Cell Fate Determination in Embryonic Ectoderm

Chenbei Chang, Ali Hemmati-Brivanlou

Laboratory of Molecular Embryology, Rockefeller University, 1230 York Avenue,
New York, NY 10021

ABSTRACT: During gastrulation in vertebrates the cells of the embryonic ectoderm give rise to epidermal progenitors in the ventral side and neural progenitors in the dorsal side. Despite many years of scrutiny, the molecular basis of these important embryonic cell fate decisions have not been solved. Only recently have we witnessed swift progress in the quest for factors involved in neural and epidermal induction. Several of what seem to be bona fide *in vivo* neural and epidermal inducers have been cloned, and the mechanism of their functions in embryos is also beginning to be understood. These new molecular results have revolutionized our view on the patterning of embryonic ecto-derm and suggest that while the induction of epidermis requires instructive inductive signals, the establishment of neural fate occurs by default when epidermal inducers are inhibited. In this review, we discuss recent advances of our knowledge on epidermal and neural induction in the context of the ''default model''. We will then address the process of neurogenesis as well as recent findings on neural patterning. Emphasis is placed on, but not limited to, discoveries made in *Xenopus*, as most of our progress in understanding the ectodermal patterning is obtained from studies using this organism. © 1998 John Wiley & Sons, Inc.

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Early embryonic cells, by definition, are pluripotent. Like stem cells, many embryonic progenitor cells can assume different cell fates if placed at different positions. During development, a cell gradually loses its potential to differentiate into other cell types and becomes committed to a specific fate. Although the first cues for differentiation of a primitive embryonic body axis are set up by the mother (e.g., through uneven deposition of maternal materials in the egg), the final body plan is determined primarily by cell–cell interactions within the embryo. Signals transmitted within and across different germ layers are important to determine specific cell fates at particular embryonic positions, including cells in the ectodermal layer.

In the amphibian African frog *Xenopus laevis,* or in the chick, ectoderm normally develops into epidermis when isolated during cleavage stages (blastula) and cultured *in vitro. In vivo,* however, ectoderm from the dorsal side of a vertebrate embryo is induced to form neural tissue during gastrulation (in *Drosophila,* the nervous system derives from the ventral ectoderm) (De Robertis and Sasai, 1996). The neural plate is further patterned along both its anterior-posterior (A-P) and medial-lateral axes at neurula stages. By specific expression of molecular markers which correlate with future morphological features, the neural plate is divided into forebrain, midbrain, hindbrain, and spinal cord from anterior to posterior. On the medial-lateral level, the neural plate is patterned to form different cell types which eventually become different neurons and glial cells occupying unique dorsal-ventral (D-V) positions after neural tube closure. Multiple signals are engaged in successive cell fate specification during neural induction and subsequent patterning.

The first revelation of neural induction in the ectoderm came from studies of amphibian embryos more than 70 years ago. Spemann and Mangold (1924) discovered that when a region containing the dorsal lip of the blastopore was grafted into the

Correspondence to: A. Hemmati-Brivanlou
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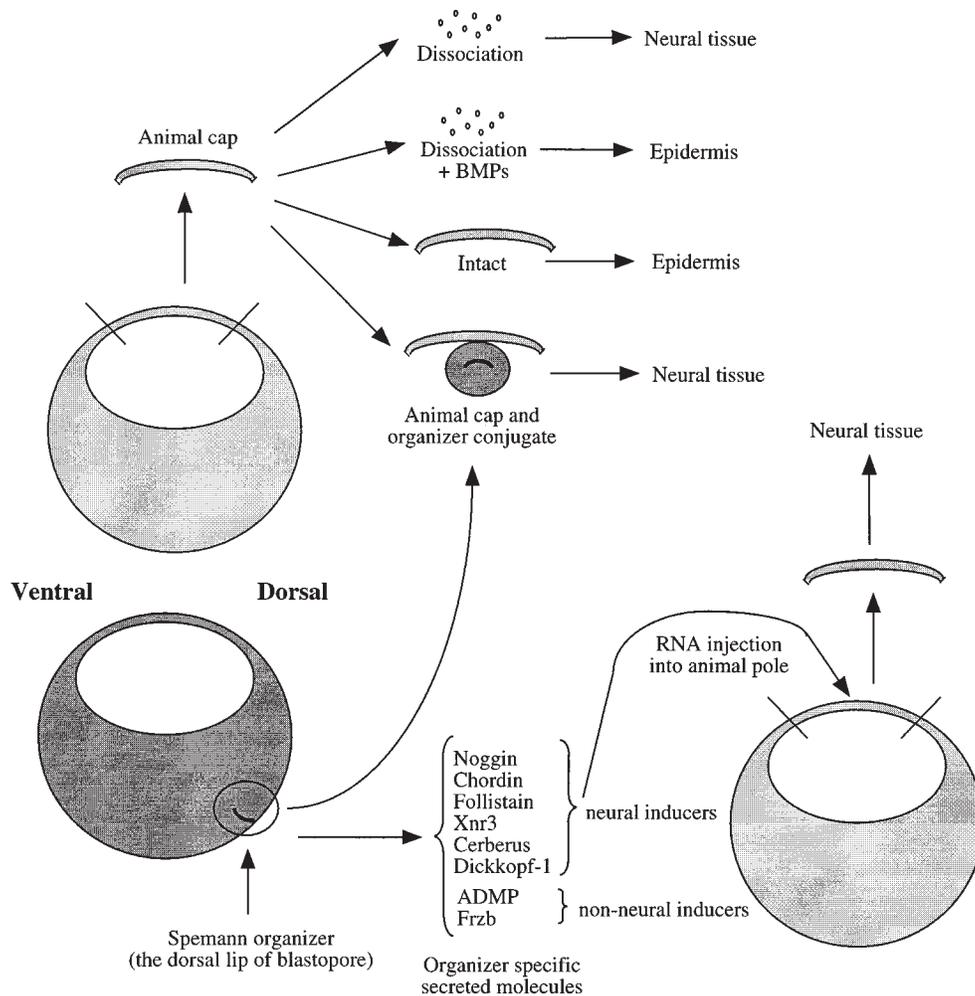
Figure 1 Ectodermal cell fate determination in *Xenopus.* In *Xenopus,* ectodermal explants (animal caps) obtained from late blastula (lighter shade embryos) to early neurula stages embryos will develop into epidermis when culture *in vitro* in buffered saline. These animal caps, however, can be induced to form neural tissue in response to signals emitted from the Spemann organizer (the dorsal lip of blastopore in early gastrula embryos, the darker-colored embryo in the figure). The action of the organizer can be mimicked by several organizer-secreted molecules such as noggin, chordin, follistatin, and Xnr3. Animal caps injected with RNAs encoding these factors express neural-specific markers without also expressing mesodermal markers (with the exception of Cerberus). Other organizer-specific soluble factors such as ADMP (anti-dorsalizing morphogenetic protein) and Frzb do not have the neural-inducing property. Neural tissue can also be induced by a prolonged dissociation (more than 3 h) of animal caps in calcium- and magnisium-free buffer. This autonomous neural induction suggests that neural may be the default cell fate for ectodermal cells. Addition of BMP proteins in dissociation assay prevents neural induction and restores the epidermal fate to ectodermal cells, implying that in intact animal caps, BMP signals are responsible for inhibiting neural cell fate and retaining cells along an epidermal development route.

ventral side of a recipient gastrula newt, the host embryo developed a secondary axis with correct A-P and D-V patterns. The ectopic dorsal axial mesoderm was derived from the grafted donor, while the secondary nervous system was recruited from the host ectoderm which would otherwise form epidermis. These results not only showed that embryonic ectoderm could be induced to form neural tissue, they also revealed a source of inducing signals in the dorsal lip region (later named the Spemann organizer) (Fig. 1). Subsequent experiments with amniote embryos (e.g., chick and mouse) uncovered

similar organizing regions that could induce a secondary nervous system when transplanted into ectopic sites (Waddington, 1933; Beddington, 1994). This argued for a conservation of neural induction mechanism in vertebrates.

The hunt for neural inducers began almost immediately after Spemann's original experiment. Despite vigorous effort, however, the search was set back first by the tendency of *Urodele* ectodermal explants to autoneuralize (neuralization with nonspecific signals, including salt) (Hamburger, 1988), and then by lack of advanced biochemical techniques at the time. Only recently, with the introduction of molecular biology into embryology, have we witnessed swift progress in the quest for neural inducers. Several of what seem to be bona fide *in vivo* neural inducers have been cloned, and the mechanism of their functions in embryos is also beginning to be understood. These new results revolutionize our view on neural inducers and favor a default model of neural induction. In this review, we discuss recent advances of our knowledge on neural induction as well as findings on neural patterning. Emphasis is placed on, but not limited to, discoveries made in *Xenopus,* as most of our progress in understanding the ectodermal patterning is obtained from studies using this organism.

NEURAL INDUCTION: PARTITIONING ECTODERMAL CELLS INTO EPIDERMAL AND NEURAL PRIMORDIA

Neural Inducers: Multiple Players

Spemann and Mangold's classical transplantation experiment (1924) laid the groundwork for neural induction. It suggested that the dorsal lip has an inducing ability which can act upon competent gastrula ectoderm to induce neural formation. When an ectodermal explant (animal cap) is cultured in buffered saline, it differentiates into epidermis, irrespective of whether the source of the explant is from dorsal or ventral side of the embryo. However, when the animal cap is combined with the dorsal lip, neural tissue is induced (reviewed in Hamburger, 1988). Thus, *in vitro* culture of competent ectodermal explants with candidate factors provides a good assay for possible endogenous neural inducers. In this assay, however, growth factors which induce dorsal mesoderm in animal caps will score positive, as secondary neural induction will occur in response to signals from the newly formed dorsal mesoderm.

It is therefore important to define the requirements for a true neural inducer.

A bona fide neural inducer should be expressed at gastrula stages when neural induction occurs. Its activity should be present in the dorsal lip region. It should be secreted from the organizer (soluble or membrane bound factors) and be able to act on the ectoderm in a non–cell autonomous manner. Finally, it should induce neural formation directly, without first inducing mesoderm.

Several neural inducers have been discovered in *Xenopus* which fulfill all or most of the above criteria. Interestingly, these genes were isolated in quite different ways and represented seemingly unrelated gene families. Three of them stand out as likely endogenous neural inducers. Noggin was the first gene to be reported with direct neural inducing activity (Lamb et al., 1993). Injected as RNA or supplied as a protein, noggin induces the expression of several neural-specific genes, such as the neural cell adhesion molecule gene (NCAM). No mesodermal markers are induced. Moreover, zygotic noggin expression starts at late blastula stages in a region which will become the Spemann organizer in gastrula embryos (Smith and Harland, 1992). Noggin is therefore expressed in the organizer before the dorsal lip is morphologically distinct (Fig. 1). Interestingly, noggin was cloned not with the aim of identifying neural inducers, but rather to uncover molecules in an expression library that could rescue *Xenopus* embryos ventralized by ultraviolet (UV) treatment (UV irradiation blocks cortical rotation, a step required in *Xenopus* to establish dorsal-ventral body axis) (reviewed in Gerhart et al., 1989). A second neural inducer was discovered by a completely different method. Follistatin, a previously known activin antagonist, was isolated from *Xenopus* to test whether inhibition of activin or a related transforming growth factor-β (TGF-β) ligand might be required for neural induction (Hemmati-Brivanlou et al., 1994). When injected as RNA, follistatin induces neural formation in the animal caps in the absence of mesoderm. Like noggin, follistatin is expressed at gastrula stages in the dorsal lip region. A third direct neural inducer, chordin, was cloned with a scheme to isolate organizer-enriched genes by differential screening of a dorsal lip library (Sasai et al., 1994). Like noggin, chordin functions to induce neural tissue directly, both as an injected RNA and as a soluble protein (Sasai et al., 1995). Homologs of all three *Xenopus* neural inducers have been found in other species, including zebrafish, chick, mouse, and human. Most notably, chordin seems to have interchangeable function

with its *Drosophila* homolog short gastrulation (sog) (Holley et al., 1995). Loss of sog function in flies results in a decrease of ventral neurogenic ectoderm with a corresponding increase in dorsal non-neural ectoderm, suggesting a functional conservation of this neural inducer during evolution.

Other genes with neural inducing ability have also been cloned from *Xenopus.* Xnr3, a *Xenopus* homolog of the mouse nodal gene that is localized in the epithelial layer of the organizer region, was discovered to stimulate neural gene expression without concomitant mesoderm induction (Smith et al., 1995; Hansen et al., 1997). Cerberus, a gene expressed in the anterior endomesoderm of the organizer, is also able to induce neural tissue (Bouwmeester et al., 1996). The induction, however, is accompanied by heart mesoderm formation and may therefore be indirect. A mouse homolog, Cerberus-like, functions similarly in *Xenopus* animal caps to induce heart mesoderm and neural tissue (Belo et al., 1997).

Signaling through the fibroblast growth factor (FGF) receptor has also been proposed to mediate neural induction. A dominant negative FGF receptor, when introduced into animal caps, blocks neural induction by coinjected noggin or chordin RNA (Launay et al., 1996). The same truncated receptor, however, does not prevent neural formation in whole *Xenopus* embryos (Kroll and Amaya, 1996). Basic FGF (bFGF or FGF-2) has been reported to have direct neural-inducing activity by two groups (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). In these studies, however, animal caps are dissociated briefly or prevented from healing by incubation in low calcium and magnesium buffer (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). Animal caps cultured in these conditions are likely sensitized to neural induction (see below), as bFGF would not induce neural fate using standard animal cap assay (Cox and Hemmati-Brivanlou, 1995). Two novel factors, FRL1 and FRL2 (FGF receptor ligand), have been found to signal through the FGF receptor (Kinoshita et al., 1995). FRL1 can induce the neural marker NCAM without muscle actin expression when its RNA is injected in the animal caps at low doses. At high doses, however, FRL1 induces dorsal mesoderm. The neural induction property of FRL1 thus needs to be examined more carefully. Furthermore, FRL1 is expressed in all germ layers including the dorsal lip and ventral mesoderm at gastrulation stages (Kinoshita et al., 1995), suggesting that it might have functions other than neural induction.

An important criterion for bona fide neural in-

ducers is that they should function non–cell autonomously. Among all the factors that have neural-inducing ability, only noggin, chordin, and bFGF have been shown to do this when supplied as proteins, although it requires a high concentration of noggin and chordin proteins in animal cap assay (Lamb et al., 1993; Piccolo et al., 1996). The high dose requirement for neural inducers at the protein level may suggest a synergistic action of their neural-inducing activity *in vivo.* It will be important to see if *Xenopus* follistatin, as well as Cerberus and Xnr3, can induce neural tissue directly when provided as proteins, and if different neural inducers can function synergistically. FRL1, like bFGF, is not secreted from injected oocytes. FRL1 and bFGF nonetheless can induce mesoderm in a non–cell autonomous fashion when naive animal caps are grafted onto oocytes injected with RNA encoding either gene (Kinoshita et al., 1995). It will be relevant to see if neural induction by FRL1 can also occur in this assay.

The Default Model of Neural Induction

Since Spemann and Mangold's transplantation experiment (Spemann and Mangold, 1924), it has generally been considered that ectoderm will develop along an epidermal pathway unless instructed by organizer factors to differentiate into neural cells. This concept of an instructive signal was, however, challenged by recent data, and as a result, a new model for neural induction was proposed.

In the late 1980s, three independent groups discovered that when animal caps or whole embryos were dissociated for an extended time, the cells changed their fates from epidermal to neural, based on neural specific marker expression (e.g., NCAM) and morphology (e.g., extension of neural fibers from cell bodies) (Sato and Sargent, 1989; Godsave and Slack, 1989; Grunz and Tacke, 1989) (Fig. 1). The dissociated ectoderm was not exposed to the dorsal lip beforehand or to any added other factors during the process. As *Xenopus* ectoderm normally is refractory to autoneuralization, the results were very puzzling at the time. Two groups proposed that some neural inhibitory factors might exist in intact animal caps which were lost during dissociation (Godsave and Slack, 1989; Grunz and Tacke, 1989). These were among the first to show that in *Xenopus* neural formation can occur autonomously without any inducers. The notion that neural induction may occur by preventing an inhibitory event *in vivo* was reiterated with a fuller understanding of its meaning years later. In a study of mesoderm

induction, a truncated form of the type II activin receptor was made (Hemmati-Brivanlou and Melton, 1994). When it was injected into animal caps, activin-mediated mesoderm formation was blocked. Surprisingly, the injected animal caps adopted a neural instead of an epidermal fate. Neural induction by the truncated receptor took place in the absence of any mesodermal markers (Hemmati-Brivanlou and Melton, 1994). This result was unexpected, as no neural inducers from the organizer region were present; however, it paralleled the observation of autonomous neural formation in dissociated cells. Realizing the significance of these results, the authors proposed a default model of neural induction (Hemmati-Brivanlou and Melton, 1994).

In this model neural is the ground, or default, cell fate. Without any extracellular signals, all ectodermal cells will develop into neural tissue. In intact animal explants, however, signals from endogenous TGF-β family, to which activin belongs, prevent neural specification and maintain cells in an epidermal fate. Removal of the inhibitory actions of TGF-β members by dilution or actively blocking through dominant negative components of its signal pathway can lead to neural formation in the ectoderm.

It remains to be determined whether a default neural fate applies to all cells in blastula embryos. When vegetal cells are injected with a truncated type II activin receptor, they adopt a neural fate and migrate out of their normal environment of prospective endoderm to form structures in the anterior neural tube (Hemmati-Brivanlou and Melton, 1994). Furthermore, vegetal cells received follistatin RNA injection start to express pan-neural marker NCAM (Henry et al., 1996). These results suggest that vegetal cells can differentiate into neural tissue if TGF-β signals are inhibited. Data from noggin injections, however, show that vegetal cells receiving noggin RNA still develop into endoderm (Smith and Harland, 1992). It will therefore be important to determine under what conditions vegetal cells will develop into neural tissue and whether vegetal cells dissociated in culture medium will take on a neural fate. The results will help to answer the question of whether a single neural default fate prevails for all cells in early embryos or whether there exists an autonomous endodermal differentiation pathway for cells in the endoderm germ layer.

Bone Morphogenetic Proteins (BMPs) as Neural Inhibitors and Epidermal Inducers

The default model of neural induction predicts the existence of inhibitory signals in animal caps which function to block neural formation. To search for this inhibitor, Wilson and Hemmati-Brivanlou (1995) used a cell dissociation assay. Reasoning that dilution of an extracellular TGF-β factor is responsible for neural induction in dissociated cells, they started to add back soluble factors of this family to the dissociated animal caps in hope of restoring epidermal fate. By adding back activin in the cell dissociation system, neural induction was inhibited; the cells, however, began to express mesodermal instead of epidermal markers. A further look at other ligands in this family revealed that BMP4 was able to suppress expression of neural markers and induce epidermal keratin expression in dissociated cells even at a low dose (Wilson and Hemmati-Brivanlou, 1995). Both activities were blocked when animal caps were injected before dissociation with RNA coding for the truncated type II activin receptor. BMP2 and BMP7, close relatives of BMP4, are also reported to be neural inhibitors and epidermal inducers in the context of dissociated cells, although the activity of BMP7 is very weak (Suzuki et al., 1997a). These data suggest that endogenous BMPs may be responsible for maintaining epidermal specification in intact animal caps. Consistent with this idea, various treatments that block BMP activities all lead to neural formation in ectodermal explants, including truncated type I BMP receptors (ALK2 and ALK3) (Sasai et al., 1995; Suzuki et al., 1995, 1997a; Xu et al., 1995), dominant negative BMP4 and BMP7 ligands (Hawley et al., 1995), as well as antisense BMP4 RNA (Sasai et al., 1995).

BMP4 transcripts are excluded from dorsal ectoderm (future neural plate) in gastrula embryos, although BMP2 and perhaps BMP7 RNA are present (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). In animal caps, neural inducers do not down-regulate transcription of BMP4 at gastrula stages, suggesting that other regulators exist *in vivo* to exclude BMP transcripts from the dorsal ectoderm at these stages. Recently, a new ligand of the TGF-β family, growth and differentiation factor-6 (GDF6), was found to be down-regulated early by neural inducers noggin and follistatin during gastrula stages (Chang and Hemmati-Brivanlou, unpublished). GDF6, like BMPs, induces expression of epidermal keratin and represses neural markers in the cell dissociation assay. Other GDFs may have a similar ability. The neural inhibitors and epidermal inducers may thus constitute a large number of BMP/GDF-like ligands. The increasing list of neural inhibitors matches that of neural inducers and brings up interesting issues of specificity and redun-

dancy in neural induction. It will be pertinent to see if differences exist in localization and inhibitory activities of different members of BMP/GDF ligands and how they correlate with the different activities of neural inducers.

Neural Inducers Function to Interfere with BMP Signals

The discovery of BMPs as neural inhibitors and epidermal inducers suggested that neural inducers may work *in vivo* to block BMP signaling. Genetic studies in *Drosophila* strongly supported this model by showing that short gastrulation (sog, a chordin homolog) genetically antagonizes the dorsalizing activities of decapentaplegic (dpp, a BMP2 and BMP4 homolog) (Ferguson and Anderson, 1992a; François et al., 1994). BMP4 has also been found to inhibit chordin-induced neural formation in *Xenopus* (Sasai et al., 1995). This antagonism might be achieved at many levels in the signal transduction pathway, from ligands binding to receptors and stimulation of intracellular signal transducers to regulation of responsive gene transcription. Recent biochemical studies demonstrate that most known neural inducers work by preventing BMPs from interaction with their receptors. Both noggin and chordin bind directly to BMPs; this binding is specific, as neither protein binds to the related ligands activin or TGF-β1 (Zimmerman et al., 1996; Piccolo et al., 1996). Noggin binds to BMP4 and BMP2 with a high affinity (a K_d of 19 pM for BMP4) and to BMP7 somewhat weakly. Chordin associates with BMP2 and BMP4 as well as with BMP4/7 heterodimer with an affinity 10-fold lower than that of noggin. By binding to BMPs, both noggin and chordin prevent BMPs from contacting their receptors, thus inhibiting signal transduction along the BMP pathway. Follistatin has previously been shown to interfere with BMP7 function (Yamashita et al., 1995), and it, too, may bind to BMP4, although the affinity of follistatin for the BMPs has not been determined and the consequence of this binding is unclear (Wilson and Hemmati-Brivanlou, 1995; Fainsod et al., 1997).

Other neural inducers such as Cerberus and Xnr3 may work in a similar way. Cerberus-dependent neural induction is inhibited by BMP4 (Bouwmeester et al., 1996). It is unclear at what level Cerberus and BMP4 interact, but it remains possible that Cerberus binds directly to BMP4. Xnr3-induced neural formation is also blocked by BMP4 or an activated BMP receptor (Hansen et al., 1997). Xnr3, a member of the TGF-β family, may exert its neuralizing effect by forming heterodimers with BMPs, preventing them from binding to their receptors. Alternatively, Xnr3 or Xnr3/BMP dimers may associate with TGF-β family receptors without transducing a functional BMP signal. For FGF, antagonistic interactions with BMPs have been reported in several situations; e.g., FGF opposes the antiproliferative effect of BMP2 during limb bud outgrowth and blocks BMP4 from inducing apoptosis in interdigital membrane during digit formation (Niswander and Martin, 1993; Ganan et al., 1996). It is possible that FGF may inhibit BMP signals during neural induction, though probably at a level downstream of the ligand. This hypothesis is supported by a recent report on inhibition of BMP signaling by epidermal growth factor (EGF) (Kretzchmar et al., 1997). EGF, acting through a tyrosine kinase receptor and Erk family of MAP kinase, stimulates phosphorylation of the BMP signal transducer Smad1 on specific sites (see below). This results in failure of nuclear translocation of this protein and inhibition of transcription activation by Smad1. The FGF signal transduction pathway is similar to that of EGF in that both stimulate Erk MAP kinases. The inhibition of Smad1 by FGF may therefore be a mechanism for FGF to block BMP signal transduction.

Neural induction in other species is less understood, although evidence is accumulating to suggest a similar mechanism. In ascidians, a BMP2/4 homolog is found to divert cell fate from neural to epidermal when injected as RNA in embryos, indicating a neural inhibitory function of BMP in this species (Miya et al., 1997). *Drosophila* SOG and DPP counteract each other's action in patterning ectodermal fates. In zebrafish, several mutants have been found to affect dorsoventral axis formation. Among those, *dino* displays a ventralized phenotype with reduced neural tissue, while the *swirl* mutant is dorsalized (Hammerschimdt et al., 1996a,b). These mutant phenotypes correlate with BMP4 expression pattern in fish embryos and can be phenocopied by overexpression (*dino*) or suppression (*swirl*) of BMP4. The *dino* mutant is rescued by injection of noggin or a truncated BMP receptor RNA and *dino* maps to the zebrafish chordin homolog chordino gene locus (Schulte-Merker et al., 1997). As in *Xenopus,* the fish chordin homolog is expressed in the organizer region (Miller-Bertoglio et al., 1997). The gene *swirl* is found to be zebrafish BMP2 homolog (Kishimoto et al., 1997). Therefore, a similar BMP antagonist action seems to be responsible for D-V axis determination in zebrafish. In chick, although implanted

chordin-expressing COS cell pellets, which produce diffusible chordin proteins around the site of implantation, are not sufficient to induce neural tissue in extraembryonic region, the cell pellets do have an effect on the size of the neural plate when implanted at the neural plate border region. Chordin-expressing cells seem to stabilize expression of early neural markers (Streit et al., 1998). In mammalian P19 embryonic carcinoma cells, inhibition of TGF-β signals by a truncated type II activin receptor leads to neuronal differentiation (Hoodless and Hemmati-Brivanlou, 1997). These data suggest that a conserved BMP antagonistic mechanism may exist in all species to regulate neural induction.

The fact that neural induction occurs as a result of inhibition of the BMP pathway explains why neural inducers have been cloned by a variety of schemes with diverse purposes, because the patterning of mesoderm in embryos also employs a strategy of BMP inhibition on the dorsal side. When BMP is overexpressed, whole embryos are ventralized, while suppression of BMP signals by a truncated BMP receptor in the ventral side leads to production of a secondary dorsal axis (Dale et al., 1992; Jones et al., 1992, 1996; Graff et al., 1994; Schmidt et al., 1995). The discovery of BMP inhibition during neural induction reveals that the embryo uses a conserved mechanism in patterning the D-V axis in two and probably all three germ layers, and that the same molecules that mediate dorsal mesoderm formation are involved in dorsalization of ectoderm as well.

One issue related to neural inducers is whether all secreted factors localized in the organizer have neuralizing activity. Although most of the reported soluble factors found in this region can induce neural formation, not all organizer-specific genes confer dorsal properties to other tissues. In fact, one gene, Anti-dorsalizing morphogenetic protein (ADMP), a BMP3 homolog, ventralizes whole embryos and down-regulates noggin and follistatin expression when injected into embryos (Moor et al., 1995). ADMP is expressed exclusively in the dorsal lip at gastrula stages, and it is not known why it has opposite functions to the organizer and how its activity is regulated.

Other issues remain open on neural induction, including whether neural inducers function only to block BMP signals and whether neural induction occurs only by BMP inhibition. For the first part, it seems that blocking the BMP signal is sufficient for noggin and chordin to induce neural tissue, and it appears to be the only function of these proteins in neural induction. An antibody which prevents nog-

gin from binding to BMPs also blocks its neural inducing activity, while nonblocking antibodies do not interfere with noggin function (Zimmerman et al., 1996). In *Drosophila,* the sog:dpp double mutant displays the same phenotype as the dpp mutant, implying that SOG may function only through DPP (Holley et al., 1996). In regard to the second issue, it is not clear whether neural induction can occur without BMP inhibition. Tissues other than the organizer have been reported to induce neural formation in ectoderm. Both somite and neural plate explants have neuralizing ability when recombined with animal caps, although a close apposition of the tissues is required (Jones and Woodland, 1989; Hemmati-Brivanlou et al., 1990; Servetnick and Grainger, 1991a). Since several known neural inducers are not expressed in somite or neural plate at the RNA level in *Xenopus,* it is not known what mediates this neural induction event. Other unidentified BMP antagonists may exist in these tissues; or, alternatively, separate signaling pathways may exist to induce neural formation independent of BMP inhibition. It is interesting to note that molecules involved in another signal pathway, wnt, may play some role in neural induction. Several genes that block wnt signal transduction, including a secreted factor dickkopf-1 (dkk-1), a dominant negative Xwnt8 ligand and the cytoplasmic negative regulator GSK3 (glycogen synthase kinase 3), can induce neural tissue in animal cap assays (Itoh et al., 1995; Glinka et al., 1997, 1998). Other wnt inhibitors, such as soluble wnt receptor Frzb, do not do so (Leyns et al., 1997; Wang et al., 1997). Furthermore, a wnt signal transducer, *Xenopus* dishevelled gene, can also induce neural formation in animal caps (Sokol et al., 1995). It is unclear how neural induction occurs in these situations, why both inhibitors and transducers of wnt can induce neural tissue, and whether neural induction also involves inhibition of BMP pathway. In *Drosophila,* wnt homolog wingless is found to repress dpp transcription during leg development (Jiang and Struhl, 1996; Theisen et al., 1996). It will be pertinent to see if a similar mechanism exists in vertebrate.

REGULATION OF NEURAL INDUCTION

Ectodermal Competence

Neural induction not only requires a source of neural inducers, but also requires competent ectoderm. Competence is loosely defined as the ability to respond to inducing signals, and has been used in

different contexts. In one aspect, a cell can respond to an inducer only during a certain developmental period before it loses its responsiveness, and the duration of the responsive time defines competence. A cell can be competent to respond to different inducers at different times. In *Xenopus,* for example, competence of ectodermal explants to respond to mesodermal inducing signals precedes competence to respond to neural inducers. Animal caps obtained at mid to late gastrula stages lose their responsiveness to mesoderm inducers but can still respond to neural inducers until early neurula stages (Jones and Woodland, 1987; Servetnick and Grainger, 1991b). In another aspect, competence has also been used to refer to the amplitude of a response a cell can achieve. Factors that do not have an inducing ability but can augment a cell's response to an inducer have been defined as competence factors. In neural induction, both protein kinase C (PKC) and G-protein pathways have been proposed to affect a cell's competence (Otte et al., 1991, 1992; Pituello et al., 1991; Otte and Moon, 1992; Pruitt, 1994). Neither PKC nor G protein alone induces neural tissue, but they can synergize with neural inducers to achieve a higher level of induction. In these cases, however, it is not addressed whether PKC or G protein is required for neural induction (e.g., if abolishing their function will block neural induction), nor whether their activities correlate with competent time of ectodermal cells to respond to neural inducers. PKC-α, for example, which is proposed to enhance ventral ectodermal cell competence, is actually expressed in ectodermal cells after they lose neural competence (Otte and Moon, 1992). It is therefore possible that PKC and G protein may directly regulate neural induction without affecting competence state of responding cells. This brings up the important issue as what can be considered as competence factors and what competence factors can do *in vivo.* A related issue is whether competence is an intrinsic property of a cell that runs automatically, or whether factors extrinsic to a cell can affect its competence state. There is no clear definition on these issues. For this review, we will define competence as the ability of a cell to respond to an inducing signal in an all-or-none fashion, irrespective of its magnitude. Competence may be regulated by extrinsic factors (competence factors) if these factors cannot induce a response but can change a cell's responsiveness to an inducer. Competence factors are necessary for an inductive event in that no responses occur in the absence of these factors. In this sense, the expression or activi-

ties of competence factors should correlate with the ability of a cell to respond to an inducer.

The idea that competence can operate as an autonomous cell program comes from studies in *Xenopus.* Animal caps obtained from gastrula embryos lose their responsiveness to mesodermal inducers, while caps explanted at early neurula stages stop responding to neural inducers. When animal caps are removed from blastula embryos and aged *in vitro* to gastrula or neurula stages, they lose their respective competence to mesodermal or neural inducers at the same time as caps obtained directly from these later-stage embryos (Sharpe and Gurdon, 1990; Servetnick and Grainger, 1991b). The data suggest that the *in vivo* endogenous environment around the ectoderm does not contribute much to ectodermal competence, which seems to run in a cell-autonomous fashion. Certain chromatin components, such as somatic subtypes of linker histone H1, have been shown to affect loss of mesodermal competence *in vivo* (Steinbach et al., 1997). Overexpression of somatic H1 RNA causes the animal caps to cease their response to activin-dependent mesoderm induction 1.5 h earlier than the uninjected controls, while reduction of somatic H1 by ribozyme injection (which results in destruction of H1 RNA and as a consequence inhibition of H1 synthesis) prolongs the responsiveness of the caps to activin up to neurula stages. In embryos, maternally deposited oocyte-specific H1 is replaced by somatic histone H1 progressively after the midblastula transition, and it appears that the accumulation of somatic H1 is critical for loss of mesodermal competence. Neural competence was not tested in this experiment, but it could be that somatic H1 also plays a role in the loss of neural competence. The level of somatic H1 keeps increasing to replace oocyte-specific H1 until early neural stages—a time when ectoderm loses its neural competence. It is possible that the ratio of somatic to oocyte-specific H1 may set the thresholds at which time mesodermal and neural competence are lost. Cell-autonomous accumulation of somatic H1 is also consistent with the observation of autonomous loss of competence by ectodermal explants.

On the other hand, extrinsic factors have been suggested to regulate ectodermal competence from studies of chick neural induction. In chick, a carbohydrate epitope named L5 was found to correlate with ectodermal competence to neural induction. While ectopic neural tissue can be induced by a grafted Hensen's node (organizer equivalent) at other sites, it is never induced in a region lacking L5 expression (Streit et al., 1997). The growth factor

hepatocyte growth factor/scatter factor (HGF/SF), expressed in the node, can induce and maintain L5 expression in the area opaca long after its normal expression fades away. This extended period of the L5 expression seems to correlate with a prolonged competence of the area opaca to node neural inducing signals, and an antibody to L5 blocks neural induction by the node (Roberts et al., 1991; Streit et al., 1995, 1998). Expression of HGF/SF and L5 does not induce the general neural marker Sox2, indicating that these factors are not neural inducers per se. Furthermore, L5 is expressed in a broad region at gastrula stages, including the future neural plate as well as epidermal region (all have the potential to be induced by the node to form neural tissue), arguing against L5 induction as a part of neural induction itself. At later stages, L5 is restricted only to the nervous system, suggesting that neural inducers may help to retain L5 expression (Roberts et al., 1991). All these data suggest that HGF/SF and L5 may play a role in the neural induction process by regulating neural competence, a step prior to actual neural induction. *Xenopus* HGF is also cloned, but its function in neural competence has not been reported (Nakamura et al., 1995). In addition, it is not known whether incubation of *Xenopus* ectodermal explants with chick HGF factor will prolong its neural competence, and no L5 equivalent glycoprotein marker has been identified in *Xenopus.* It will be important to determine whether *Xenopus* and chick use similar neural competence and induction mechanism, or when they have different requirements in neural induction process.

Neural competence may also be viewed from another angle in light of our recent understanding of neural induction as a default state. As neural inducers function to antagonize BMP signals and permit a default neural fate to develop, competence may thus measure the accumulation of BMP signals (discussed in Wilson and Hemmati-Brivanlou, 1997). Continuous exposure to BMP signals through blastula and gastrula stages may set an irreversible change in ectodermal cells, resulting in commitment of these cells to the epidermal fate. Further antagonizing BMP signals after gastrula stages may then be not sufficient to revert the cell fate.

BMP as a Morphogen to Pattern Ectoderm

The ectoderm contains broad neural and epidermal domains at the end of gastrulation. Cells at the bor-

der of the two domains acquire different fates. Cement gland forms at the most anterior border of the neural plate and epidermis. Caudal to it develop other border-specific cell types, such as placodes (later forming sensory organs, e.g., lens and otic vesicles) and neural crest cells. Patterning of ectodermal cells at the border may require different signals, a gradient of a single signal, or a combination of both. The finding that a neural crest-specific marker Slug is turned on at the border region at gastrula stages in *Xenopus* (Mayor et al., 1995), a time concomitant with neural induction, suggests that initial specification of the neural plate and the border cell fates may involve a single event employing a morphogen (this does not exclude further interaction between the neural plate and epidermis to establish border cell types, see below (also reviewed by LaBonne and Bronner-Fraser, this issue). A morphogen refers to a diffusible extracellular signal molecule which, when distributed in a gradient, specifies different cell fates at different concentrations. It is a pivotal concept in pattering embryonic domains (reviewed by Lawrence and Struhl, 1996). Many factors are identified to function as morphogens to determine different cell types in a dose-dependent fashion, such as DPP in D-V patterning and in wing development in *Drosophila* and hedgehog in patterning of vertebrate ventral neural tube (Ferguson and Anderson, 1992b; Wharton et al., 1993; Roelink et al., 1995; Nellen et al., 1996). In *Xenopus,* it was discovered that BMP4 can work as a morphogen to induce different ectodermal cell fates at different doses (Wilson et al., 1997).

Animal cap cells assume a neural fate when dissociated for an extended time. When BMP4 is supplied at a relatively high concentration during dissociation, all neural markers are completely suppressed and epidermal keratin gene expression is activated. If BMP4 is added at an intermediate dose, however, cement gland markers are turned on (Wilson et al., 1997). Similar progressive fate changes can be obtained using intact animal caps. When different doses of BMP4 inhibitors, such as noggin and a truncated BMP type I receptor, are introduced into the animal caps, cells change their gene expression pattern from epidermal to cement gland to neural, depending on the concentration of injected BMP4 inhibitors (Wilson et al., 1997). Similarly, a neural crest marker Slug is induced at the intermediate doses of these BMP antagonists in the animal caps (Morgan and Sargent, 1997). These results indicate that BMP4 can act as a morphogen to determine distinct cell types according to its concentra-

tion, and suggest that *in vivo,* cells can interpret the strength of BMP signals and adopt different fates accordingly.

In vivo, a gradient of BMPs activity may be established by limited diffusion of neural inducers. The ectodermal cells closest to the source of neural inducers (BMP antagonists), those overlying the dorsal mesoderm, form neural plate. Cells further away from the source receive progressively more BMP signals and in turn assume neural crest and epidermal fates. Possible shifts in the BMP gradient by treatments such as UV irradiation may underlie the mechanism for a corresponding shift in size of the neural plate according to the degree of UV ventralization.

Smad Proteins as BMP Signal Transducers and Inhibitors

In a search for signal transduction components in the BMP/Dpp pathway, a genetic screen was performed in *Drosophila* to look for second-site enhancers of a weak dpp allele. One gene, Mad (mothers against dpp), was found to have mutant phenotypes strikingly similar to that of dpp (Sekelsky et al., 1995). MAD acts downstream of DPP and seems to transduce DPP signals (Newfeld et al., 1996, 1997). It is homologous to the nematode *C. elegans* genes sma2, sma3, and sma4, which also function in a TGF-β signaling pathway involving the type II kinase receptor Daf-4 (Sekelsky et al., 1995; Savage et al., 1996). Cloning and characterization of vertebrate homologs of Mad/sma (designated as Smad for sma and Mad) have led to a breakthrough in our understanding of how signal transduction occurs in this pathway and the possible roles Smads play in ectodermal patterning (Fig. 2).

Smads constitute a large protein family (so far, nine members have been reported) that transduce specific signals for different TGF-β ligands (reviewed by Heldin et al., 1997). Smad2 and Smad3 act downstream of activin and TGF-β and can induce dorsal mesoderm in *Xenopus* animal caps (Graff et al., 1996; Baker and Harland, 1996; Macias-Silva et al., 1996). Smad1, Smad5, and possibly Smad9 (previously named Smad8) seem to function in the BMP pathway, and all of them have a ventral mesoderm-inducing ability (Graff et al., 1996; Thomson, 1996; Suzuki et al., 1997c; Chen et al., 1997b). Smad1 has been shown to rescue neural induction by a truncated BMP receptor in intact animal caps, and both Smad1 and Smad5 can mimic BMP4 in inhibiting neural formation and inducing epidermal keratin in the cell dissociation assay.

Moreover, Smad1 and Smad5, like BMP4, can act in dissociated cells to induce different cell type markers, depending on their concentration (Wilson et al., 1997; Suzuki et al., 1997c). These data suggest that Smad1 and Smad5 (and maybe Smad9) mediate BMP signaling during ectodermal patterning [Fig. 2(A)]. Another Smad protein, Smad4, does not transduce specific signals for any TGF-β ligands. Instead, Smad4 is a common partner for signal-transducing Smads. A mutant Smad4 blocks both activin and BMP4 mesodermal induction in *Xenopus* animal caps (Lagna et al., 1996). Both Smad1- and Smad5-dependent neural inhibition is also blocked by the mutant Smad4 (Suzuki et al., 1997c). In mammalian cell culture, Smad4 forms a heteromer with Smad1 upon BMP4 activation and the Smad1/4 complex is translocated into cell nuclei to affect BMP-regulated gene expression [Fig. 2(A)]. The same mechanism applies to the signal transduction in the activin/TGF-β pathway by Smad2 and Smad3 (reviewed by Heldin et al., 1997).

Recently, several new members of the Smad family have been cloned, including Smad6, Smad7, Smad8, and Drosophila Dad (Tsuneizumi et al., 1997; Nakao et al., 1997; Hayashi et al., 1997; Imamura et al., 1997; Topper et al., 1997; Hata et al., 1998; Nakayama et al., 1998). Interestingly, these proteins act as inhibitors of TGF-β signal transduction [Fig. 2(B)]. Smad7 seems to inhibit all TGF-β ligands with no discrimination, such as TGF-β1, activin, Vg1, and BMP4. Smad6, on the other hand, seems to function selectively to inhibit only the BMP pathway without blocking activin signal transduction. Smad8 preferentially blocks BMP signaling, although it may also be involved in other pathways. Overexpression of Smad6, Smad7, or Smad8 is sufficient to induce neural markers in the absence of mesoderm in *Xenopus* ectodermal explants, and all genes can induce a partial secondary axis when injected in the ventral side of embryos. It is not known how Smad8 functions as an inhibitory Smad, but for Smad6 and Smad7, different mechanisms seem to be employed (Hayashi et al., 1997, Hata et al., 1998). Smad7 can bind nonselectively to many TGF-β type I receptors. This high-affinity binding, even in the presence of ligands, prevents activation of Smads by the type I receptors (phosphorylation, heteromer formation with Smad4, and nuclear translocation). Thus, Smad7 inhibits general signal transduction by many TGF-β ligands. Smad6 also binds to type I receptors, but it does not seem to function at this step. The inhibitory activity of Smad6 correlates not with its ability to bind to type
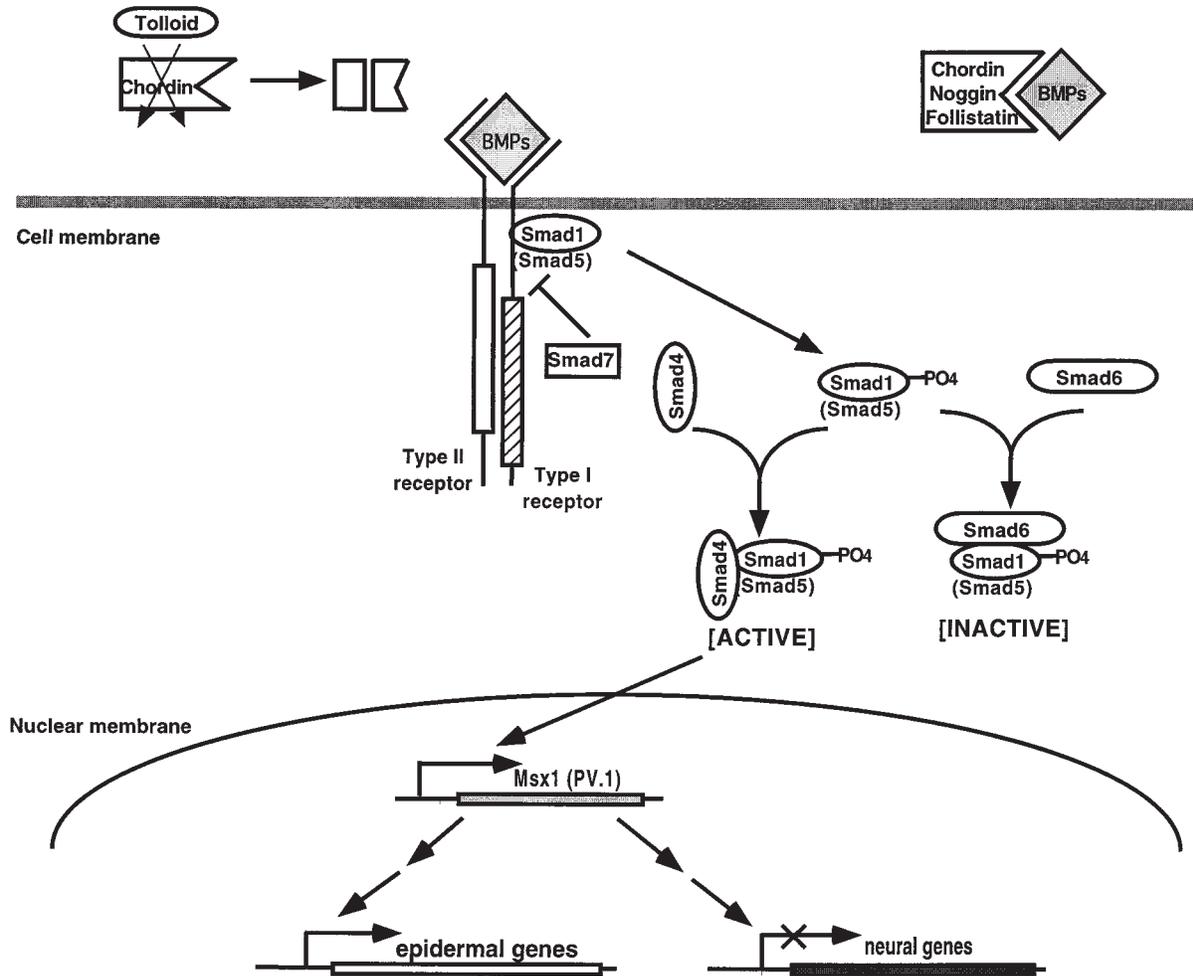
Figure 2   Models for neural induction and inhibition in *Xenopus*: antagonist interaction between neural inducers and BMPs. In *Xenopus* ectoderm, when BMP antagonists are not present or are degraded (e.g., chordin can be digested by tolloid metalloprotease), signals from BMPs are transmitted through type I and type II BMP receptors to stimulate intracellular Smad proteins. Smad1 and Smad5 (maybe Smad9 also) are specific signal transducers in the BMP pathway. Activated Smad1 forms a complex with Smad4 and is translocated into cell nuclei upon ligand stimulation. Smad1/4 complex may then activate specific DNA binding factors in some ways, e.g., Msx1 and PV.1. These transcription factors will mediate BMP-dependent effect to activate epidermal-specific gene expression and to repress neural-specific gene transcription. Neural inducers such as noggin and chordin bind to BMPs directly and prevent their interaction with BMP receptors. In addition, Smad7 can prevent Smad1 activation by interfering the access of Smad1 to activated type I kinase receptors, while Smad6 inhibits Smad1/Smad4 complex formation by binding to Smad1. Therefore, BMP signal transduction can be blocked both outside a cell with neural inducers and inside a cell by inhibitory Smads. Inhibition of BMP signaling leads to derepression of neural-specific genes.

I receptors, but with its ability to associate with the BMP signal transducer Smad1 (Hata et al., 1998). Smad6 interacts specifically with Smad1, but not Smad2 or Smad3, in the presence of their respective ligands. Smad6 competes with Smad4 for Smad1 binding, and the relocation of Smad1 from Smad1/4 to Smad1/6 complexes seems to inactivate Smad1

[Fig. 2(B)]. It is not known if Smad6 interferes with nuclear translocation of Smad1 or inhibits Smad1-dependent transcription directly.

In ectodermal patterning, inhibitory Smads may play an important role in balancing epidermal and neural induction by blocking activities of Smad1/Smad5/Smad9. Both Smad6 and Smad7 are ex-

pressed widely in early *Xenopus* embryos, making it difficult to know where exactly the active sites are for these proteins' function. Interestingly, Smad7 and Smad8, as well as Dad, can be activated at the transcriptional level upon ligand stimulation, and the increase in Smad7 transcript is an immediate early response to TGF-β signaling (Tsuneizumi et al., 1997; Nakao et al., 1997; Nakayama et al., 1998). One explanation for this effect is that Smad7/Smad8 (and maybe Smad6) are involved in negative feedback regulation of signals to control the final outcome. Smad6/Smad7/Smad8 may attenuate the strength of a signal to help establish a functional gradient of the ligand. Alternatively, these inhibitory Smads may be involved in termination of a signal after it is transduced into nuclei. Moreover, the selective inhibition of the BMP pathway by Smad6 may help to mediate the balance of different TGF-β signals *in vivo*. The common partner Smad4, released by Smad6 through titrating Smad1 out of the Smad1/4 complex, could be reused by other pathway-specific Smads (Smad2, Smad3, etc.) and thus help to shift the response to a particular signal. It will therefore be interesting to see if Smad6 can also distinguish between Smad1, Smad5, and Smad9, all of which transduce signals from BMPs. It will also be relevant to see if the TGF-β family neural inducer, Xnr3, can induce Smad6/Smad7/Smad8 transcription and/or stimulate their activities.

Transcription Factors

Bone morphogenetic protein signals are transduced through Smad proteins into cell nuclei, leading to the regulation of BMP-responsive gene expression. A direct biochemical link between Smad transducers and downstream transcription factors has not been illustrated for the BMP pathway. In *Drosophila,* Mad protein has been reported to bind to a promoter element in a Dpp-responsive gene (Kim et al., 1997). Smad4 has also been shown to bind DNA directly upon ligand stimulation (Yingling et al., 1997). The importance of the binding to promoter elements by these Mad/Smad proteins, however, is not clear. In the case of Smad4, gene activation is not dependent on the Smad4 binding site, as mutations of the sequence that abolish Smad4 binding do not eliminate reporter gene activation by TGF-β1. This implies that Smads may work with other transcription factors to regulate gene expression.

In *Drosophila,* a zinc finger transcription factor, Schnurri, mediates multiple DPP-regulated events (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). Schnurri mutants display a phenotype similar to dpp, and the expression pattern of Schnurri resembles that of dpp and the dpp receptors. Schnurri may therefore be the direct target of DPP signal transduction. It is not known if vertebrate Schnurri homologs, such as PRDII/MBP/HIV-EP1, can act downstream of BMPs to mediate signal transduction in ectodermal patterning. Other transcription factors that can mediate BMP responses have been identified in *Xenopus,* including a homeobox protein Msx1 (Davidson, 1995; Suzuki et al., 1997b) and a new family of homeobox transcription factors containing Xvent-1, Xvent-2/Vox/Vbr-1/Xom and PV.1 (Gawantka et al., 1995; Onichtchouk et al., 1996; Schmidt et al., 1996; Papalopulu and Kintner, 1996a; Ladher et al., 1996; Ault et al., 1996). All these genes can be up-regulated by BMP4, and overexpression of these factors in *Xenopus* results in ventralized phenotypes, as does BMP4. Msx1 and PV.1 are able to counteract the neuralizing activity of a truncated BMP receptor to restore epidermal fate in intact animal caps (Ault et al., 1997; Suzuki et al., 1997b). These data, plus the expression pattern of all the above homeobox genes in the ventral and/or posterior region of *Xenopus* gastrula embryos, suggest that they mediate BMP-dependent patterning effects *in vivo*. It is unclear how activated Smad proteins regulate functions of these transcription factors in response to BMP signals. In the case of Smad2 in activin pathway, a winged-helix transcription factor FAST-1 can associate directly with Smad2 in Smad2/4 complex to stimulate gene expression (Chen et al., 1996, 1997a). It is possible that transcription factors in the BMP pathway work in a similar way to interact with Smad1/Smad5/Smad9 to regulate downstream genes [Fig. 2(A)].

In addition to transcription factors that are responsible for neural inhibition and epidermal induction effects of BMPs, DNA-binding proteins that can stimulate neural gene expression are also identified in *Xenopus* embryos [Fig. 2(B)]. Neural plate-specific markers (expressed both in neurons and glias), such as NCAM, can be activated in the animal caps by a zinc finger transcription factor, Zic-related-1 (Zic-r1, Mizuseki et al., 1998) or by a POU domain-containing DNA binding factor, X1POU2 (Witta et al., 1995). Both Zic-r1 and X1POU2 are expressed in the prospective neurectoderm at gastrula stages, suggesting that they may participate in neural induction *in vivo*. Other transcription factors may also be involved in formation of neural tissue in a partially redundant fashion.

For example, a protooncogene c-ski induces a cell autonomous partial neural axis in *Xenopus* embryos and activates NCAM expression in animal caps (Amaravadi et al., 1997). However, mutant mice lacking a functional ski gene still contain neural structures, although they have other defects in neurulation (Berk et al., 1997). These multiple transcription factors may form a cascade to stimulate neural specific gene expression (also see below on neurogenesis).

In vivo, the determination of the epidermal and neural cell fates may rely ultimately on the balanced interaction of the opposing activities of the above-mentioned transcription factors. In epidermal cells, BMP-regulated DNA-binding proteins may repress expression of neural-specific transcription activators to block neural formation (e.g., Zic-r1) (Mizuseki et al., 1998). Alternatively, BMP-dependent transcription factors may suppress neural gene expression directly by blocking access of neural-specific activators to DNA [Fig. 2(A)]. In the absence of BMP signals, neural inhibitory transcription factors are rendered inactive. Neural genes are then activated by DNA-binding proteins such as Zic-r1 [Fig. 2(B)]. The model predicts that blocking inhibitory action of BMP-regulated transcription factors is sufficient to induce neural tissue, in the absence of other stimuli. This idea is consistent with a default model of neural induction (see above) and is supported by the discovery that Zic-r1 is up-regulated by cell dissociation (Mizuseki et al., 1998). Furthermore, a dominant negative mutant T-box–containing transcription factor, Brachyury, is found to convert ectodermal cells from an epidermal to a neural fate directly (Rao, 1994), suggesting that preventing inhibitory function of T-box–containing transcription factors can induce neural formation without other stimuli.

Tolloid Metalloprotease

The restricted neural induction in the dorsal ectoderm is due in large part to the localized expression of neural inducers in the organizer region. The organizer develops into dorsal mesoderm, such as notochord, which secrets diffusible neural inducers that help to establish the size of the neural plate. Recently, however, a new level of regulation was discovered which acts functionally upstream of neural inducers and may play an essential role in confining induction of neural tissue in the dorsal ectoderm.

Tolloid (tld), a *Drosophila* BMP-1-related metalloprotease, is found to oppose sog gene action in dorsal patterning of *Drosophila* embryos (Shimell

et al., 1991; Marques et al., 1997). Tolloid is a secreted protein, and a proteolytic-processing step is required for its function. Mature TLD is able to cleave the sog gene product in the presence of DPP. Like TLD, the *Xenopus* tolloid homolog Xolloid cleaves chordin directly and specifically without having any effect on noggin or follistatin (Piccolo et al., 1997). The protease activity, however, does not require the presence of BMPs. Chordin, either alone or in a complex with BMPs, is processed equally efficiently; and the cleavage of chordin inactivates the neuralizing activity of the protein by releasing functional BMP proteins. *In vivo* injection of Xolloid RNA ventralizes whole embryos, while injection of a dominant-negative Xolloid mutant results in dorsalization of embryos. The Tolloid homolog in zebrafish also has similar proteolytic and biological activities (Blader et al., 1997). Interestingly, the expression of Tolloid in both *Drosophila* and *Xenopus* is not localized. In *Xenopus*, Xolloid transcripts are distributed evenly in both dorsal and ventral halves of early embryos at gastrula stages. The apparent paradox may be explained in part by the discovery that most of the *Drosophila* TLD protein extracted from whole embryos is in the unprocessed, inactive form (Marques et al., 1997). The tight control of tolloid processing itself may be regulated by D-V axis determinants, such as components in the BMP pathway in *Xenopus.* It is unclear why embryos establish this extra layer of regulation of chordin function, as expression of chordin is localized. One explanation is that the tolloid product helps to restrict the action of secreted and diffusible chordin/SOG protein to establish a functional gradient of BMP/DPP along the D-V axis. This model suggests that a mechanism for the dominant-negative Xolloid to induce dorsalization of embryos is by shifting the gradient of BMPs. It is not known if proteases also exist for other neural inducers, such as noggin.

We have discussed some of the recent discoveries in the field of neural induction and inhibition, especially in relationship with the BMP pathway. Other factors also exist which may regulate this process at different levels. For example, the translation elongation initiation factor 4AIII (eIF-4AIII) seems to act in the BMP signaling pathway. Expression of eIF-4AIII blocks neural induction in dissociated cells, while another translation elongation factor eIF-4E cannot do so (Weinstein et al., 1997). In the near future, other factors involved in neural induction and inhibition pathways are likely to be uncovered, and the mechanism by which these factors work will then be illustrated with more clarity.

NEURAL PATTERNING AND NEUROGENESIS: FINE-TUNING OF NEURAL CELL FATES ACCORDING TO THEIR POSITIONS
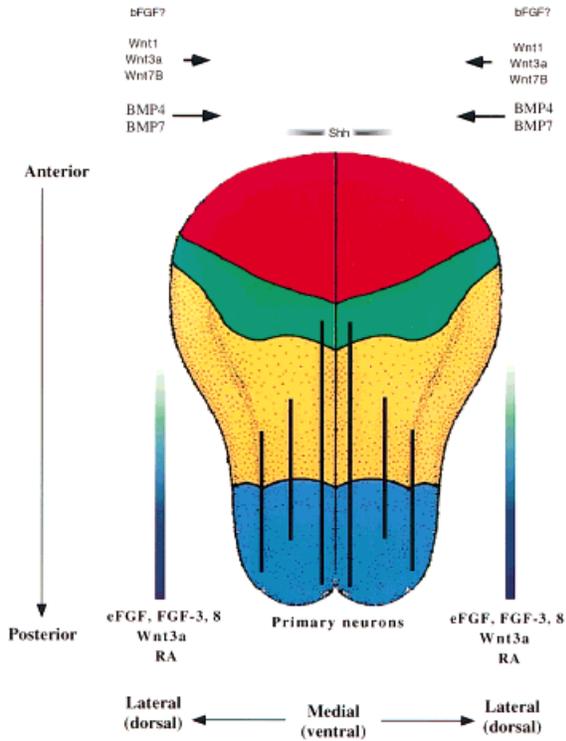
A-P Neural Patterning

Anterior-posterior neural patterning occurs soon after neural induction at open neural plate stages. Neural cells with equivalent developmental potentials are induced to express position-specific genes along the A-P axis in broad domains corresponding to forebrain, midbrain, hindbrain, and spinal cord. Within the boundary of each domain, finer subdivisions are observed by morphology and the expression of specific marker genes. The different gene expression patterns in the segmented hindbrain, for example, illustrates the existence of A-P differences within this domain. It seems that signals transmitted both from the underlying mesoderm (vertical signals) and from the organizer region through the neuroectodermal layer (planar signal) are important to pattern the A-P neural axis (reviewed by Doniach, 1992; Ruiz i Altaba, 1992).

The organizer is a source of signals for A-P neural patterning. When ectodermal explants are conjugated with the organizer *in vitro*, not only is neural tissue induced, but this tissue is also patterned to express multiple genes at different A-P levels. Interestingly, organizer tissue from early gastrula embryos induces complete anterior and posterior markers, while organizers from later-stage embryos induce progressively more posterior neural genes. Two models have been proposed to explain these observations (reviewed by Slack and Tannahill, 1992; Doniach, 1992; Ruiz i Altaba, 1992). In Mangold's (1933) model, different neural inducers are thought to exist in the early and late organizer region which are responsible for induction of neural genes with different A-P character. In Nieuwkoop's (1952a, 1952b) model, neural induction was proposed to involve two steps. Anterior neural tissues are first induced by organizer factors (activation); these tissues can then be caudalized by other organizer signals, which on their own do not have neuralizing ability, to form posterior neural tissue (transformation). So far, only bFGF has been reported to induce posterior neural genes in some cases (Kengaku and Okamoto, 1995; Lamb and Harland, 1995), lending some support to the first model. On the other hand, conspicuous evidence has accumulated to prove the plausibility of the second model (e.g., Cox and Hemmati-Brivanlou, 1995; McGrew et al., 1995). The most important support

comes from the observation that all the well-characterized neural inducers produce only anterior neural tissue, which can then be posteriorized by other signaling molecules.

Fibroblast growth factor is one of the foremost candidates for a caudalizing factor. The neural inducers noggin, chordin, and follistatin all induce anterior neural markers such as the cement gland marker XAG1 and the anterior brain marker Otx2 (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995). They do not activate posterior neural genes such as the hindbrain marker Krox20 or the spinal cord–specific homeobox gene HoxB9 (Xlhbox6). When FGF is supplied in conjunction with the neural inducers, these posterior genes are induced (Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995). Furthermore, FGF can caudalize dissected forebrain to express the hindbrain marker Krox20 or stimulate HoxB9 expression in hindbrain segment, suggesting that FGF is able to confer more posterior character to intact endogenous neural tissue (Cox and Hemmati-Brivanlou, 1995). *In vivo*, several members of the FGF family, including FGF-3, FGF-8, and eFGF (embryonic FGF, close to both FGF-4 and FGF-6), are expressed at a high level at the posterior end, around the blastopore, at neurula stages (Isaacs et al., 1992; Tannahill et al., 1992; Christen and Slack, 1997). These data suggest that FGF plays an important role in the A-P patterning of the neuroectoderm (Fig. 3). Blocking FGF signal by a dominant negative receptor results in embryos with defects in trunk and tail regions, but still retaining intact heads (Amaya et al., 1991). A careful examination of these embryos, however, reveals that all A-P neural markers including Otx2, Krox20, and HoxB9 are expressed in succession along the truncated trunk (Kroll and Amaya, 1996). The result is surprising and indicates that although FGF may be involved in A-P patterning of the neural tube, other factors also exist which can compensate for the lack of FGF signaling in these embryos. Two likely candidates with caudalizing activity are the Wnts and retinoic acid. *Xenopus* Wnt3A has been shown to transform anterior neural tissue into posterior tissue when coexpressed with several neural inducers (McGrew et al., 1995). Animal caps coinjected with neural inducers and Wnt3A starts to express the hindbrain marker Krox20, and anterior markers XAG-1 and Otx2 are suppressed in these caps. A signaling molecule in the Wnt pathway, β-catenin, can mimic Wnt3A in caudalization of anterior neural tissue. Other Wnts, such as Wnt1 and Wnt7B, also have posteriorizing activities (Chang and Hemmati-Bri-

Figure 3   Positional information encoded by anteroposterior and dorsoventral patterning factors. Integration of signals from both A-P and D-V patterning factors determines specific cell fates inside the neural plate. Shown is a dorsal view of the neural plate in a neurula stage (stage 14) *Xenopus* embryo. From anterior to posterior, four regions with different gray shades represent a fate map for future forebrain, midbrain, hindbrain, and spinal cord. Sonic hedgehog (Shh) is expressed in the midline of an embryo; and by forming a protein gradient, it functions as a morphogen to specify distinct ventral neural cell fates. On the lateral side (future dorsal neural tube), cell type specification involves signals from epidermis. Both BMP4/BMP7, expressed in the epidermis, and Wnt1/3a/7B, expressed at the border of the neural plate, may play important roles in dorsal cell fate determination. At the same stages, secreted factors from FGF and Wnt families, as well as retinoid acid (RA), seem to specify the anteroposterior axis in the neural plate. The higher level of RA at the posterior end, as well as the expression of eFGF, FGF3, FGF8, and Wnt3a in the posterior region around the blastopore support their roles in A-P patterning. The combinatorial signals from both A-P and D-V axes may help to determine the precise domains (three longitudinal stripes on each side of the neural plate) of primary neuron formation. Neurogenesis employs vertebrate homologs of *Drosophila* homeobox genes in the Achaete-Scute complex, as well as Delta-Notch lateral inhibition signals. Cell fates in the neural plate are therefore specified at particular coordinates by integration of multiple signal inputs.

vanlou, 1998). Wnt3A is expressed strongly in both anterior and posterior regions of *Xenopus* neurula embryos, while Wnt7B is expressed along the whole A-P axis in the dorsal neural tube (Wolda et al., 1993; Chang and Hemmati-Brivanlou, 1998). The action of Wnts in neural patterning may therefore be regulated by other components in its signal transduction pathway. The finding that a Wnt receptor, Frizzled gene, is expressed in a graded fashion with the highest RNA level at the posterior end of neurula embryos supports this idea (Chang and Hemmati-Brivanlou, unpublished). Retinoic acid (RA) caudalizes intact embryos in a dose-dependent manner, and it affects both mesodermal and ectodermal patterning (Sive et al., 1990; Ruiz i Altaba and Jessell, 1991). In the ectoderm, RA suppresses anterior neural markers induced by noggin and promotes expression of more posterior neural genes. Blocking RA signal by a dominant negative RA receptor (RAR) leads to shifts of anterior neural genes to a more posterior position or a reduction of posterior neural gene expression in embryos, while an active form of the receptor increases expression of posterior markers at the expense of anterior ones (Blumberg et al., 1997; Kolm et al., 1997). No accurate measurement of *in vivo* RA concentration along the A-P axis is available, although by an indirect assay, RA seems to be higher at the posterior end of *Xenopus* neurula embryos (Chen et al., 1994). It is not clear if RA patterns the whole A-P axis or functions only in a specific domain such as in the hindbrain region. *In vivo*, the three types of caudalizing factors may act together to pattern the A-P axis. These signals are transmitted into nuclei to affect activities of transcription factors, including vertebrate homologs of *Drosophila* caudal gene and Hox gene complexes, which then mediate downstream effects (Conlon, 1995; Pownall et al., 1996; Epstein et al., 1997).

D-V Neural Patterning

The initial patterning along the medial-lateral axis in dorsal ectoderm, as for the A-P axis, occurs also at the open neural plate stages. By the time the neural plate closes dorsally to form the neural tube, medial cells assume the ventral positions to develop into floor plate and ventral types of neurons, while the most lateral cells populate the dorsal structures and become neural crest, roof plate, and dorsal types of neurons. Signals from both the underlying mesoderm and the epidermis seem to influence the D-V patterning, and many factors have also been identified which play a role in this process (reviewed

by Graham, 1997; also see LaBonne and Bronner-Fraser, this issue). Although most of our knowledge on regulation of the D-V patterning comes from studies in chick or mouse at the spinal cord level, similar mechanisms seem to operate in the brain region and may be applied to other vertebrates.

In the spinal cord, signals from the underlying notochord seem to mediate ventralization of the neural tube (Yamada et al., 1991, 1993; Placzek et al., 1991). When notochord is grafted to the dorsal side, a secondary floor plate as well as motor neurons form in place of the dorsal neural tube. When notochord is eliminated from its normal position, the floor plate fails to develop and dorsal neural tissue expands to take over the ventral region. The secreted molecule sonic hedgehog (shh) is likely responsible for this ventralization event (Echelard et al., 1993; Roelink et al., 1994). When shh is expressed on the dorsal side, floor plate markers are expressed in these ectopic positions. Shh is expressed initially in the notochord, and later is found in the floor plate itself. An autoproteolytic activity of hedgehog gives rise to a functional N-terminal fragment which is apparently attached to the surface of cell membranes (Porter et al., 1995). The localized expression and limited diffusion of this protein help to create a shh gradient in the ventral neural tube. Sonic hedgehog then acts as a morphogen to determine different ventral cell fates at different concentration thresholds, from floor plate to motor neurons (Roelink et al., 1995; Johnson and Tabin, 1995) (Fig. 3).

On the dorsal side, signals from the epidermis influence the neural cell fates. *In vitro*, conjugates of neural plate and epidermal explants give rise to dorsal neural cells (e.g., roof plate and neural crest cells) which are not present when either type of explant is incubated alone (Selleck and Bronner-Fraser, 1995; Dickinson et al., 1995). *In vivo*, similar induction of dorsal cell types occurs when neural plate explants are grafted into the ventral epidermis region, or vice versa (Moury and Jacobson, 1989). Therefore, the interaction between the neural plate and epidermis is important for patterning the dorsal neural tube. Several BMPs, which are expressed in the epidermis at the open neural plate stages, seem to mediate this process. BMP4 and BMP7 have been shown to induce neural crest markers as well as markers expressed in the dorsal aspect of the neural tube in chick (Liem et al., 1995). When BMP antagonists noggin and follistatin are added in the neural plate and epidermis conjugates, expression of dorsal neural markers is inhibited (Liem et al., 1997). Although in many cases neural crest, roof plate, and dorsal neurons are induced together, it seems that induction of roof plate and specification of dorsal neurons take place progressively. The epidermally derived BMPs, such as BMP4 and BMP7, activate other TGF-β ligands in the roof plate, including dorsalin, activin, BMP4, BMP5, and GDFs, which are subsequently involved in dorsal neuron fate determination (Basler et al., 1993; Liem et al., 1997). Recently, Wnt family members have also been implicated in the D-V patterning of the neural tube. Wnts may be involved in both cell fate determination and expansion of dorsal progenitor cells. In *Xenopus*, coexpression of Wnt1, Wnt3a, or Wnt7B with neural inducers in animal caps can induce expression of neural crest and dorsal neural markers (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998). *In vivo* overexpression of these Wnts enlarges neural crest domains. The induction of dorsal cell fates by Wnt1 and Wnt3a occurs in the absence of cell division, indicating that these Wnts (or other Wnt members) may specify dorsal cells directly instead of merely stimulating survival or multiplication of these cells (Saint-Jeannet et al., 1997). In mice, Wnt1 and Wnt3a double knockouts display reduced but not eliminated neural crest cell population, suggesting additional roles of Wnt1 and Wnt3a in expansion of dorsal neural cells as well as partial redundancy of other Wnts (Ikeya et al., 1997). In *Xenopus*, Wnt7B is expressed before onset neural crest induction and is later restricted to the dorsal midline of the neural tube, while both Wnt1 and Wnt3a are expressed in the dorsal midline of the spinal cord in many vertebrate embryos (Wolda et al., 1993; Parr et al., 1993; Hollyday et al., 1995; Chang and Hemmati-Brivanlou, 1998). The expression pattern of the Wnts in the dorsal neural tube is consistent with their potential roles in this region. It is not known how BMPs and Wnts work together to pattern the neural tube. It is possible that the two signals synergize with each other; alternatively, one pathway may work downstream of another to determine neural cell fates (Fig. 3). In addition, FGF has also been implicated in specification of neural crest cells. A truncated FGF receptor blocks neural crest-specific gene Slug expression in embryos without affecting pan-neural gene Sox-2, while coexpression of noggin and FGF stimulates Slug transcription (Mayor et al., 1995, 1997). The effect of FGF on other dorsal cell fates is not known, nor are its effects on expression of Wnts and BMPs.

The opposing signals from the ventral sonic hedgehog gradient and the dorsal TGFβ/Wnt pathways are interpreted in the neural tube by specific expression of a whole array of transcription factors

along the D-V axis. From the ventral to the dorsal neural tube resides a fork head transcription factor, HNF-3β (floor plate), a zinc finger protein, Gli1 (floor plate and motor neurons), Pax6 (ventral neural tube), Pax3, Pax7, and Msx1 and Msx2 (dorsal neural tube). These transcription factors further turn on other DNA-binding proteins which help to establish final identities of cells according to their specific D-V positions in the neural tube (reviewed by Gruss and Walther, 1992; Tanabe and Jessell, 1996; Ruiz i Altaba, 1997).

Neurogenesis

Neurons arise in the patterned neural plate in defined regions, and the process in which neurons are generated is neurogenesis. Neurogenesis begins in *Xenopus* at the open neural plate stages, when primary neurons in the caudal neural plate can first be detected by expression of β-tubulin that demarcates the three longitudinal stripes at the medial, intermediate, and lateral position, corresponding to future motor neurons, interneurons, and sensory neurons, respectively (Oschwald et al., 1991). The production of primary neurons at these precise positions may be subjected to similar A-P and D-V patterning events, but currently it is unknown how the accurate spatial information of the primary neurons is determined along the medial-lateral axis. The actual production of neurons in vertebrate, however, may involve many conserved molecules as well as a mechanism similar to that in *Drosophila*. In flies, proneural genes containing HLH proteins of the Achaete-Scute complex (AS-C) are expressed initially in all cells in a proneural cluster, and all have equal potential to develop into neuroblasts. AS-C also stimulates expression of the neurogenic gene Delta, which interacts with Notch to inhibit AS-C expression and neuroblast formation in neighbor cells. Through this lateral inhibition a single cell in a proneural cluster will eventually become the neuroblast. The remaining cluster cells take an epidermal fate. In vertebrates, homologs of *Drosophila* proneural genes as well as neurogenic genes have been identified, and they seem to play a similar role in production of primary neurons during neurogenesis. In *Xenopus*, an Achaete-Scute homolog, XASH3, is able to increase the number of primary neurons and enlarges the neural plate at the expense of the epidermal and neural crest cells (Zimmerman et al., 1993; Turner and Weintraub, 1994; Ferreiro et al., 1994). Other HLH proteins, including neurogenin (Ngn) and NeuroD, are also able to activate neurogenesis (reviewed by Lee, 1997). Both Ngn

and NeuroD are expressed in *Xenopus* at open neural plate stages in three longitudinal stripes that coincide with the later tubulin-expressing domains. Overexpression of Ngn or NeuroD induces ectopic expression of primary neurons in *Xenopus* neural plate (Lee et al., 1995; Ma et al., 1996). Ngn expression precedes that of neuroD, and Ngn unidirectionally activates neuroD in the ectodermal explants, suggesting that neurogenin acts upstream of neuroD in neuron production. In addition to HLH proteins, a zinc finger transcription factor, MyT1, has been reported to play an important role in *Xenopus* neurogenesis (Bellefroid et al., 1996). MyT1 is expressed in similar domains as Ngn, but at a later time. Blocking MyT1 function perturbs normal neurogenesis and prevents ectopic neurogenesis induced by Ngn, implying that MyT1 may be an essential component in the Ngn pathway. The primary neuron production stimulated by these proneural genes is inhibited by the Delta-Notch signaling pathway (Coffman et al., 1993; Chitnis et al., 1995). Overexpression of Delta1 or an activated version of Notch in *Xenopus* reduces primary neuron differentiation. *In vivo*, Notch is expressed broadly in the neural plate, while Delta 1 expression is limited to the three stripes which give rise to the primary neurons in *Xenopus*, consistent with an inhibitory role of Delta-Notch pathway in primary neuron production.

It is unclear how neurogenesis is connected to neural induction. Several neural inducers (e.g., follistatin) (Hemmati-Brivanlou et al., 1994) can induce both pan-neural gene NCAM (expressed both in neurons and glias) and the neuron-specific β-tubulin at the neurula stages in *Xenopus* ectodermal explants. Transcription factors that mediate neural induction, such as Zic-r1, X1POU2, and Xski, can also induce β-tubulin expression in animal caps (Witta et al., 1995; Amaravadi et al., 1997; Mizuseki et al., 1998). The activation of proneural genes by these neural inducers, with the exception of Zic-r1, has not been studied in detail. It remains possible that neuron is the intrinsic fate for cells in the neural plate. Inhibitory actions, including Delta-Notch pathway, may be required to divert cells from neuron formation. A close examination of possible neuronal cell fates in dissociated animal caps at molecular level may help to make the link between neural induction and neurogenesis.

In summary, we have discussed neural induction and patterning events during embryonic ectodermal cell fate determination. Multiple signal cascades take place during this process, and they may all be required to regulate a single event. Conversely, one

particular signal pathway can be used at a different time and place to regulate different cell specifications. BMP signaling, for example, is involved in epidermal fate determination at gastrula stages, while it specifies dorsal neural cells at later stages after neural tube closure. One theme recurrent in many regulatory events is the formation of an activity gradient of a secreted factor, which can act as a morphogen to determine different cell fates. Both BMPs and hedgehog employ this mechanism, and it may also account for some actions of the Wnt and FGF ligands in caudalizing the neural tube. It is also important to keep in mind that A-P and D-V patterning events take place at the same time, and they may influence each other in cell fate specification. For example, although primary neurogenesis is regulated by medial-lateral patterning to set the three stripes of primary neurons at specific positions in the neural plate, signals involved in A-P specification can influence the timing of neurogenesis. Normally, posterior neurons are produced earlier than anterior neurons in *Xenopus*. A caudalizing factor, retinoic acid, can promote neuron production in anterior neural tissue at an early developmental stage (Papalopulu and Kintner, 1996b). In another case, the expression of Pax3, a dorsal and posterior-specific neural gene, is also subjected to a complex array of A-P and D-V signals (Bang et al., 1997). The cells in the neural plate may therefore need to incorporate simultaneously the signaling inputs from both anteroposterior and dorsoventral patterning factors to have a correct readout of their coordinates and express a set of markers at the right time and in the right place.

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