The avian embryo: a powerful model system for studying neural induction

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ABSTRACT **Neural induction is the process during early embryonic development whereby the mesoderm of the embryo elicits a change of fate in cells of the overlying ectoderm, from epidermal to neural. Since its discovery in 1924 by Spemann and Mangold, who used newt embryos, most research on this developmental event has been conducted with urodelean and anuran amphibians. This is because of the ease with which they can be manipulated and because of the recent availability of cell type- and region-specific molecular markers. With the recent isolation and characterization of suitable markers in the chick embryo, and the equal ease with which it can be manipulated, the way is now open for amniote embryos to join amphibians as an experimental system for neural induction studies. Another advantage of the avian embryo is that it possesses a peripheral extraembryonic region, which although it does not contribute to embryonic tissues at all, is competent to respond to neural-inducing signals, thereby providing developmentally naive cells for in vivo and in vitro assays. Here, I review recent advances that make the chick embryo a system uniquely suited for the study of neural induction at both the cellular and the molecular level.— Stern, C. D. The avian embryo: a powerful model system for studying neural induction. *FASEB J.* 8: 687-691; 1994.**

Key Words: embryonic axis • chick embryo • primitive streak • Hensen's node

THE WORDS NEURAL INDUCTION EVOKE, FOR many scientists, reminiscences of the pioneering experiments of Hans Spemann and Hilde Mangold, published in 1924 (1). These authors showed conclusively, using two differently pigmented species of newt embryo (*Triturus*), that a region of the gastrula (the dorsal lip) is unique in being able to induce the formation of an ectopic, second nervous system when transplanted to a different region of a host embryo. Only a few years later, in the early 1930s, C. H. Waddington demonstrated that amniote embryos, including ducks, chicks, and rabbits, could also be made to generate a second nervous system by transplanting the tip of the primitive streak, a region known as Hensen's node (2, 3). Since that time, many experiments on neural induction have been performed using both amniotes and the anamnia. Progress has been relatively slow, however, mainly because of the lack of good early and objective markers. More recent, spectacular advances in the field of mesoderm induction have led to the identification of members of the fibroblast growth factor (FGF)[2] and transforming growth factor-β/activin families of growth factors as mesodermal-inducing molecules (reviewed in ref 4). The expectation has therefore naturally risen that similar advances will be made soon in identifying "the" neural-inducing factor. However, it now appears that such a factor will be more difficult to identify. Here I will consider some possible reasons for this slow progress and make the case that much can be learned about neural induction by using avian embryos as a main experimental model.

THE SEARCH FOR A NEURAL INDUCER

Experiments in amphibians: the animal cap assay

Since Spemann's time, several new strategies have been devised to study neural induction in amphibians, and *Xenopus* has replaced the newt as the main experimental species. One reason for the latter change is that the animal cap (North pole) of the Urodele blastula undergoes neural differentiation very readily. In the 1930s, for example, several studies reported that many heterologous inducers can elicit neural differentiation from newt ectoderm, and these included high and low pH, alcohol and vital dyes, as well as crude extracts from organs from different species (reviewed in ref 5).

Spemann and Mangold (1) first conducted their experiments by implanting donor tissue onto a host embryo, either within the ectodermal cap or inside the blastocoelic cavity. More recently, a new strategy has become more widespread, the animal cap assay. This is derived from the experiments of Nieuwkoop (6) on mesoderm formation. He showed that if the animal cap of the *Xenopus* embryo is cut out and grown alone in saline, only epidermis develops. However, if combined with vegetal (South pole) tissue, both epidermis and mesoderm, as well as some neural tissue, form. For this reason, isolated animal caps have been used since then to test the ability of soluble growth factors to cause it to differentiate into mesodermal derivatives, and this assay led to identification of the FGFs and activins as mesodermal inducers. Workers with amphibians have now adopted this assay to look for neural inducers, but using animal caps isolated from later-stage (early/mid-gastrula, or stages 10–11) embryos. Some putative neural-inducing molecules and pathways have been reported.

Some candidate-inducing molecules in amphibians

Harland and his colleagues (7) recently reported the cloning of a gene encoding a secreted factor, which they called nog-

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[2]Abbreviations: FGF, fibroblast growth factor; NCAM, neural cell adhesion molecule; HGF/SF, hepatocyte growth factor/scatter factor; CNS, central nervous system.

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gin, expressed specifically in the amphibian dorsal lip. It was isolated on the basis of its ability to rescue embryos that had been ventralized by UV-irratiation (7). Indeed, noggin can dorsalize mesoderm, that is, increase the amount of dorsal tissues like notochord at the expense of more ventral mesoderm like blood and mesenchyme. The organizer is considered the most dorsal of all the tissues in the amphibian gastrula, and the appearance of neural tissue could be a measure of the presence of the organizer. Consistent with this, when animal caps from gastrula-stage embryos are treated with noggin, they form neural tissue, which led to the recent suggestion that noggin is a neural inducer (8). Harland and colleagues (7) were careful to examine whether noggin also induced mesoderm, using RNAase protection assays with a variety of mesodermal markers. They found none. But it is possible that some of the most dorsal tissues (such as endoderm and prechordal plate), for which there are no reliable cDNA probes, had been induced by the factor. This possibility still needs to be ruled out before noggin can be considered a bona fide neural-inducing molecule.

Other experiments in *Xenopus* in which part of the activin signaling pathway was disrupted by the construction of dominant-negative mutants for one of the activin receptors (XAR1), suggest that these pathways inhibit neural induction because such embryos develop with no mesoderm but with ample neural tissue (9). These authors have suggested that a neural fate may be the default pathway of development in amphibian ectoderm, and that activin acts as a negative regulator of this fate. In support of this hypothesis, they have recently found that an endogenous inhibitor of activin, follistatin, also causes the ectoderm to differentiate into neural tissue (A. Hemmati-Brivanlou and D. A. Melton, see "note added in proof"). Clearly, further research will be required to elucidate the relationships between noggin and the activin signaling pathways in neural induction, and indeed to establish whether other factors are involved.

There are two major problems when searching for neural inducers with the animal cap assay. First, the animal cap of the amphibian early gastrula contains at least some cells normally fated to become neural plate (see, for example, refs 10, 11). These cells may already have been exposed to the natural inducers for some time before their isolation, complicating interpretation of the results. This offers an explanation for the observations that: *1)* simple dissociation of the animal cap can elicit neural differentiation both in *Xenopus* and in Urodeles (e.g., ref 12), and *2)* animal caps obtained from dominant-negative activin receptor mutants show a natural tendency to become neuralized without further treatment (9). The second problem is the lack of completely specific early neural markers in amphibians. Most workers use the expression of mRNA encoding the neural cell adhesion molecule (NCAM) as a general neural marker, but there is a low level of maternal NCAM message present in the egg, and it is also expressed in other regions that never become neural. Other markers that recognize particular regions of the nervous system are also not specific to the neuroectoderm, such as the homeobox gene *XlHbox1/Hox3.3/Hoxc-6*, which is also expressed in the underlying mesoderm (13).

Experiments on avian embryos

Avian embryos offer a system where some of these problems might be overcome and where neural-inducing factors may also be sought. One important difference between amphibians and avian embryos is that the latter also contain a region of epiblast/ectoderm that lies outside the normal fate map, and contributes cells only to the extraembryonic membranes. This region is called the area opaca, and it has been shown (14, 15) to respond to a graft of Hensen's node (**Fig. 1*A***) by producing a complete nervous system, expressing regional markers (Fig. 1*C*–E). Therefore, the area opaca epiblast can be considered a collection of cells, which from the neural point of view are naïve.

A second advantage of avian embryos is the recent availability of at least one early expressed, pan-neural marker. This is the L5 epitope, recognized by monoclonal antibody 487 (16, 17): immunoreactivity is confined to the elevating neural plate as soon as this is morphologically distinguishable at stages 7–8, and remains in the neural axis after it has closed to form a tube (Fig. 1*F*).

The dorsal lip and Hensen's node

The evidence that the amniote equivalent of the amphibian dorsal lip is Hensen's node, at the tip of the primitive streak (Fig. 1*A*), initially came from Waddington's (2, 3) transplantation experiments: a functional test demonstrating the organizer property of the node. But there are also molecular markers expressed in the dorsal lip of *Xenopus* (18) and in the node of chick and mouse embryos (19, 20), such as the homeobox gene *goosecoid*. There is also detailed information about the distribution of cell fates in these regions of amphibian and amniote embryos suggesting that they are homologous structures (e.g., refs 21, 22); both contain presumptive notochord, gut endoderm, and somite cells as well as some cells that contribute to the floor plate region of the neural tube (Fig. 1*G*). It is not yet known whether neural-inducing ability is associated with any particular presumptive cell type, or whether the node/dorsal lip possesses this ability independently of the fates of the cells contained in it.

L5 and competence to respond to neuralizing signals

As soon as the neural plate forms in the chick embryo, immunoreactivity with the L5 antibody is restricted to this region. This specificity persists to later stages, when the neural tube has formed (Fig. 1*F*). However, during gastrulation, before the appearance of a visible neural plate, the carbohydrate epitope recognized by this antibody is distributed more widely, starting from the mid-primitive streak stage (16). At this stage, the antibody decorates the region that is competent to respond to a graft of Hensen's node. To investigate whether the L5 epitope is involved in some aspect of the response of competent epiblast to such a graft, Hensen's node was grafted together with hybridoma cells secreting this antibody; in the presence of the hybridoma cells, neural induction is inhibited (16). Thus, the L5 antigen, or the molecules carrying it, may be required by epiblast cells in the response to neural induction. Moreover, the gradual restriction of immunoreactivity to more and more central regions of the embryo mimics a similar restriction in competence of the epiblast to respond to a graft of Hensen's node (15). Therefore, the L5 epitope is a marker of neural competence.

The peptide factor hepatocyte growth factor/scatter factor may induce or maintain neural competence

When human or mouse cells secreting the peptide hepatocyte growth factor (also known as scatter factor, or HGF/SF; ref 23) are grafted onto an early chick embryo, an ectopic neural plate is sometimes seen to form in the vicinity of the graft (24). This led to the suggestion that HGF/SF may have neural-inducing properties. In tissue culture, treatment of extraembryonic epiblast explants with recombinant human

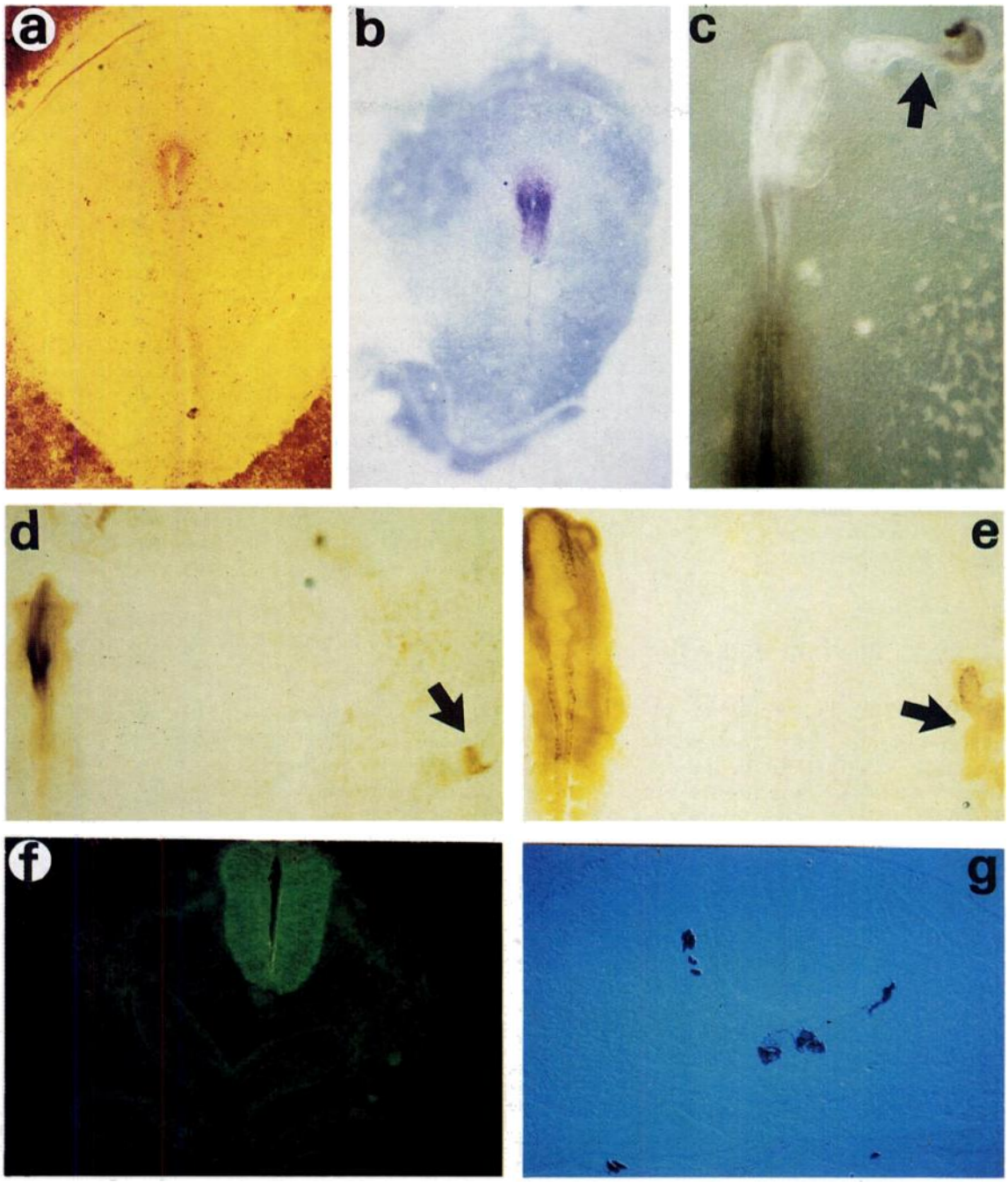

Figure 1. *a)* Chick embryo of about 14 h incubation, at the full primitive streak stage. The primitive streak can be seen, defining the future anteroposterior axis of the embryo. At its tip, the thickening corresponds to Hensen's node. *b)* An embryo at the same stage as that shown in *a,* after whole-mount in situ hybridization to reveal the expression of the homeobox gene *goosecoid:* expression is restricted to Hensen's node. *c–e)* Examples of the result of grafting Hensen's node onto the extraembryonic region of a host embryo, and expression of three different region-specific markers: the homeobox gene *Hoxc-6* is expressed in the posterior nervous system of the host and induced (arrow) embryo (*c*); the homeobox gene *engrailed-2* is expressed in the metencephalic region of the host and ectopic (arrow) nervous system (*d*); a monoclonal antibody against a phosphorylated, neurofilament-associated antigen recognizes two identifiable groups of neurones, one in the diencephalon and one in the hindbrain at this stage—the induced nervous system (arrow), generated by an older node, contains only the more posterior (rhombencephalic) group (*e*). *f)* Transverse section through an embryo of about 2 days' incubation, stained by indirect immunofluorescence with monoclonal antibody L5, which at this stage is specific for the neural tube. *g)* Transverse section through an embryo in which a few cells had been labeled with the carbocyanine dye, DiI, at the same stage as in *a,* which was subsequently incubated for 36 h. The fluorescence of the dye was used to photoconvert the substrate diaminobenzidine to an insoluble, black precipitate. Cells descended from those originally labeled are found in the medial portion of the somites, in the notochord, and in the endoderm. From ref 20, with permission of Cell Press, Inc. *b*); (*c–e, f,* and *g*) from refs 15, 16, and 22, respectively, with kind permission of the Company of Biologists Ltd.

or mouse HGF/SF sometimes causes the differentiation of neurones (C. D. Stern et al., unpublished results).

Such explants, after overnight culture in the absence of factors, lose their expression of the L5 epitope. However, when cultured with HGF/SF at concentrations as low as 4 ng/ml, strong L5 immunoreactivity is seen throughout the explant after 24 h (C. D. Stern et al., unpublished results). This finding suggests that HGF/SF may act by either enhancing or maintaining the competence of the epiblast to respond to neural-inducing signals.

As L5 immunoreactivity and competence both gradually become condensed to progressively more central regions of the epiblast during development, it would seem likely that Hensen's node produces a factor that delays the loss of competence from the nearest regions of the epiblast in a concentration-dependent way. Could this factor be HGF/SF? Consistent with this possibility, in situ hybridization experiments reveal that the node is the only region of the primitive streak-stage embryo that expresses mRNA encoding this factor (C. D. Stern et al., unpublished results). Further experiments will be required to test this hypothesis more directly, but these considerations suggest that the functions of Hensen's node may include maintenance of the neural competence of neighboring epiblast to respond to neural-inducing signals.

REGIONALIZATION OF THE NERVOUS SYSTEM

Experiments in amphibians: the "Keller sandwich"

When an organizer is grafted onto a host amphibian embryo as Spemann and Mangold (1) did, or indeed when animal caps are treated with soluble inducing factors, the response consists of more than neural and/or mesodermal differentiation of cells. A complete or almost complete embryonic axis is formed, which prompted Spemann to name his discovered inductive interaction "primary induction." In experiments using amphibians, induction and the process that might be termed regionalization (the acquisition of regional characteristics by the induced tissue) do not seem to be separable experimentally. Whenever induction takes place, there is also visible development of axial structures that can be recognized both by morphology and by the expression of several region-specific markers.

Attempts to separate induction from regionalization led to a new strategy for isolating explants from *Xenopus* embryos: the Keller sandwich (25). Here, the animal cap is isolated as if for an animal cap assay, but the explant extends into the dorsal side of the embryo, to the organizer region itself. The explants obtained in this way are known as "open-faced" Keller sandwiches, in contrast to the "double-decker" version, in which two such caps are juxtaposed either in the same dorsoventral orientation or with one of them reversed. Using this assay, it has been possible to analyze to some extent the relative contributions of vertical (from underlying mesoderm) and planar (from within the plane of the explant) signals to induction and regionalization (for a recent review, see ref 26). The main conclusion from these experiments is that vertical and planar signals both contribute to pattern the induced structures, and one view is that the former act first and in a more general way whereas planar interactions act to refine the original pattern.

The same sandwich technique also helped to confirm Waddington's original observation that inducing signals can act across species. In Waddington's experiments, the operated species included the rabbit, chick, and duck—all amniotes. In the more recent experiments it was shown that chick Hensen's node is able to induce and to pattern even amphibian animal caps when sandwiched between them (27). Similarly, Blum et al. (19) showed that when the tip of the mouse embryo, which contains the organizer region (28), is grafted into the blastocoelic cavity of a *Xenopus* embryo, a second axis is generated in the frog host.

Induction and regionalization can be separated experimentally in avian embryos

Are induction and regionalization inseparable? Previous studies (e.g., refs 29–31) have concluded that young nodes induce anterior structures whereas old nodes induce more posterior structures. Hara (32) combined epiblast with different regions of the head process and concluded that different regions of the chordamesoderm induce different regions of the central nervous system (CNS). Results obtained from amphibian embryos support this conclusion (see ref 5). For example, anterior mesoderm induces the expression of the anterior markers *XIF3* (33) and *En2* (34) whereas posterior mesoderm induces the expression of the posterior marker *XlHbox6* (33).

Recent results (15) show that Hensen's nodes that still contain presumptive head process (up to stage 4) induce the CNS, which includes the forebrain and the midbrain/hindbrain (En2-expressing) region. Once the presumptive anterior notochord cells have left the node (stage 5), the CNS induced by such a node no longer expresses fore- or midbrain markers but does express markers characteristic of the posterior hindbrain and spinal cord. This supports the conclusion that the information required to form the diencephalon and the midbrain/hindbrain region is conveyed by the presumptive chordamesoderm cells that underlie the presumptive anterior CNS, and suggests that the presumptive notochord cells remaining in older nodes may be responsible for regionalizing more posterior CNS. Taken together, these findings suggest that the notochord may be a source of some regionalizing signals.

The neuroepithelium itself can also convey regionalizing signals. This was shown most clearly by recent experiments in which metencephalic neuroepithelium (En2-expressing) was grafted onto the diencephalon (En2-negative). The diencephalic neuroepithelium surrounding the graft acquired metencephalic characteristics and started to express En2 (35). This experiment alone provides strong evidence that neuralizing and regionalizing signals can be separated experimentally in the chick embryo: even an already induced, fully formed neural tube can still acquire new regional characteristics in response to signals from neighboring cells, and these signals can flow within the plane of the neuroepithelium itself.

How many regionalizing signals?

One question that emerges from these considerations is: Are there as many different signals as there are regions in the CNS? Perhaps surprisingly, some experiments suggest that this might be the case. Stage 6 nodes generate occipital CNS, but the CNS generated by stage 4+ to 5 nodes includes anterior as well as posterior hindbrain (15). Finally, stages 2–4 nodes uniquely induce anterior CNS including the diencephalon and the midbrain/hindbrain, En2-expressing region.

The anterior and posterior boundaries of expression of *XlHbox1* in the mesoderm of *Xenopus* embryos are initially in line with those in the overlying nervous system (36). Based on this finding, they suggested that the mesoderm imparts

positional information to the nervous system in a manner that resembles homoiogenetic induction because the same state is transmitted between the inducing and responding tissues. So, if regionalization does require as many signals as there are regions, how many such signals/regions are there in the embryo? Will the number of signals equal that of the genes whose expression is regionally restricted? The answers to these questions no doubt will emerge from research on both amphibian and avian embryos in the not-too-distant future. FJ

Note added in proof: Since preparation of the manuscript, the following articles have been published.

Hemmati-Brivanlou, A., and Melton, D. A. (1994) Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. *Cell* **77**, 273-281

Hemmati-Brivanlou, A. Kelly, O. G., and Melton, D. A. (1994) Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295

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