of the inner cell mass that face the blastocoel flatten and partition themselves from the remainder of the inner cell mass. These cells eventually form an epithelium and represent the murine hypoblast or primitive endoderm. The remaining cells within the inner cell mass become the primitive ectoderm or the epiblast. The cells of the primitive endoderm divide and some of the progeny migrate to cover the surface of the mural trophoectoderm, where they are known as the parietal endoderm. The cells of the primitive endoderm that remain in contact with the inner cell mass constitute the visceral endoderm.

By 5 days after fertilization (referred to as 5 days post coitum or 5 dpc), the blastocyst hatches from the zona pellucida and implants into the uterine wall. During this time the polar trophoectodermal cells have accumulated to form a pyramidal mass of cells. The outermost surface of the mass (i.e., the surface that faces the uterine wall) invades the uterine wall, forming the ectoplacental cone; the remainder of the polar trophoectoderm forms the extraembryonic ectoderm, namely, the ectoderm of the chorion. Cells of the mural trophoectoderm also invade the uterine walls, leaving behind the parietal endoderm. The latter becomes adherent to a thickened basement membrane called Reichart's membrane. At this stage in development, the endoderm of the embryo proper encases an epiblastic core; during subsequent turning of the embryo, this configuration is reversed, so that the ectoderm comes to lie on the outside of the embryo and the endoderm, on the inside, the typical situation present in the other vertebrate model organisms.

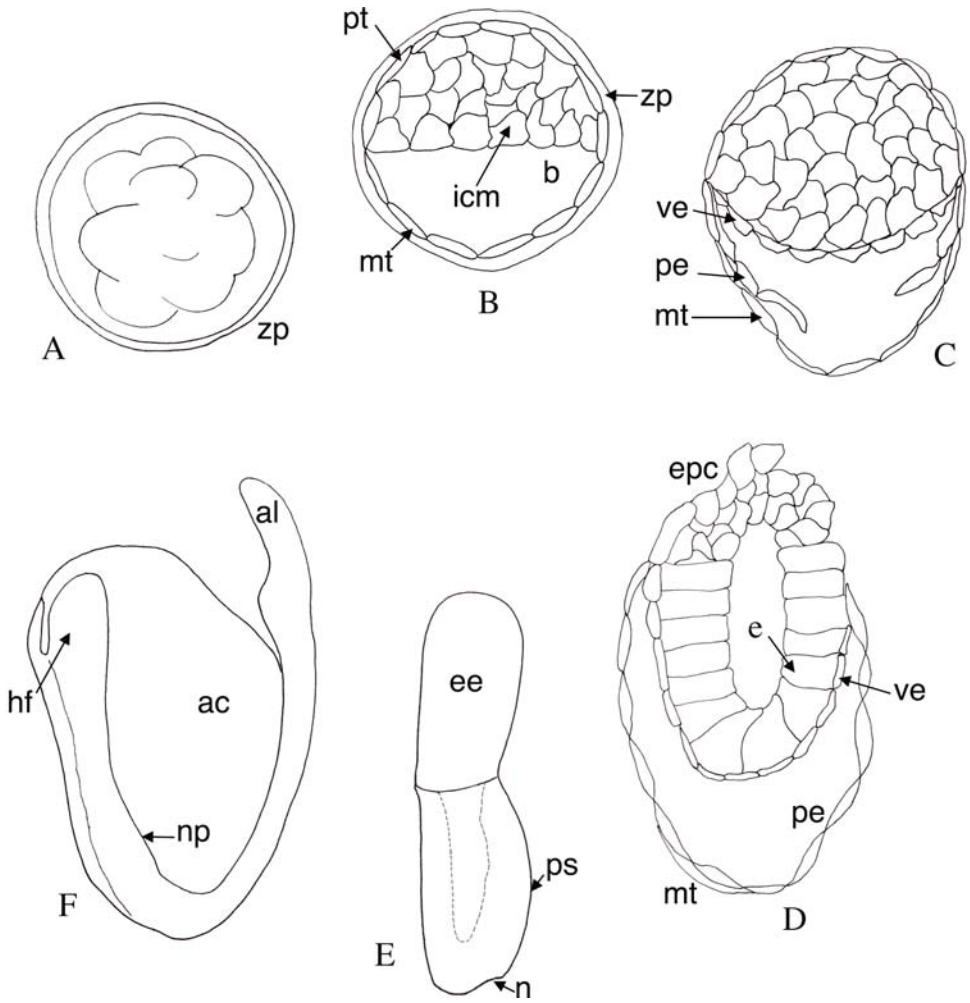

FIGURE 6. Mouse development leading up to neurulation. Diagrams of embryos at (A) morula, (B–D) blastocyst, (E) gastrula, and (F) neurula stages. Once fertilized, the mouse embryo cleaves within the confines of the zona pellucida (zp), an extracellular membrane important in preventing premature implantation and lost at the blastocyst stage (C). At the third cell division, the cells of the embryo undergo compaction to form the morula (A). With formation of the blastocyst (B), the inner cell mass (icm) and trophoblast can be identified; the latter becomes subdivided into mural trophectoderm (mt) and polar trophectoderm (pt). The inner cell mass will form the embryo proper, as well as contribute to the extraembryonic tissue. The cells of the inner mass that face the blastocoel (b) form the hypoblast or primitive endoderm. The latter gives rise to the visceral endoderm (ve) and parietal endoderm (pe; C). The remaining cells of the inner cell mass form the epiblast (D). By the late blastocyst stage (D), the epiblast has cavitated and now forms a cylindrical structure encased in visceral endoderm; the composite is known as the egg cylinder. The polar trophectoderm now forms a structure known as the ectoplacental cone (epc). The primitive streak (ps) of the mouse is initiated at the caudal end of the egg cylinder, and like the chick primitive streak, it is the site of ingression of cells that will form the mesoderm and definitive endoderm (E). The streak extends rostrally and eventually forms a knot of cells, known as the node (n), the organizer of the mouse embryo. To view embryos at this stage, the trophoblast is typically removed revealing the extraembryonic ectoderm (ee) and cup-shaped blastoderm containing epiblast on the inside of the cup and endoderm on the outside (E). At neurula stages (F), the neural plate (np) has formed and the body plan is apparent. The neural folds jut forward as the head folds (hf). Two extraembryonic membranes are visible at this stage: the amnion and allantois (al). The former encloses the developing embryo within the amniotic cavity (ac). Modified from Cruz (1997).

As implantation is occurring, the epiblast (i.e., the primitive ectoderm) cavitates to form the amniotic cavity, and growth transforms the conceptus into the egg cylinder. It is likely that the constraints of the uterine wall cause the epiblast (and adherent visceral endoderm) to assume this shape, reminiscent of a round-bottomed shot glass. During gastrulation, the epiblast will give rise to the embryo proper and also to the extraembryonic mesoderm (of the allantois and chorion).

Gastrulation of the mouse embryo commences with the formation of the primitive streak, at around 6 dpc, in the epiblast. It is during these stages that similarities with chick gastrulation become apparent. Like in the chick embryo, epiblast cells migrate through the primitive streak to form the mesoderm and definitive endoderm. As development proceeds, the streak elongates until, at 7.5 dpc, it reaches its maximal length. The distal tip of the streak is known as the node, the equivalent of Hensen's

node in the chick, the dorsal lip in amphibians and the embryonic shield in fish; the node shares many of the same properties as the organizer in the other models and as such, it constitutes the murine organizer (Beddington, 1994; see also Box 1). The cells that migrate through the node become axial tissues, whereas those emanating from the rostral streak just caudal to the node give rise to paraxial mesoderm and endoderm. The definitive endoderm, as in the chick, displaces the hypoblast/visceral endoderm rostrally during its formation. The rostral displacement of the visceral endoderm plays an important role in the patterning of the embryo, which is more fully described in the subsequent chapter, with the anterior visceral endoderm acting in the generation of the forebrain (Thomas and Beddington, 1996), and the node acting in the induction of the neural plate caudal to the level of the midbrain.

NEURAL INDUCTION

The identification of the organizer prompted a vigorous search for the biochemical nature of the neural-inducing signal, a quest that has lasted over 75 years. In the intervening period, studies were undertaken to address the nature of the inducing signal. Unsurprisingly, virtually all of the work was performed in amphibian embryos; their heritage, ease of culture, and establishment (through the work of Spemann and Mangold) of a simple assay for neural induction made the choice straightforward.

One of the main controversies was whether the induction signal acted vertically, emanating from the involuted dorsal mesoderm and acting upon the overlying ectoderm, or whether the signal acted in the plane of the ectoderm, emanating from the dorsal ectoderm prior to its involution into the interior of the embryo during gastrulation. Spemann's subsequent experiments suggested that the vertical signaling predominated. Using the "einsteckung" method, he inserted the organizer into the blastocoel of the embryo, finding that a secondary axis could be induced (Geinitz, 1925). Extending these results, he found that whereas dorsal mesoderm was able to induce a secondary axis, dorsal ectoderm could not (Marx, 1925). In subsequent experiments, Holtfreter found that when the animal ectoderm was wrapped around pieces of notochord, neural tissue was induced (Holtfreter, 1933a). Similar experiments in the chick (Smith and Schoenwolf, 1989; van Straaten *et al*., 1989) showed that the notochord acts vertically on the overlying ectoderm. This strengthened the argument for vertical signals emanating from the dorsal axial tissue. Holtfreter also devised an experimental scheme unique to amphibian embryos (Holtfreter, 1933b). When blastulae are placed in a high salt solution, cells do not involute into the interior during gastrulation; instead, they expand outward to form what is known as an exogastrula—a mass of mesoderm and endoderm attached to an empty sac of ectoderm. In such cases, vertical signals cannot occur, as the two tissues are never juxtaposed vertically. Holtfreter found that no morphologically recognizable neural tissue was present in exogastrulae, indicative of the need for vertical signaling. This experiment has revisited using molecular markers. Kintner and Melton (1987), using *Xenopus* embryos, found that although the neural tissue was not morphologically apparent, neural markers such as N-CAM could be detected. This led to the argument that a planar signal initiated neural induction. An alternative explanation is that the dorsomost mesoderm and endoderm of *Xenopus* is placed under the dorsal blastopore lip during pre-gastrula movements; thus, these cells are in a position to signal vertically even in exogastrulae (Jones *et al*., 1999). Unfortunately, there are currently little data distinguishing planar from vertical signaling in amniotes; however, the current thinking is that both modes of neural induction can occur.

Although much headway has been made into the identification of the tissues producing the neural-inducing signal, as well as the timing of neural induction, the identity of the inducing signal remained elusive. In early studies, it was discovered that neural induction could be initiated by a variety of tissues, ranging from the extract of a fish swim bladder to guinea pig bone marrow (Grunz, 1997). This proved quite exciting; perhaps, it would be easier to purify the signal from adult tissue, which was present in far greater mass and lacked yolk, which made amphibian tissues difficult for biochemical purification studies. Tiedemann showed that the phenol phase of an extract of an 11-day chick embryo was able to neuralize animal caps, demonstrating that proteins were the likely candidate for the inducing signal (Tiedemann and Tiedemann, 1956). Saxén (Saxén, 1961) and Toivonen (Toivonen and Wartiovaara, 1976) separated organizers juxtaposed to animal caps by using filters that excluded cell–cell contact. Their results showed that neuralization could still occur in the absence of direct cell–cell contact, indicating that the responsible protein was diffusible.

This is not quite the case in *Xenopus*. The *Xenopus* animal cap is resistant to induction by "nonspecific" neural inducers (Kintner and Melton, 1987), and it is also resistant to autoneuralization; however, these attributes have been more of an asset than a liability, as *Xenopus* tissues allow a more stringent test of candidate neural inducers. Thus, most modern studies on the molecular nature of the neural-inducing substance have used this amphibian and have relied heavily on the animal cap assay (Fig. 7).

The Default Pathway

As discussed below, neural fate is a default state, resulting from an inhibition of a non-neural fate within the ectoderm. There are some layers of complexity, but the majority data that have been gathered so far points to an inhibition of the inducing signal for the non-neural ectoderm. This is clearly true for amphibian (*Xenopus*) neural induction. However, the case for antagonistic signals inducing the nervous system of chickens and mice is less clear.

An indication that the neural fate may be a default one in the amphibian came from a number of studies where the *Xenopus* blastula animal cap was dissociated into single cells (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). By culturing the animal cap in media free of calcium and magnesium ions, the animal cap dissociates into a suspension of cells. If the ions are immediately added back, the animal cap cells

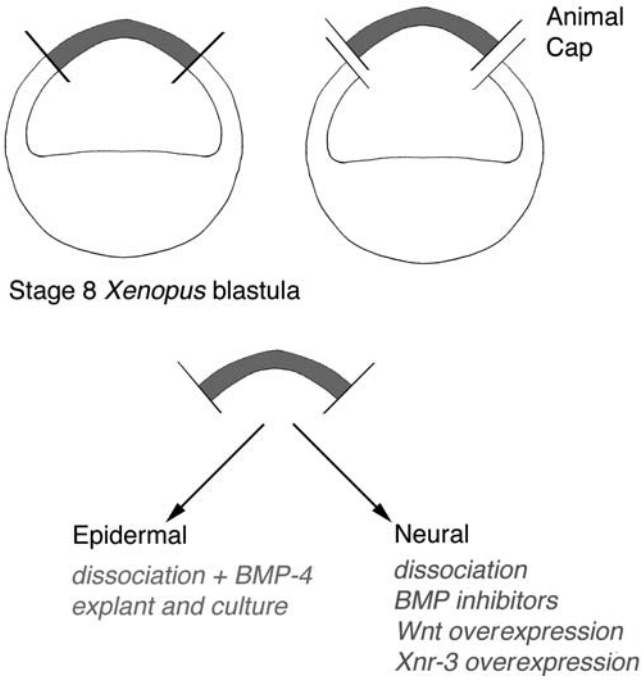

FIGURE 7. Neuralization of the *Xenopus* animal cap. Shown are the effectors required to cause the isolated animal cap of a blastula-staged *Xenopus* embryo to change its fate from epidermal to neural. Modified from Wilson and Edlund (2001).

reassociate and form epidermis, similar to the intact cap. If the reassociation is delayed, the fate of the animal cap cells once they are reassociated is neural. These results suggested that intact blastula animal caps had an activity that maintained non-neural character, an activity that was diluted out during dissociation. Grunz also made the finding that this activity was located in the extracellular matrix (Grunz and Tacke, 1990).

Noggin was first isolated as an activity able to rescue dorsal development in *Xenopus* embryos that had been ventralized by UV irradiation of the vegetal pole (Smith and Harland, 1992). Using *in situ* hybridization, noggin was found to be expressed first in the dorsal mesoderm and later in the notochord of the embryo. Both places had already been defined as sites of the neural-inducing signal. That the molecule was secreted, made its involvement in neural induction more likely. This role was confirmed when Lamb and Harland incubated *Xenopus* animal caps in a simple salt solution containing purified noggin protein (Lamb *et al*., 1993). These caps changed their fate from epidermis to neural. What made the activity of noggin unique was that it was able to directly induce the animal cap to become neural, without the concomitant induction of mesoderm. The induction of mesoderm and neural tissue had already been described for activin, a member of the TGF-β family (Box 2). In fact, the next neural inducer identified was a known inhibitor of activin activity, follistatin (Hemmati-Brivanlou *et al*., 1994). Like noggin, it was able to directly induce neural tissue in animal caps. The fact

BOX 2. The BMP Signaling Pathway

BMP-2 and BMP-4 are members of the TGF-β superfamily, a group with a large number of members and with diverse functions during development. The transduction pathway of these genes has become well known and what follows is a simplified description of the components of the pathway. For a more in-depth review of the transduction pathway, the reader is directed to a number of excellent reviews on the subject (Massagué and Chen, 2000; von Bubnoff and Cho, 2001; Moustakas and Heldin, 2002; Fig. 8).

Transduction of the BMP signal involves two kinds of serine/threonine receptors, the type 1 and type 2. The ligand binds preferentially to the type 1 receptor, causing a conformational change that allows the association of the type 2 receptor. The juxtaposition of the type 2 receptors results in its phosphorylation of the type 1 receptor within the key glycine/serine (GS-rich) domain (Wrana *et al*., 1994). The phosphorylation of the type 1 receptor causes the recruitment of Smad to the plasma membrane (Liu *et al*., 1996). There are a number of Smad molecules in the cell, and they form two distinct classes (Attisano and Tuen Lee-Hoeflich, 2001). The receptor-regulated Smad or R-Smads, associate with the type 1 receptor via an adaptor protein, Smad Anchor for Receptor Activation (SARA) (Tsukazaki *et al*., 1998). In fact, the R-Smads themselves can be split into two subclasses; Smad2 and Smad3 transduce responses elicited by activin or TGF-β signals, whereas Smad1, Smad5, and Smad8 generally transduce the BMP response (Attisano and Tuen Lee-Hoeflich, 2001). The association between Smad and the type 1 receptor results in the serine phosphorylation of the R-Smad, releasing it from the SARA/type 1 receptor complex. The phosphorylated R-Smad can now associate with the second class of Smads, the Co-Smad, usually Smad4, or additionally in *Xenopus*, Smad10. The R-Smad/Co-Smad complex results in the nuclear translocation of these molecules (Lagna *et al*., 1996). Once in the cytoplasm, the Smads complex acts as coordinators for the assembly of a number of transcription factors and thereby modulates the transcription of specific genes.

The BMP signal transduction pathway is also subjected to intracellular antagonism, an aspect that provides negative feedback for BMP activity. As well as the R-Smads that are responsible for activating BMP responsive genes, there are at least two inhibitory Smads (I-Smads), Smad6 and Smad7, which associate with the type 1 receptor to prevent the binding of the R-Smad/SARA complex (Imamura *et al*., 1997; Tsuneizumi *et al*., 1997; Inoue *et al*., 1998; Souchelnytskyi *et al*., 1998). It seems that the expression of I-Smad is induced by BMP activity itself (Nakao *et al*., 1997; Afrakhte *et al*., 1998). Another intracellular inhibitor is BMP and Activin Membrane Bound Inhibitor (BAMBI). BAMBI shows considerable sequence homology to the BMP receptors, but lacks the intracellular kinase domain, making it a naturally occurring dominant negative receptor (Onichtchouk *et al*., 1999). Homologues have been identified in mouse (Grotewold *et al*., 2001), humans (Degen *et al*., 1996), and zebrafish (Tsang *et al*., 2000). The expression pattern correlates well with the expression of BMP-2 and BMP-4, and indeed BAMBI is induced by BMP-4 expression and is lost in zebrafish mutant for bmp-2b (Tsang *et al*., 2000).

Another feature of the BMP pathway is its ability to intersect with other signaling pathways (von Bubnoff and Cho, 2001). Particularly pertinent to this consideration of neural induction is the interaction, within the cell, with signaling from the fibroblast growth factor (FGF) family of molecules and the wingless/wnt group. Both can negatively influence BMP activity, and this is particularly germane to the role of these factors in the induction of the nervous system in amniotes.

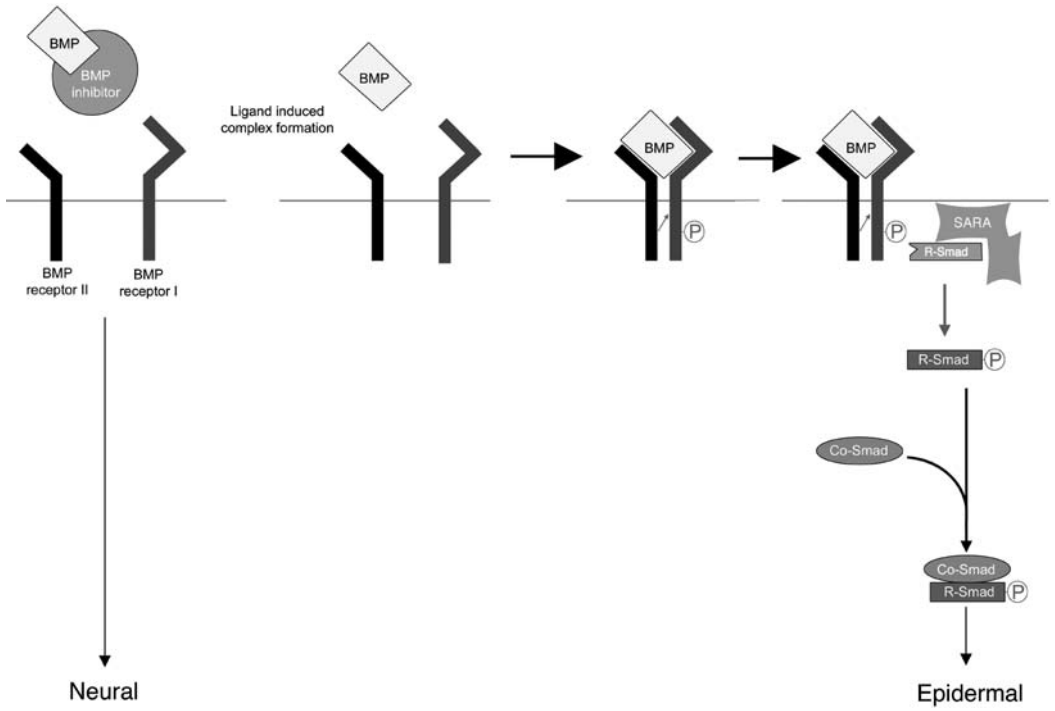

FIGURE 8. The BMP signal transduction pathway. BMP activity specifies the ectoderm as epidermal; its inhibition (e.g., by binding to a soluble inhibitor-like chordin) leads to neural induction. Ligand binding induces the type I and type II receptors to associate and causes the phosphorylation of the intracellular intermediate R-Smad, held in place by the adaptor molecule SARA. R-Smad is now free to associate with a Co-Smad, causing translocation into the nucleus, where the complex participates in the transcriptional modulation of a number of genes. Modified from von Bubnoff and Cho (2001).

that follistatin, an inhibitor of TGF-β signaling, was able to induce neural tissue suggested that inhibition of a pathway involving perhaps activin was responsible for the induction of neural ectoderm. These data were supported by studies using a truncated receptor for activin. RNA encoding the activin receptor lacking the transducing, cytosolic domain but with the extracellular and transmembrane domains, acts as a dominant negative, that is, although ligand binding can occur, it is unable to elicit a response (Hemmati-Brivanlou and Melton, 1992). As this modified molecule is present in far excess of the wild-type molecule, it has the effect of sequestering the ligand. Animal caps that express the dominant negative, truncated activin receptor follow a neural pathway of differentiation (Hemmati-Brivanlou and Melton, 1994).

This led to somewhat of a paradox. Though it seemed that neural induction was a result of activin inhibition, activin itself induced mesoderm and neural ectoderm. In actuality, the activin receptor used by Hemmati-Brivanlou and Melton was not specific for activin; rather it recognized other members of the TGF-β superfamily (Hemmati-Brivanlou and Melton, 1994). As the truncated receptor also induced dorsal mesoderm, rather than recognizing activin, another TGF-β family member active on the ventral side of the embryo could be the native ligand.

BMP-2 and BMP-4, members of the TGF-β superfamily, are both expressed in the ventral part of the embryo (Dale *et al*., 1992; Jones *et al*., 1992). Consequently, their potential role in neural induction was placed under scrutiny, which grew more intense with the discovery of chordin, another secreted molecule capable of inducing neural tissue. Chordin was discovered by virtue of its expression in Spemann's organizer. Later, it is expressed in the axial tissue of the prechordal mesoderm and notochord, all structures capable of neural induction (Sasai *et al*., 1994). Examination of the primary sequence of chordin provided further insight into the mechanism of neural induction. It was found that chordin shows considerable homology to the fruit fly *Drosophila* gene *short of gastrulation* (*sog*). Genetic analysis in *Drosophila* had already shown that *sog* acted as an antagonist to another gene, *decapentaplegic* (*dpp*), which is homologous to the vertebrate genes BMP-2 and BMP-4. The similarities with flies are not limited to the sequence (Holley *et al*., 1995). In flies, eliminating *dpp* converts the epidermal cells of the fly into neuroectoderm. Overexpression of *dpp* changes the fate of neuroectodermal cells into epidermal (Biehs *et al*., 1996). In the amphibian, BMP-4 is also expressed in the non-neural ectoderm, consistent with it being an epidermal inducer. Moreover, when BMP-4 is added to dissociated animal cap cells, neural induction is prevented regardless of how long reassociation is delayed (Wilson and Hemmati-Brivanlou, 1995). Overexpressing BMP-4 RNA on the dorsal side of the embryo results in an embryo with a loss of neural ectoderm. However, it should be noted that dorsal mesoderm, the primary neural-inducing tissue, is also missing (Dale *et al*., 1992; Jones *et al*., 1992). The data pointed to neural induction occurring by inhibition of the BMP pathway, and indicated that perhaps not only chordin, like its *Drosophila*

counterpart *sog*, but also noggin and follistatin acted as antagonists of BMP activity. Indeed chordin, noggin, and follistatin bind to BMP-4 and the closely related BMP-2 (Piccolo *et al*., 1996; Zimmerman *et al*., 1996; Iemura *et al*., 1998), and from genetic analysis in *Drosophila*, where chordin or noggin were ectopically expressed in various fly mutants in components of the BMP pathway, the site of action of chordin and noggin was placed upstream of the receptor, in the extracellular matrix (Holley *et al*., 1995, 1996). An additional number of extracellular, secreted antagonists of BMP activity have been found. These molecules, such as Cerberus, Gremlin, and Xnr-3 (*Xenopus* nodal related-3), all induce neural fates in the animal cap of the *Xenopus* embryo (Smith *et al*., 1995; Bouwmeester *et al*., 1996; Hsu *et al*., 1998).

Further support for the idea that BMP inhibition is germane to the induction of neural tissue came from inhibiting the intracellular components of the BMP signal-transduction pathway (Box 2). As well as the truncated activin receptors, acting as dominant negative forms of the endogenous receptor, which have been shown to bind BMP-2 and BMP-4, negative forms of the Smad molecules have been shown to promote neural differentiation in the animal cap (Liu *et al*., 1996; Bhushan *et al*., 1998). Indeed, even negative forms of the transcription factors that form the nuclear response to BMP signaling have been shown to neuralize the animal cap (Onichtchouk *et al*., 1998; Trindade *et al*., 1999). Many of these experiments have been repeated in the zebrafish embryo, with similar, if not identical, results (e.g., Imai *et al*., 2001).

Complexities and Questions

That BMP inhibition, emanating from the organizer, is responsible for neural induction has been well demonstrated in anamniote (fish and frog) embryos. However, the data from the chick and mouse are confusing and challenge this idea.

Is the Organizer Responsible for Neural Induction?

The role of the chick and mouse equivalents of the organizer—Hensen's node and the node, respectively—in neural induction has been questioned over the years. In the chick, neural induction can occur even after the node is surgically ablated (Waddington, 1932; Abercrombie and Bellairs, 1954). This result was interpreted as showing that Hensen's node, though sufficient for neural induction, was not necessary. However, subsequent studies have shown that after extirpation, the node is reconstituted quickly owing to a series of complex inductive interactions (Yuan *et al*., 1995; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1998, 1999; Joubin and Stern, 1999). Genetic ablation of the node and notochord in the mouse and fish also has little effect on the induction of neural tissue (Gritsman *et al*., 1999; Klingensmith *et al*., 1999). Recently, it has become clear that neural induction in all vertebrates occurs earlier than previously thought, beginning before the appearance of a morphologically distinct organizer. For example, in chick, neural induction begins before the appearance of Hensen's node, as determined by the stage at which explants of *prospective* neural ectoderm first express neural markers (Darnell *et al*., 1999; Wilson *et al*., 2000). In *Xenopus*, neural induction is initiated before gastrulation. Using the clearance of the expression of components of the BMP signaling pathway as a marker for when neural induction is occurring, it has been shown that neural induction occurs during late blastula stages of *Xenopus* embryogenesis (Hemmati-Brivanlou and Thomsen, 1995; Faure *et al*., 2000).

In fish containing the mutation one-eyed-pinhead (oep), the embryonic shield and dorsal mesoderm do not form. Despite this, these mutants still express chordin, indicating that some neural-inducing activity still persists (Gritsman *et al*., 1999). The situation in the mouse HNF-3β mutant is more striking. Even in the absence of a node and axial mesoderm, and despite the lack of expression of many markers of the mouse organizer, the rostral streak, from which the node derives, is still capable of neural induction (Klingensmith *et al*., 1999).

Is BMP Inhibition Sufficient for Neural Induction?

Experiments again in the chick first questioned the hypothesis that BMP inhibition mediates neural induction. Streit and coworkers showed that neural tissue could not be induced by clumps of noggin- or chordin-expressing cells, even though grafts of Hensen's node in parallel experiments induced neural tissue (Streit *et al*., 1998). In the same study, Streit *et al*. (1998) showed that cells expressing BMP-2 or BMP-7 failed to inhibit neural plate formation. However, Wilson and coworkers showed that BMP-4 was able to induce epidermis in explants of the chick embryos fated to become neural ectoderm (Wilson *et al*., 2000). The difference between these sets of data seem to be the stage at which the experiments were performed, with the experiments using expressing cells being done at mid-gastrula stages, and the explant-induction experiments being done at blastula to early-gastrula stages. In the mouse, null mutants of BMP-2 (Zhang and Bradley, 1996), BMP-4 (Winnier *et al*., 1995), and BMP-7 (Dudley *et al*., 1995) do not alter their pattern of neural induction. However, there is probably functional redundancy between these molecules, with one compensating for the loss of another (Dudley and Robertson, 1997). Compound mutants have not yet been established to address this issue.

The expression patterns in the chick of the BMP inhibitors noggin, follistatin, and chordin are not strictly correlated with tissues that contain neural-inducing ability (Connolly *et al*., 1995, 1997; Streit *et al*., 1998). Taken with the data from mice doubly mutant for noggin and chordin, which still have neural tissue (Bachiller *et al*., 2000), this seems to indicate that BMP inhibition is not required for neural induction in amniotes. However, as discussed above, there are other inhibitors of BMP signaling, both extracellular and intracellular, which may account for neural induction (von Bubnoff and Cho, 2001; Muñoz-Sanjuan and Hemmati-Brivanlou, 2002). For example, support for the idea that BMP inhibition induces neural character in the chick embryo comes from an inspection of the localization of phosphorylated Smad1, -5, and -8. Using an antibody that recognizes the activated form of these Smads as an indication of BMP signaling,

Faure *et al*. (2002) showed that there is no BMP signaling activity in the forming neural plate. An argument has also been made that BMP inhibition merely stabilizes and reinforces neural cell fates, and that other families of signaling molecules are the primary neural inducers (Streit and Stern, 1999). Until the full complement of molecules that can induce neural tissue is known, and a full understanding of the signaling networks is understood, this question will not be fully resolved.

The Role of Other Signals in Neural Induction

Fibroblast Growth Factors (FGF)

Both the FGF family and the wnt family have been shown to play a role in the induction of neural tissue. This role is distinct from their roles in patterning of the neural tube, which are discussed in the subsequent chapter. In *Xenopus*, FGF can actually induce neuralization of animal cap cells that have undergone brief dissociation, a procedure that diminishes the amount of BMP activity (Kengaku and Okamoto, 1993). Furthermore, blocking FGF signaling using a truncated FGF receptor makes the animal cap refractory to neuralization by low amounts of chordin (Launay *et al*., 1996). In chick, the role of FGF in neural induction has received considerable attention. Streit *et al*. (2000) reported that an FGF-responsive gene, Early Response to Neural Induction (ERNI), marks the territory in the chick epiblast fated to become neural, and it rapidly induced FGF expression. By using an FGF receptor antagonist, SU5402, Wilson *et al*. (2000) showed that neural differentiation could be blocked in chick epiblast explants normally fated to become neural ectoderm. The exact role of the FGF pathway in neural induction is unclear. Some of the data point to a role for FGF signaling in aiding the clearance of BMP activity from the neural plate; indeed, downstream effectors of the FGF pathway have been shown to inhibit the nuclear accumulation of the R-Smad/Co-Smad complex (Kretzschmar *et al*., 1997, 1999). FGF may also induce neural tissue by a mechanism independent of BMP inhibition. An investigation of Smad10, a Co-Smad, in *Xenopus*, has yielded some relevant data (LeSeur *et al*., 2002). Smad10, a component of the BMP signaling pathway, actually induces neural tissue within the animal cap. More surprisingly, by removing Smad10 protein using antisense oligonucleotides, neural tissue is never formed in the affected embryos. Using co-injection studies, it has been found that Smad10 cannot inhibit the BMP pathway, indicating some other mechanism for its function. One such mechanism is the identification of a site in the Smad10 protein that becomes phosphorylated and activated as a result of FGF signaling (LeSeur *et al*., 2002).

An alternative view suggests that FGF signaling provides the ectoderm with competence to become defined as neural. There is precedence for this; Cornell *et al*. (1995) have shown that FGF signaling acts to define the competence of tissue to respond to mesoderm induction by TGF-β signals in *Xenopus*, the very same tissue that can respond to neural-inducing signals.

In fact, it is likely that both a competence-defining role early in development and a later neural-stabilizing role will be shown for the FGF family. However, like many of the controversies surrounding neural induction, we will have to wait until all the players and the way they interact are known before adequate resolution can be achieved.

Wnts

The role of the wnt family of molecules has also been investigated during the induction of neural ectoderm. In the chick, wnt overexpression converts the epiblast fated to become neural to become epidermal (Wilson *et al*., 2001). Conversely, in presumptive epidermal tissue fated to form epidermis, wnt inhibition causes the explant to take on a neural fate. In addition, at a sub-threshold concentration of wnt inhibitors, below the level required for neural induction in the epidermal epiblast explants, BMP inhibition and FGF signaling were able to induce neural ectoderm. One proposed mechanism is that wnt signaling causes an upregulation of BMP expression (Wilson *et al*., 2001), and thereby induces epidermal fate, although in *Xenopus*, additional data suggest that wnt expression downregulates BMP expression (Baker *et al*., 1999; Gomez-Skarmeta *et al*., 2001). However, wnt signaling may also regulate the strength of the transduced BMP signal via activation of the calmodulin/Ca^{2+} pathway (Zimmerman *et al*., 1998; Scherer and Graff, 2000). This may explain why BMP inhibition cannot induce neural tissue in epidermal epiblast explants. If the level of abrogation of BMP signaling is not complete, the sensitized transduction pathway can still receive an input, resulting in epidermal cell fates. If, however, wnt signaling is also inhibited, reception is desensitized and when combined with BMP inhibition, can lead to neural cell fates. Interestingly, two naturally occurring inhibitors of wnt signaling, FrzB and Sfrp-2, are expressed in the presumptive neural plate at around the stages that neural induction has been proposed to be occurring (Ladher *et al*., 2000).

Insulin-Like Growth Factor

The insulin-like growth factor (IGF) family can also neuralize the *Xenopus* animal cap (Pera *et al*., 2001). The necessity for IGF signaling has also been shown using a truncated IGF receptor. In these embryos, neural induction mediated by noggin is inhibited. The authors propose that the IGF pathway may act downstream of BMP inhibition during neural induction, and that as well as a passive role for BMP inhibition, neural induction may not be a default as previously thought. Instead, it may also require an active signal, induced as a result of BMP inhibition.

Summary of the Molecular Events of Neural Induction

As discussed above, the main mechanism by which the neural ectoderm is induced is via the inhibition of the BMP pathway. Other factors do play a role, namely the FGF family and the wnt family. As yet it is unclear what the exact roles of these molecules are, whether they are required as competence factors or whether they act to aid the clearing of BMP signals and their reception from the neural plate.

Once induced, the neural ectoderm—also known at this juncture as the neuroepithelium—still has a daunting journey ahead of it to form the central nervous system: it must roll up into a tube, which is subsequently patterned. We will describe in the next section the mechanism by which the specified neural ectoderm becomes a tube; other chapters later in this book deal with the elaboration of the neural tube into the adult central nervous system.

NEURULATION

The process of neural induction results in a plate of cells running along the rostrocaudal length of the embryo. The medial part of the neural plate will eventually form the ventral part of the neural tube, and the lateromost edges will be brought together to form the dorsal part of the tube during the process of neurulation. The end result of neurulation is a hollow nerve cord.

Neurulation can be subdivided into a number of events, each requiring different interactions. First, neurulation occurs in two phases called primary and secondary neurulation. When one speaks of neurulation, they are typically referring to primary neurulation, a process that occurs in four stages defined as formation, shaping, and bending of the neural plate, and closure of the neural groove (Figs. 9 and 10). Each stage will be described in turn. This discussion focuses primarily on the chick embryo, as most of the mechanistic studies have been performed on this embryo. For a more in-depth discussion, the reader is directed to several reviews (Schoenwolf and Smith, 1990; Smith and Schoenwolf, 1997; Colas and Schoenwolf, 2001).

Formation of the Neural Plate

The neural plate is a thickened region of the ectoderm located medially within the embryo. The thickening forms by an apicobasal elongation of ectodermal cells, an action known as cell pallisading. The thickening of the neural plate is not a result of an increase in the number of cell layers; the neuroepithelium remains pseudostratified (see Fig. 9A). It has been shown that thickening of the neural plate is an intrinsic property of the ectodermal cells once they have been induced as neural (Schoenwolf, 1988).

Shaping of the Neural Plate

During shaping, different cell behaviors convert the neural plate from a relatively short (in the rostrocaudal axis) and squat (wide in the mediolateral plane) structure to one that is long and narrow (see Fig. 10). This results from a combination of continued cell elongation, convergent extension, and cell division, as well as the caudalward regression of the primitive streak (Schoenwolf and Alvarez, 1989; Schoenwolf *et al*., 1989).

Neuroepithelial cells continue their apicobasal elongation during shaping, a process initiated shortly after neural induction and resulting in formation of the neural plate. As a result of cell elongation, a concomitant narrowing of the neural plate occurs, as neural plate cells maintain their individual volumes. Convergent extension movements further exaggerate the narrowing of the neural plate; that is, cells of the neural plate intercalate in the mediolateral plate, effectively causing the neural plate to lengthen rostrocaudally while narrowing simultaneously. Cell division also contributes to the lengthening of the neuroepithelium; about half of the division planes are oriented such that they place the daughter cells into the length of the neural plate rather than adding to its width (Sausedo *et al*., 1997). Isolation experiments have shown that the cell behaviors causing shaping of the neural plate are autonomous to the neural plate. In other words, such changes in cell behavior within the neural plate generate intrinsic forces for its shaping. However, for the shaping of the neural plate to occur completely normally, normal gastrulation movements also must occur, as the axis develops in the wake of the regressing Hensen's node.

Bending of the Neural Plate

Bending involves the establishment of localized deformations of the cells of the neuroepithelium and the subsequent elevation of the two flanks of the neuroepithelium, converting it from the neural plate to the neural groove. Bending is actually driven by two distinct types of movement: furrowing and folding (Colas and Schoenwolf, 2001). Furrowing is a behavior intrinsic to the hinge points within the neuroepithelium. There are three hinge points within the neural plate: a single median hinge point, found along the neuroaxis (except at the future forebrain level) and coincident with the floor plate of the neuroepithelium; and the paired (right and left) dorsolateral hinge points, found primarily at levels where the brain will form (Fig. 11; Schoenwolf and Franks, 1984). Neuroepithelial cells within the hinge points undergo wedging, that is, apical constriction with a concomitant basal expansion, driven in part by the basalward interkinetic movement of the nucleus (see Fig. 11; Smith and Schoenwolf, 1987, 1988). This acts not only to deform the neuroepithelium, creating a furrow, but it also provides points around which the neural plate can rotate during folding; that is to say, true to their nomenclature, the hinge points do act like hinges during neurulation.

Folding is a more complicated process and is driven by the non-neural ectoderm. The net result is rather like closing a pair of calipers. The easiest way to close calipers is to apply a force laterally at the tip of the calipers, and eventually the tips will meet, folding around the hinge. Like calipers, the neural plate elevates and folds by forces generated laterally in the non-neural ectoderm. This force results, in part, from cell shape changes in the non-neural ectoderm (Alvarez and Schoenwolf, 1992; Sausedo *et al*., 1997). These cells undergo apicobasal flattening, thus effectively increasing their surface area. Folding itself can be divided into three distinct events (Fig. 12), occurring while the lateral epithelium provides a medialward force (Lawson *et al*., 2001). The first is epithelial kinking, where cells at the interface between the neural and non-neural ectoderm deform, with each

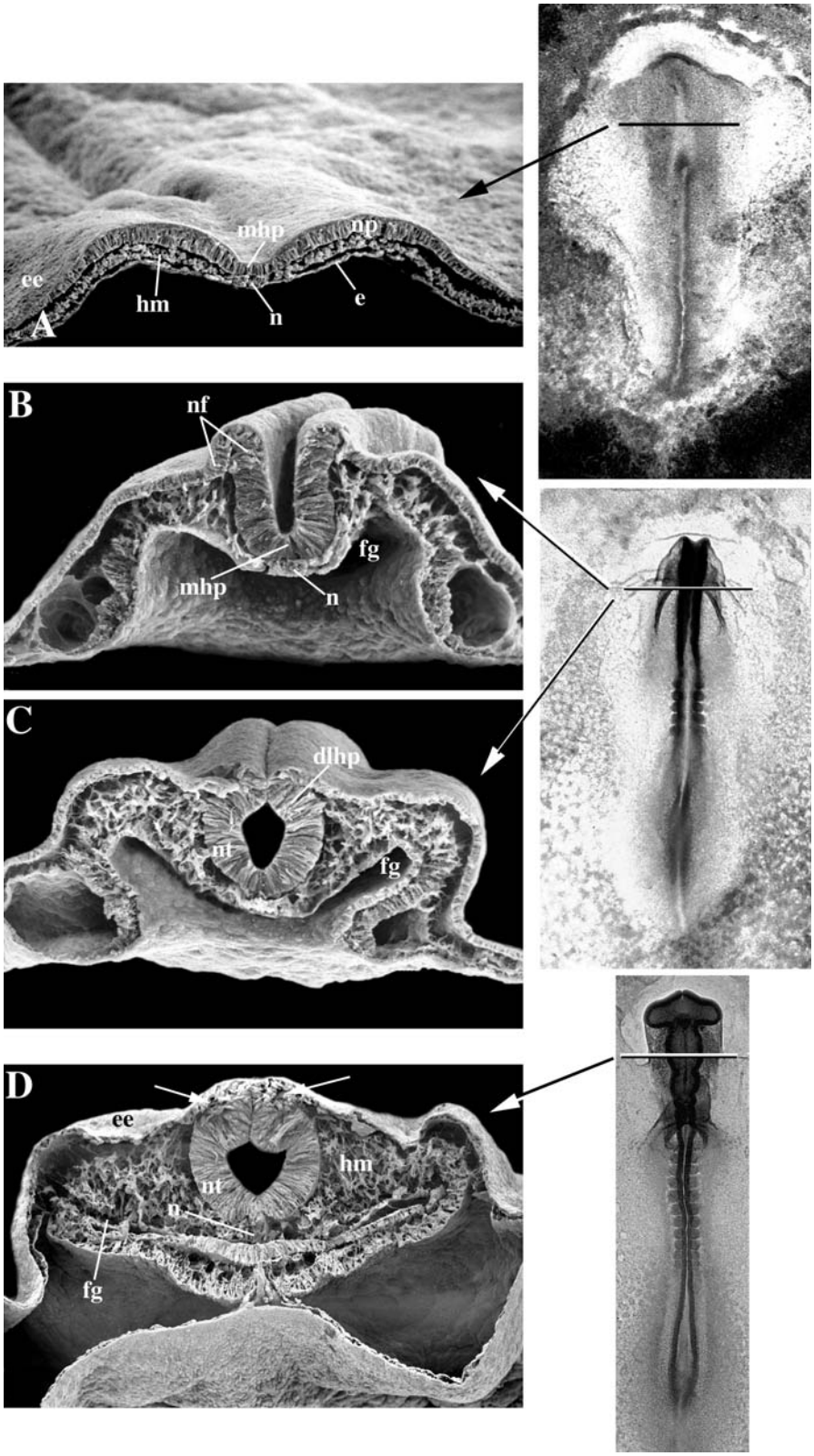

FIGURE 9. Whole mounts (for orientation; transverse lines indicate levels of cross-sections identified by long arrows) and scanning electron micrograph cross-sections of the neuroepithelium during neurulation. Shown are changes in the neuroepithelium that occur during the (A) formation, (B, C) shaping, and (A–C) bending of the neural plate, and closure of the neural groove (C, D). Details are provided in the text. dlhp, dorsolateral hinge point; e, endoderm; ee, epidermal ectoderm; fg, foregut; hm, head mesenchyme; mhp, median hinge point; n, notochord; nf, neural fold; nt, neural tube; arrows (D), neural crest cells. Modified from Schoenwolf (2001).

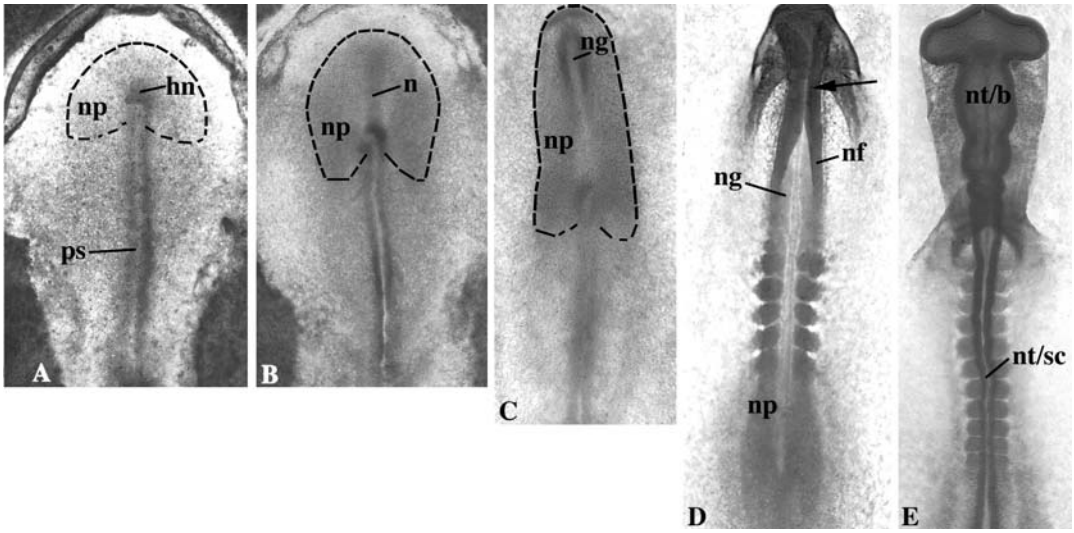

FIGURE 10. Whole mount embryos viewed from their dorsal side during neurulation. A–E indicate progressively older, yet partially overlapping, stages of neurulation, beginning with (A) formation of the neural plate, (B–E) shaping of the neural plate, (B–E) bending of the neural plate, and (D, E) closure of the neural groove. The neuroepithelium at the time of its formation is a relatively short and squat structure, as seen in surface view. However, during convergent extension movements that commence concomitant with regression of Hensen's node, the neural plate lengthens rostrocaudally and narrows mediolaterally. hn, Hensen's node; nf, neural fold; ng, neural groove; np, neural plate; ps, primitive streak; dashed lines, lateral borders of neural plate. Modified from Smith and Schoenwolf (1997).

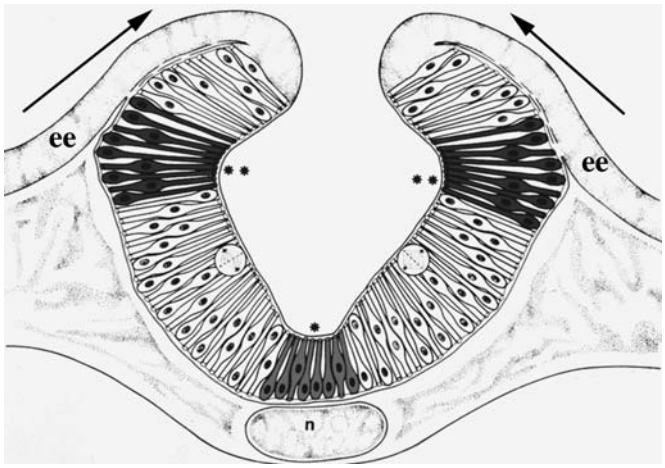

FIGURE 11. Cell behavior in the neural plate during its bending. Shown is a diagram of a cross-section through the neural tube during bending. Highlighted (darker shading) are the three hinge points: the median hinge point (asterisk), coincident with the floor plate of the neural tube, and the dorsolateral hinge points (double asterisks), found in the future brain level of the neuroaxis. ee, epidermal ectoderm; n, notochord; arrows, directions of expansion of the epidermal ectoderm. Modified from Schoenwolf and Smith (1990).

forming an inverted wedge that is apically expanded and basally constricted. The next step is epithelial delamination. This involves the deposition of extracellular matrix at the neural fold interface (i.e., the space between the two ectodermal layers of each neural fold) and a re-orientation of neural and non-neural cells around the interface, such that their basal surfaces abut. The final step is epithelial apposition, which occurs in the brain region. This is essentially a rapid expansion of the neural folds, with extension of the area of epithelial delamination in the mediolateral plane and further deposition of extracellular matrix along the expanding width of the neural fold interface. Additionally, the non-neural ectoderm intercalates and undergoes oriented cell division, thereby contributing to the mediolateral forces generated in the epidermal ectoderm.

Tissue isolation experiments have been used to identify the cell types responsible for generating the forces of folding (Schoenwolf, 1988). Removal of the lateral, non-neural ectoderm results in the loss of folding, but furrowing of the neural plate within the hinge points still occurs (Hackett *et al*., 1997). If the mesoderm and endoderm lateral to the neural plate are removed, but leaving the non-neural ectodermal layer intact, both folding and furrowing occur (Alvarez and Schoenwolf, 1992). Thus, the non-neural ectoderm is both necessary and sufficient for folding to occur.

Closure of the Neural Groove

Bending brings the tips of the neural folds into close contact at the site of the dorsal midline of the embryo. During closure, the two tips attach and fuse. Each component of the tip must fuse correctly, such that the non-neural epithelium forms a continuous sheet overlying the newly formed roof plate of the neural tube and the associated neural crest. The exact mechanism of this concluding step of neurulation is not well understood, and the molecules that mediate adhesion, epithelial breakdown, and fusion are not known.

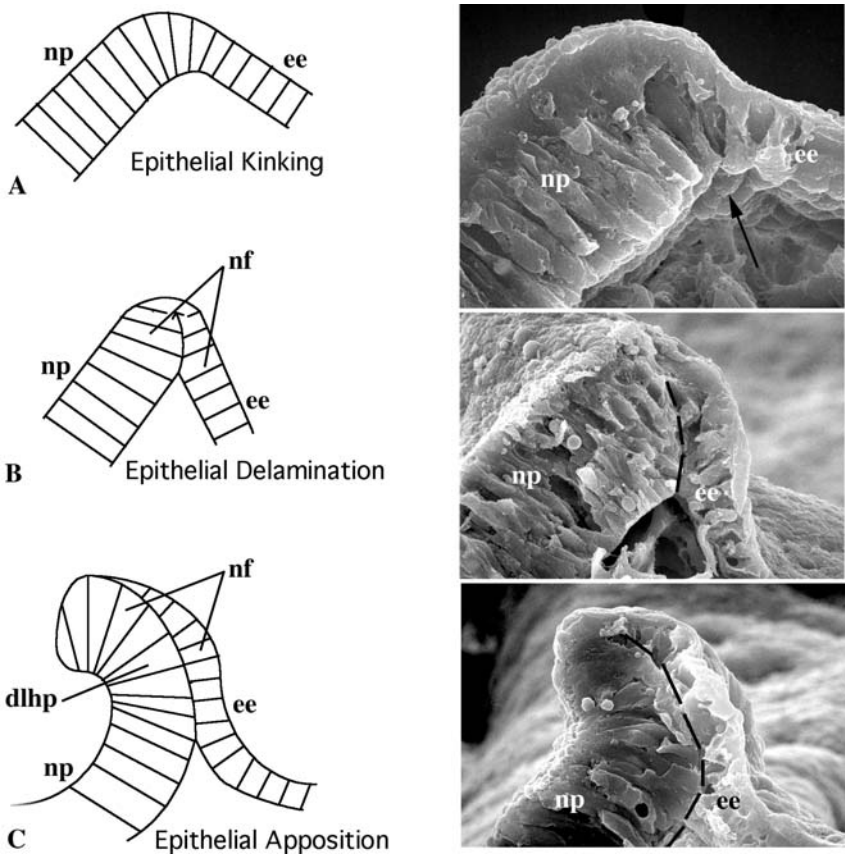

FIGURE 12. Formation of the neural folds. The scanning electron micrographs and accompanying diagrams highlight the formation and morphogenesis of the neural folds, in particular, (A) epithelial kinking, (B) delamination, and (C) (in the brain region) apposition. dlhp, dorsolateral hinge point; ee, epidermal ectoderm; nf, neural folds; np, neural plate; dashed lines, interface between the two ectodermal layers of the neural fold. Modified from Lawson *et al*. (2001).

Secondary Neurulation

At caudal levels of the neuraxis of birds and mammals (e.g., the lumbar and sacral regions), the neural tube develops in a manner distinct from more rostral regions. Caudal neural tube formation occurs through a process known as secondary neurulation. Rather than the rolling up of a flat plate of cells, as is the case in primary neurulation, secondary neurulation consists of the cavitation of a solid epithelial cord of cells in the tail of the embryo.

Secondary neurulation begins when cells within the tail bud condense to form an epithelial cord of cells, known as the medullary cord (Schoenwolf, 1979, 1984; Schoenwolf and DeLongo, 1980). The outer cells of the medullary cord then undergo elongation, forming a pseudostratified columnar epithelium similar to that of the neural plate during primary neurulation. This pseudostratified epithelium then becomes polarized, resulting in the formation and fusion of small lumina at the apices of the outer layer, around a central core of mesenchymal cells. These inner cells are removed during cavitation, by cell rearrangements and perhaps limited apoptosis. Cavitation results in the formation of a single, secondary lumen, which will join with the primary lumen of the rostral neural tube.

SUMMARY

The future central nervous system is derived from an unspecified sheet of ectoderm, with fate being instructed by signals emanating, in the main, from a specialized region of the early embryo, the organizer. The organizer secretes signals that have the net effect of inhibiting the BMP pathway, be it by extracellular antagonism or by intracellular modulation of the ability of the cell to perceive BMP signals. Other factors also play a role in neural induction, for example, the FGF family of molecules, but their exact role in neural induction remain unknown. As more players are identified in what undoubtedly will be a signaling network leading to neural induction, the exact molecular mechanism of neural induction can be established.

Once induced, the neuroepithelium rolls into the neural tube. One model, and one that has gained widespread acceptance, is the hinge point model. In this model, both extrinsic (i.e., outside the neural plate) and intrinsic forces cooperate and synergize in bending the neural plate. Although the cellular behaviors of much of this process have been well characterized, the molecular bases for these behaviors have so far proved elusive. The relationship between induction of the neuroepithelium and its

subsequent morphological movements is of particular interest to the developmental neurobiologist.

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