

Vertebrate Embryonic Cells Will Become Nerve Cells Unless Told Otherwise

Review

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The past few years have witnessed a significant change in the understanding of how the vertebrate nervous system forms during embryogenesis. More than seventy years since Spemann and Mangold first demonstrated the phenomenon of neural induction, the molecular mechanisms underlying neural induction now appear to be at hand. Two independent approaches, one focusing on a “default” or “ground-state” model for neural induction and the other culminating in the discovery of secreted neural inducing factors (noggin, follistatin, and chordin), have not only brought to a successful conclusion the search for Spemann’s neuralizing factor, but also illuminated its mechanism of action. It now appears that neuralization of embryonic cells occurs when cells do not receive other inducing signals telling them to form epidermis, mesoderm, or endoderm. This concept of neuralization allows for a reinterpretation of the classical views on both neural and epidermal specification. The secreted growth factor BMP4 (Bone Morphogenetic Protein) plays a pivotal role wherein BMP signaling induces epidermal differentiation. It is the absence of BMP signaling, accomplished by BMP antagonists including noggin, follistatin, and chordin, that leads to the formation of neural tissue.

Lessons from History

The concept of neural induction was established in 1924 by Spemann and Mangold’s grafting experiments using salamander gastrula (Spemann and Mangold, 1924). During gastrulation, prospective ectodermal cells, located on top of the embryo (animal pole) make a choice between two fates: epidermal and neural. The prospective neural plate is defined by two boundaries: the epidermal–neural boundary in the animal pole and the neural–mesodermal boundary in the equatorial region (Figure 1). The blastopore lip, where cells first invaginate during gastrulation, marks the prospective dorsal side where the neural plate forms. Transplantation of a dorsal blastopore lip, which consists of mesoderm and endoderm, from an early salamander gastrula to the ventral side of another early gastrula causes formation of a second nervous system (Figure 2). The second nervous system develops not from the transplanted tissue, but from ventral ectoderm, which in an undisturbed embryo forms epidermis. Spemann named the dorsal blastopore lip the “organizer,” and proposed that in normal development this region induces and organizes a correctly patterned nervous system in neighboring dorsal ectoderm

(Spemann and Mangold, 1924). In the absence of this influence, as on the ventral side, the ectoderm differentiates as epidermis. Thus, in the following decades, the development of epidermis was assumed to be a fall-back, or “default” fate for gastrula ectoderm, requiring no cell–cell communication, whereas neural specification was thought to be contingent upon the receipt of a positive signal (in molecular terms, a signal that activates a signal transduction pathway) from neighboring cells.

A considerable effort over several decades failed to identify the gene products responsible for neural induction in the embryo. The idea of a positive signal involved in neural induction so dominated thinking in the field that the significance of results inconsistent with this idea were not widely appreciated. For example, several researchers found that when cells of ectodermal explants (also called “animal caps”), were dissociated, they can form neural tissue whereas intact or whole animal caps form epidermis (Grunz and Tacke, 1989; Godsave and Slack, 1991). In these experiments dorsal mesodermal tissue (the organizer) is absent and neuralization occurs in a cell autonomous fashion, in contradiction with the requirement for a positive signal derived from the organizer. With hindsight these results using simple cell dissociation provided a strong hint that neural inhibitory signal(s) within the ectoderm, or the whole embryo, prevent neuralization, and in the case of animal caps, these neural inhibitors or antagonists drive cells toward an epidermal fate.

Neural, Not Epidermal, as the Default State of Embryonic Ectoderm

It is important to distinguish between direct and indirect effects in understanding assays for neural induction.

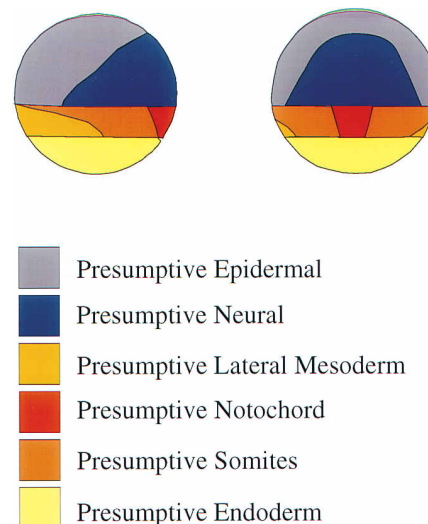


Figure 1. Fate Map of the *Xenopus* Gastrula

(Left) Lateral view.

(Right) Dorsal view.

The presumptive neural plate is delineated by presumptive epidermis and mesoderm. Animal pole is at the top.

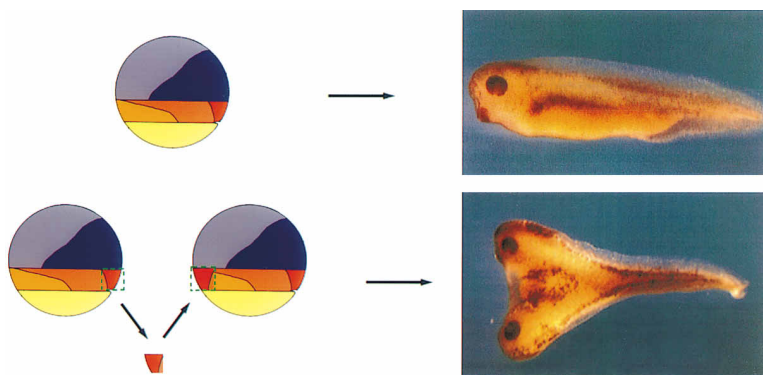


Figure 2. Classical Transplantation Experiment by Spemann and Mangold

(Top) The fate of a normal embryo.

(Bottom) The transplantation experiment.

The transplantation of an organizer from a donor to the ventral side of a host embryo induces a complete secondary axis and giving rise to an embryo with two main body axes. This experiment demonstrates that all the information necessary and sufficient to induce a dorsal axis, including the entire nervous system, is contained within the cells of the organizer (boxed here in green).

Several growth factors with mesoderm inducing activity, including activins and Vg1, can cause formation of neural tissue when added to ectodermal explants, but this neuralization is indirect because some of the treated cells first form dorsal mesoderm. The mesodermal cells then mimic the action of the organizer and induce neural tissue in the surrounding cells. The first *direct* molecular neuralizing treatment to be described was a truncated type II activin receptor (Δ 1XAR1, here referred to as tAR), designed to test for a requirement of activin, a member of the TGF β growth factor superfamily, in mesoderm induction (Hemmati-Brivanlou and Melton, 1992). Analysis of the expression of several tissue specific markers following injection of tAR yielded the surprising observation that a general neural marker, neural cell adhesion molecule (NCAM), was turned on in ectodermal explants following inhibition of activin type II receptor signaling. These explants express the activins and their receptors and, as mentioned above, would make epidermis when cultured alone. In addition, it was found that the dominant negative activin receptor could also neuralize cells located at the bottom of the embryo or vegetal pole, cells normally fated to become endoderm. This result suggested that neuralization by inhibition of the type II receptor signaling is not confined to cells of the ectoderm but can be generalized to other germ layers. In terms of the specificity of the effect, it was noted that while tAR does not interfere with receptor tyrosine kinase signaling (such as FGF), tAR could inhibit signaling of other TGF β factors. It is now appreciated that TGF β receptors are heterodimers and thus interfering with a particular pathway may affect signaling from other members of the family. Indeed, it was subsequently determined that tAR blocks more than just activin signaling, and appears to inhibit other TGF β s including Vg1 and BMPs (Schulte-Merker et al., 1994; Hemmati-Brivanlou and Thomsen, 1995).

The fact that tAR expression directly initiated nerve cell formation was significant because not only did it occur in the complete absence of dorsal mesoderm, but more importantly, it demonstrated that neuralization can occur by inhibition of signaling. Moreover, injection of tAR showed that cells in any germ layer would become neural if TGF β signaling was blocked (Hemmati-Brivanlou and Melton, 1994). Since both cell dissociation and expression of a dominant negative activin receptor in intact ectodermal explants can be interpreted as an interference with the communication between cells,

these results suggested that individual cells of the early gastrula animal cap are predisposed to form neural tissue in the absence of further signals. In this view, epidermal (but not neural) specification requires a positive cell signaling within the prospective ectoderm. When this signaling is interrupted experimentally by cell dissociation or molecular antagonists, neural tissue forms. Neural induction by the organizer in vivo could work in the same way, that is, by blocking epidermal induction within the animal cap (Hemmati-Brivanlou and Melton, 1994; Figure 3). This view contrasts with the commonly held textbook model wherein neural induction requires a positive signal. The term “neuralization” more aptly describes the situation than “neural induction”; indeed, it is the epidermis that is induced.

Predictions of the Default or Ground State Model for Neuralization

The model for neural specification described above makes two important predictions. First, the signal from the organizer is an antagonistic secreted signal that inhibits the activity of a neural inhibitor/epidermal inducer. This antagonism occurs specifically in the dorsal ectoderm during gastrulation. Second, the hypothesis that epidermal rather than neural specification requires positive cell signaling among ectodermal cells, predicts that epidermal fate can be induced in ectodermal cells. These predictions have very recently received experimental support from several fronts.

BMP4 Inhibits Neuralization and Induces Epidermis

The neuralizing activity of the truncated activin receptor, and the observation that an activin antagonist, follistatin, has direct neural inducing activity (see below) pointed to activin as an endogenous neural inhibitor. Nonetheless, these data provided no direct evidence that activin could specify or induce epidermis. To address this prediction, a complementation assay was used where cells of the animal cap were dissociated and incubated in the presence or absence of activin or BMP4, which are both TGF β ligands inhibited by the truncated activin receptor. While activin did inhibit neuralization of dissociated ectodermal cells by inducing mesoderm, activin did not induce expression of epidermal markers. In contrast, BMP4 not only inhibited neuralization but induced epidermal fate. The two activities of BMP4, neural suppression and epidermal induction, always occur together, leading to the conclusion that they represent a single

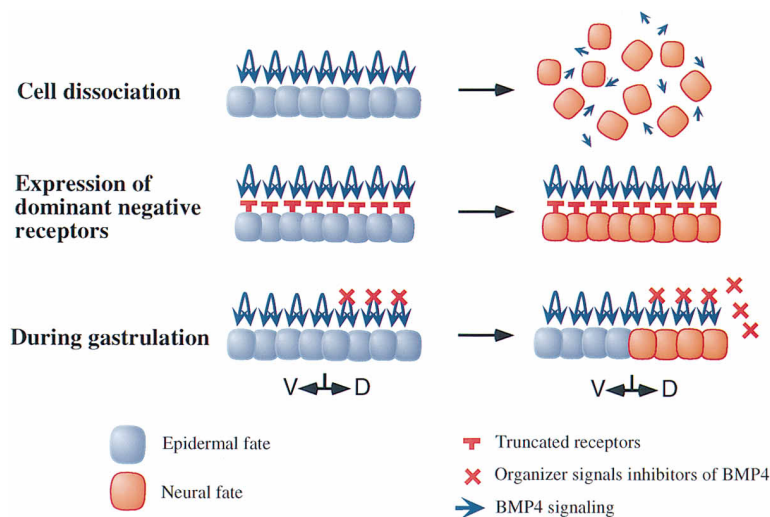


Figure 3. Schematic of the “Default Model” of Vertebrate Neuralization

(Top) In normal intact ectodermal explants (animal caps), BMP4 (the blue arrows) induces and maintains the epidermal fate. Upon dissociation this secreted neural inhibitor/epidermal inducer is diluted and ineffective, and thus the neural fate is unveiled by derepression.

(Middle) Expression of tAR or tBR interferes with the cells ability to receive the BMP4 signal. The epidermal fate can no longer be maintained and the neural fate is unveiled.

(Bottom) In the embryo, the ectoderm has a dorsal-ventral polarity. Secreted factors, such as noggin, chordin, and follistatin, interfere directly with the BMP4 signal (in the case of noggin and chordin, by direct binding to BMP4). The consequence of this interference is that BMP4 can no longer have access to its receptor and therefore can no longer induce or maintain the epidermal fate and thus neural tissue forms on the dorsal side.

action, as expected from the neuralization model (Wilson and Hemmati-Brivanlou, 1995). Induction of epidermis is inhibited if the dissociated cells express the truncated activin receptor. These findings demonstrated that epidermis is an induced fate rather than the default state of the ectoderm.

BMP4 is expressed at the appropriate time and place to be the endogenous neural inhibitor/epidermal inducer. In situ hybridization shows that BMP4 RNA is present in the entire animal cap at the start of gastrulation, as well as in ventral and lateral marginal zone (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). At later stages, transcripts disappear from the portion of the ectoderm that becomes the neural plate, suggesting that repression of BMP4 transcription is one of the mechanisms by which BMP4 activity can be inhibited in the prospective neuroectoderm. A BMP4 receptor is also expressed in the animal cap. Thus the pattern of BMP4 transcription is consistent with its proposed functions in epidermal induction and the suppression of neural development.

Additional Evidence for the Default Model of Neuralization in Vertebrates

Dominant Negative BMP Receptors and Ligands

As would be predicted from the default model, antagonists of BMP4 signaling lead to neuralization. For example, a truncated type I BMP4/2 receptor, tBR, induces neural tissue directly in intact animal cap explants as does the truncated activin receptor, tAR (Suzuki et al., 1994; Xu et al., 1995). However, while tAR blocks all TGF β s tested so far, tBR seems to be more specific in that it does not inhibit activin or Vg1 signaling. In addition, dominant negative forms of ligands such as BMP4 and BMP7, but not activin, induce neural tissue directly in ectodermal explants (Hawley et al., 1995). The fact that BMP4 dominant negative ligand can also induce neural markers suggests that either other BMPs can fulfill the same neural inhibitory activity or that dominant negative BMP ligands have a pleiotropic inhibitory effect on all BMPs.

Noggin, Follistatin, Chordin, and Others

Isolation of the first *endogenous* direct neural inducing factors was reported shortly after the characterization of tAR activity. A functional screening strategy using ventralized (UV irradiated) embryos allowed for the identification of noggin. Because of its localized expression in the organizer and its neural inducing effect, noggin was proposed to be the instructive positive signal defined by Spemann’s experiments, and thus presented a serious challenge to the double inhibition mechanism which is the trademark of the default model. In fact the cloning of the noggin receptor was much anticipated as a way into the signal transduction involved in neural induction (Lamb et al., 1993).

On another front, an obvious extension of the demonstration of neuralizing activity by tAR was to examine the embryonic distribution and activities of other activin antagonists in embryos. Follistatin, an inhibitor which binds activin, also expressed in the organizer, was shown to turn on neural markers directly (Hemmati-Brivanlou et al., 1994). However, as it was the case for tAR, the specificity of follistatin for activin was uncertain. Recently it was shown that follistatin can interfere with the function of BMP7 (Yamashita et al., 1995; see below), and can dorsalize ventral mesoderm (Sasai et al., 1995).

Another important gene expressed in the organizer, chordin, was originally isolated in a differential screen for dorsal specific genes (Sasai et al., 1994). Chordin, a secreted factor and the vertebrate homolog of the *Drosophila* gene *short gastrulation (sog)* has direct neural inducing ability. Though the possibility of an antagonism between chordin and BMP4 was noted, chordin was also suggested to be a positive neural inducing signal derived from the organizer with a possible receptor and a signal transduction pathway (Sasai et al., 1995). Just like noggin and follistatin, chordin can also rescue ventralized UV embryos and dorsalize mesoderm.

Biochemical Mechanisms for Neuralization

Two significant papers recently published in *Cell* shed light on the mechanism of neural tissue formation by

noggin and chordin. Biochemical studies demonstrated that both chordin and noggin directly bind BMP4 (Piccolo et al., 1996; Zimmerman et al., 1996). The binding affinity is higher for noggin–BMP4 (20 pM) than for chordin–BMP4 (300 pM), but chordin protein seems to be more abundantly expressed in the organizer. Interestingly, in both cases, this binding can be competed efficiently with BMP2 and to a lesser degree with BMP7. The consequence of this binding for both noggin and chordin is that the neural inhibitor/epidermal inducer BMP4 can no longer access its receptor; thus, BMP4 signaling, which would otherwise occur throughout the animal cap, is inhibited on the dorsal side and neural fate is unveiled. Finally, the interaction of both noggin and chordin seems to be specific to BMP2 and BMP4 since they both fail to bind activin or TGF β 1.

While the biochemical mechanism of neuralization by noggin and chordin seems to be solved, the case for follistatin is unresolved. First, it is clear that follistatin directly binds activin with very high affinity (Nakamura et al., 1990). Activin, however, does not have an epidermal inducing activity; instead, it inhibits neural formation by pushing the cells toward a mesodermal fate. It is thus possible that activin mediates cell fate choices at the ectodermal-marginal zone boundary. Also, there is evidence that follistatin can inhibit BMP7 activity (Yamashita et al., 1995). Because there is evidence that heterodimers of BMP4/7 have a much higher activity than BMP4 or BMP7 homodimer, and that there is overlap of expression for BMP4 and BMP7 in the ventral side of the embryo (reviewed by Hogan, 1996), it is tempting to speculate that the inhibition of BMP4 by follistatin is mediated by its binding to BMP7. Alternatively, because RNA injections with follistatin are done at the two cell stage and the animal cap explants are removed about 4 hours later, at blastula stages, it could be argued that an intact activin pathway is required for BMP4 signaling, whose disruption eliminates BMP4 activity.

It is also noteworthy that three other secreted factors FGF, FRL1 (Harland, 1994; Kinoshita et al., 1995), and Xnr3 have been reported to have direct neural inducing activity. The mode of action of both FGF and FRL1, which is an FGF related factor, is unclear. Xnr3, however, is a member of the TGF β family localized in the organizer with direct neural inducing activity also mediated through a BMP4 inhibition (Hansen et al., 1996).

Evolutionary Considerations

In an interesting turn of events, recent studies of ectodermal patterning in vertebrate embryos may have helped us understand the situation in *Drosophila*. The strategy of neuralization by inhibition of an inhibitor seems to have been conserved from arthropods to mammals.

In *Drosophila*, the homolog of BMP4 is *decapentaplegic* (*dpp*) and the homolog of chordin is *short gastrulation* (*sog*). DPP/BMP4 and SOG/chd can functionally substitute for each other in both organisms despite the fact that the nervous system forms on the ventral side in *Drosophila* and on the dorsal side in *Xenopus* (Holley et al., 1996). In addition, it has recently been shown that noggin can inhibit DPP function in the *Drosophila* embryo (Holley et al., 1996). There is as of now no noggin or follistatin characterized in the fruit fly.

On the other end of the spectrum, homozygous knock-out mice for BMP4 or the BMP receptor (BMPR1) die mostly at gastrulation stage at the time when these types of cell fate decisions are being made (for review, see Hogan, 1996). Even though this is a negative result, it highlights the pivotal role that BMP4 seems to play. It is also important to remember that since more than one BMP4 inhibitor is present in vertebrates, it is likely that the knock out of single BMP4 antagonist will have no obvious neural phenotype.

Is the Inhibition of BMP Signaling Sufficient for Neuralization?

Although noggin, follistatin, and chordin can neuralize by antagonizing BMP4 epidermalizing activity, is it possible that, in addition, they transduce a signal via a receptor, as was originally postulated for noggin and chordin? The default model would predict not. There are indeed three lines of evidence that strongly argue against the existence of receptors for follistatin, noggin, or chordin, at least in the pathway mediating neuralization, and that inhibition of BMP signaling is sufficient to unveil the neural fate. First, there is the evidence from cell dissociation experiments discussed above: when embryonic cells are dissociated for several hours they will make neural tissue (Grunz and Tacke, 1989; Godsave and Slack, 1991), and the addition of BMP4 will inhibit this effect and induce epidermis (Wilson and Hemmati-Brivanlou, 1995). The second evidence comes from experiments recently performed in *Drosophila*. Holley et al. (1996) demonstrate that at least for SOG/chd, and perhaps for noggin, binding DPP/BMP is their only function. They showed that while noggin inhibits DPP and phenocopies a *dpp*⁻ mutation, it can only operate outside of the cell, and in the presence of an activated DPP receptor, its effect is abolished. More compelling is the fact that the double *sog*⁻ *dpp*⁻ mutant has the same phenotype as the *dpp*⁻ mutant. If SOG had any other function than just inhibiting DPP, the double mutant phenotype should have been different than that of *dpp*⁻ alone. The final line of evidence comes from the fact that, while chordin can reverse the osteogenic induction caused by BMP4 in 10T1/2 cell lines, it cannot block the one mediated by retinoic acid (Piccolo et al., 1996). Taken together, these observations strongly suggest that inhibition of BMP signaling is sufficient for neuralization.

Contribution from many groups working with the amphibian system has provided a molecular solution to the problem of vertebrate neural induction originally defined by Spemann and Mangold. The challenge for the future will inevitably include the establishment of a link between the early neural specification process, described above, and the function of neurogenic genes operating downstream of these signaling events, ultimately leading to the generation of a mature neuron.

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