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Neural induction and antero-posterior patterning in the amphibian embryo: past, present and future

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Abstract. Neural induction and patterning in competent ectoderm occurs during gastrula and early neurula stages in response to signals from dorsal mesoderm. The earliest views of antero-posterior (A-P) patterning were modified beginning in the 1930s, as complexities concerning the timing of the pattern-forming process and potential sources of the patterning signals were revealed. In the 1950s and 1960s several different models for A-P patterning were proposed, all of which, however, bear a number of similarities, including a two-component system for generating A-P axial information in the embryo. Early attempts to identify neural-inducing molecules were largely unsuccessful due to technical limitations in biochemical analyses and concerns about assaying neural responses. The advent of modern molecular genetic technology has permitted more precise tests of a number of classic observations about the timing of A-P patterning and the sources of patterning signals. While some early observations have been confirmed, a number of new concepts have emerged in recent years, particularly concerning the source of patterning signals in the embryo. Striking progress has been made in identifying putative neural-inducing molecules, and recent experiments have begun to suggest how these might contribute to A-P patterning. While the successes in recent years have been revealing, many of the classic issues concerning neural induction and patterning remain essentially as they were when first defined many decades ago. The power of modern molecular genetics, however, should permit many of these issues to be significantly clarified in the decades to come.
Key words. Neural; induction; anterior; posterior; determination; development; embryo.

Introduction

The mechanisms leading to determination of the vertebrate central nervous system (CNS) have been intensively studied since the seminal experiments of Spemann and Mangold [1] defined a conceptual framework for this process. Most of these studies have been performed on amphibian embryos because of the relative ease with which one can examine and experimentally manipulate early developmental events in these organisms. Work done in other vertebrate systems such as the chick, and more recently zebrafish, has also contributed greatly to our current understanding of how the CNS is induced, and subsequently patterned (for recent reviews concerning these organisms see respectively Stern [2] and Strahle and Blader [3]). Here we shall primarily consider work done in amphibian species, focusing on the early phases of neural determination. We will first provide an overview of some of the now classic studies which serve as the foundation for our current view of neural determination and then turn to more recent work in this field relating these results to the earlier work. This discussion focuses only on the very earliest stages of neural determination, initiated when responsive, or competent, ectoderm on the dorsal side of the embryo is induced by dorsal mesoderm to become neural tissue during gastrulation. During this phase of neural induction the definitive body axes, both antero-posterior (A-P) and dorsoventral (D-V) are established as well. In our discussion we focus only on A-P axis determination since the preponderance of studies on early regionalization have focused on this aspect of axial formation. Recent work on D-V axis formation, however, has led to important new insights regarding this process [4, 5].

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Classical embryological experiments

The organizer

The ability of dorsal mesoderm to induce neural tissue was first revealed by Spemann and Mangold [1] when they transplanted the dorsal lip of the blastopore at early gastrula stages to the ventral region of a second early gastrula of a closely related newt species. The use of heteroplastic grafts allowed Spemann and Mangold to distinguish whether structures were derived from host or donor tissues based upon species differences in pigmentation. They observed that a second embryonic axis formed in the region of the graft and contained dorsal structures such as somites and nerve cord not expected to form from ventral tissue at the site of the graft. The presence of these dorsal cell types was not entirely unexpected, since at the time, the dorsal lip region was thought to possess a remarkable capacity for self-differentiation [6–8]. However, the finding that the

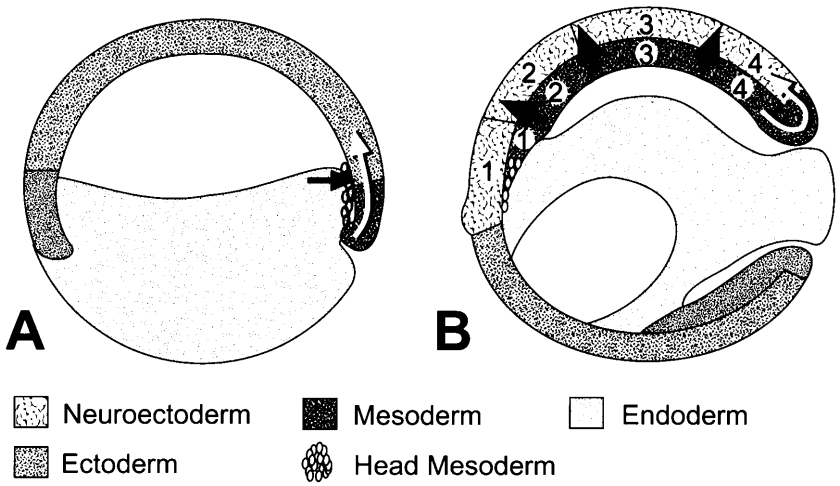

Figure 1. Simplified representation of a gastrulating *Xenopus* embryo, illustrating the possible routes by which neural-inducing and patterning signals could pass from dorsal mesoderm to the presumptive neuroectoderm. (*A*) Early gastrula (stage 10 +) showing the formation of the dorsal lip where the future prechordal mesoderm is involuting. Note the presence of head mesoderm and pharyngeal endoderm which have already come into vertical contact (black arrow) with the presumptive neuroectoderm at this early stage. Signals may also pass from dorsal mesoderm to presumptive neuroectoderm in the plane of the tissue along their common boundary (grey arrow). What is not shown on this diagram is that the area indicated as dorsal mesoderm is actually comprised of two layers. Only the inner layer forms mesoderm; the outer layer will contribute to the endoderm. (*B*) A late gastrula embryo showing the register between the A-P axial character of both the mesoderm and the neuroectoderm. Extensive vertical contact is now possible along the length of the developing axis (black arrows), while planar contact (grey curved arrow) exists only in the posterior of the embryo.

CNS within the second axis was derived largely from host tissues suggested that the neural tissue was induced by the transplanted presumptive dorsal mesoderm and was not the product of a self-differentiating transplant. Perhaps as striking as the induction of surrounding host tissue adjacent to transplanted dorsal mesoderm was the near normal A-P axis present within the transplanted and induced tissue. This suggested that dorsal mesoderm not only has the ability to induce neural tissue, but can also organize its A-P pattern. This, combined with the ability of the graft to recruit host cells into the mesodermal portion of the secondary axis, led Spemann to coin the term 'organizer' to describe this remarkable tissue [8].

The route by which neural-inducing signals move from the organizer to dorsal ectoderm during normal development has been a subject of interest since the time of the Spemann and Mangold transplantation experiments. There are two possible ways in which an organizer signal might travel: 1) vertically across the narrow extracellular space separating the two tightly apposed tissues during gastrulation; or 2) through the plane of the tissue at the juncture between presumptive dorsal mesoderm and neuroectoderm (fig. 1A and 1B). Early support for the vertical route of induction came from the work of Holtfreter [9] who performed an extensive analysis on 'exogastrulated' urodele embryos (induced by hypertonic salt solutions) in which the dorsal mesoderm, instead of involuting during gastrulation, moves outward into the culture medium. Vertical interactions are thought to be inhibited in exogastrulae since the dorsal mesoderm does not involute, and therefore should not come in vertical contact with the presumptive neuroectoderm. In examining these embryos Holtfreter found no histological evidence of neural tissue, and argued that vertical interactions are critical for neural induction and may therefore serve as the primary route for neural induction in the embryo.

The possibility that a planar signal might be involved in neural induction is discussed in a number of early papers, for example by Spemann [10]. Goerttler [11] presents evidence consistent with this signalling mechanism, based again on observations of embryos in which normal involution of mesoderm was blocked during gastrulation but in which neural tissue nonetheless still formed. Holtfreter [9] argued, however, that when exogastrulation is complete neural tissue does not form. In Goerttler's experiments exogastrulation was incomplete and transient involution or partial involution might be sufficient to transmit vertical signals that could elicit neural induction. More recent work on this controversial issue will be discussed below.

When and how axial determination within the nervous system occurs has been the subject of intense study by a multitude of investigators, but was first extensively examined by Spemann [12]. This paper has been interpreted to support a model in which different regions of the dorsal lip possess definitive A-P properties at gastrula stages which in turn confer A-P properties on induced neural tissue during gastrulation (fig. 1B). Spemann's experiments entailed transplantations using small portions of the dorsal lip taken from various stage gastrulae. Instead of grafting these into the ventral side of host embryos he inserted the graft into the blastocoel

of a second early gastrula, the classic *Einsteckung* experiment. Spemann found that there was a tendency for dorsal lips taken from early gastrulae (which will eventually underlie the head region) to induce head structures while dorsal lips taken from older gastrulae (which will eventually underlie the trunk) tended to induce trunk/tail structures. However this was not always the outcome; for example, in some cases the 'head' organizer induced trunk structures. The latter result may be due to host effects on the implant, an issue which has been recently addressed in detail by Slack and Isaacs [13]. While Spemann's experiments and those of a number of others [e.g. 14, 15] have been interpreted as evidence for the presence of regional inducing activity within gastrula dorsal mesoderm, the case is not clearcut. Nonetheless these results have contributed significantly to the framework for the classical view of neural induction: neural-inducing and regionalizing information is present in involuting dorsal mesoderm and this information is imprinted by vertical signalling to the overlying presumptive neuroectoderm as the mesoderm passes under the neuroectoderm during gastrulation (fig. 1B).

From a number of other studies of this period a more complex view of the generation of the A-P axis emerges, including evidence that determination of axial properties is only definitively established at the neural plate stage. Using the Einsteck method, the classic experiments of Mangold [16] indicated that the mesoderm of the archenteron roof was strongly regionalized with respect to its inductive ability by early neurula stages. However, even at this stage Mangold's data show that the inducing ability of the four regions analyzed is not as tightly localized as one might expect from fate maps of the different regions (e.g. eyes are sometimes induced by mesoderm posterior to the presumptive eye region). Further evidence along the same line comes from the extensive study of Ter Horst [17] assaying the inductive ability of the archenteron roof along the A-P axis in recombinants with two pieces of competent ectoderm taken from early gastrulae. She finds that the structures induced by regions of the archenteron roof are significantly regionalized, but tend to be of a more anterior character than expected based upon fate maps. The work of Sala [18] shows a similar degree of regionalization at neural plate stages.

Several studies indicate that at earlier stages (during gastrulation), A-P properties of dorsal mesoderm are not yet fixed. Tondury [19] and Waddington and Yao [20] show that when one rotates large regions of the dorsal lip of young gastrula embryos, or when presumptive trunk and head organizer regions are interchanged, a proportion of embryos are completely normal. Deuchar [21] argues that even at late gastrula stages the inducing ability of regions of dorsal mesoderm is not yet fixed. Perhaps the most extensive data arguing that the early gastrula organizer has not yet achieved its final character come from the explant experiments performed by Holtfreter-Ban (discussed in detail in ref. 22). She shows that the presumptive trunk organizer in early gastrulae is not yet mesodermalized; when cultured it forms both ectodermal and neural tissue. Holtfreter-Ban also found that the presumptive head organizer could produce both ectoderm and pharyngeal endoderm, suggesting that the A-P character of this tissue is not yet definitively established at this stage either. More recently, Kaneda and Hama [23] have shown that the trunk organizer does not acquire its final inducing ability until after involution during gastrulation. The presumptive head organizer before gastrulation behaves as an inducer of trunk structures and again only acquires its head-inducing characteristics gradually during gastrulation [24]. Taken together these reports suggest that definitive A-P axial properties in dorsal mesoderm are not determined until at least mid-gastrula stages. More recent work on the timing of regionalization is discussed in a subsequent section.

What is then responsible for the transition from a labile A-P pattern at gastrula stages to a more determined pattern at neural plate stages? A number of possible mechanisms have been proposed. Several investigations suggest that simple aging of early gastrula dorsal mesoderm permits it to acquire its final properties. Hama [25] found that recombinants between presumptive head organizer from early gastrula and blastula ectoderm formed trunk and tail structures; however, if the head organizer was first aged in vitro to mid-gastrula stages virtually no trunk-tail structures were formed. Instead, structures associated with the head were formed. Similar conclusions have been reported by Suzuki and coworkers [26]

An earlier study by Okada and Takaya [27] reaches the same conclusion and at the same time sheds light on the experiments of Spemann [12], discussed earlier, which have been used as evidence suggesting early regionalization of inducing properties within the dorsal lip. Using *Cynops pyrrhogaster* these investigators were unable to obtain head structures from transplants of the head organizer but always obtained trunk and tail structures [27]. The major difference between their work and that of Spemann was the method used for transplantation. Spemann used the Einsteck procedure while Okada and Takaya made recombinants of organizer and ectoderm which were transplanted into the ventral side of the future head region. In order to investigate these differences further Okada and Takaya performed additional transplants taking involuted and uninvoluted regions of the presumptive head organizer. Recombinants made from involuted portions of the organizer produced head structures, confirming at least the presence of the head organizer reported by Spemann. However, the uninvoluted part again only produced trunk and tail

structures. Subsequent experiments by these authors showed that head organizer activity could be gained autonomously by explanted uninvoluted organizer when aged in vitro until mid-gastrula stages when this tissue would normally have been involuted. Takaya later offered an explanation for the differences between Spemann's result and theirs [28]. Einsteck experiments require that the inserted piece of organizer tissue attach to the blastocoel roof and subsequently differentiate and induce regionally corresponding neural structures. By directly transplanting recombinants of organizer and ectoderm Okada and Takaya were able to regulate more closely the stage of the inducing tissue which was immediately excised and recombined with ectoderm. In Spemann's experiments the organizer tissue may have aged between the time of insertion and the time in which it attached to the blastocoel roof where it could then develop as a head organizer.

Other studies have argued that the determination of regional properties of dorsal mesoderm during gastrulation is due to a series of tissue interactions during this period which establishes its final nature. Waddington and Yao [20] suggest that contact between involuting head mesoderm and uninvoluted trunk mesoderm may be important in this regard. The data of Kaneda [29] provide evidence in support of interactions within the mesoderm, though it is argued that the important interaction is a planar one between different regions of dorsal mesoderm. Contact between involuting mesoderm and overlying ectoderm may also be important, as suggested by the work of Ohara and Hama [30] concerning the properties of the trunk organizer.

The two models described above for generating A-P regionalization appear to be contradictory: if autonomous aging of mesoderm is sufficient for regionalization, then interactions between regions of mesoderm and/or with overlying ectoderm would not seem to be necessary. At present there are a number of reasons why it is not possible to decide which proposal is correct, or perhaps whether a combination of the two is important. Interpretation of data in several of the studies described above is uncertain because host and donor marking was not used to distinguish different interacting tissues. In addition, all of these studies rely on assessment of morphological features to determine the regional character of induced tissues. Such qualitative judgments are subject to interpretation by the individual investigators making it difficult to compare studies directly. Clearly, further work on this intriguing problem is required

Early attempts to identify neural-inducing factors

By the early 1930s enough information had been garnered regarding the tissues involved in neural induction, and sufficiently straightforward ways to assay the process, that investigators were inspired to search for the molecules responsible for neural determination. The search proceeded with great intensity in spite of a number of mysteries about where neural-inducing signals might reside; for example induced neural tissue is itself able to induce more neural tissue in responsive ectoderm (homogenetic induction: ref. 16). The first reports [31] showed that a number of killed tissues still exhibited neural-inducing ability, suggesting that simple chemical factors, resistant to such treatments as extensive heating, were responsible. Surprisingly, evidence accumulated that almost any tissue, even from organisms evolutionarily very distant to amphibians (e.g. boiled human thyroid: ref. 32) could act as an active inductor, while those taking a more reductionist approach could show that compounds as diverse as steroids and nucleic acids appeared to have neural-inducing capacity (for a detailed discussion see refs 33, 34). A feature that was common to inductions by heterogeneous tissues and by chemical factors was that they had an anterior, or prosencephalic, character based on gross morphology. Needham et al. [35] referred to these inductions as being due to an 'evocator' of generalized neural potential which in vivo would act in concert with 'individuators' responsible for neural regionalization. It was argued that the evocator was present in all tissues and was only normally activated in the organizer, thus explaining the potential of many tissues to act an neural inductors.

An even more surprising and initially quite controversial report was one by Barth [36] in which the presumptive neuroectoderm of *Ambystoma punctatum*, when removed from the embryo, was shown to undergo neural differentiation in the absence of dorsal mesoderm, questioning the very validity of neural induction by dorsal mesoderm. The ability of ectoderm which had never been underlain by dorsal mesoderm to differentiate into neural tissue was rather unexpected because of the previously held view of the importance of vertical signals for neural induction and the fact that other investigators had not found such 'autoinduction' in other species. Holtfreter [37] in a parallel series of experiments repeated Barth's work on *A. punctatum* and compared these results with a series of experiments performed on *Triton torosus*. Holtfreter was able to confirm the autoneuralization of the *A. punctatum* ectoderm, but did not observe this with *T. torosus* ectoderm or presumptive neuroectoderm, and suggested that the phenomenon of autoneuralization was not widespread among amphibian species. Barth had also reported that autonomously induced neural tissue had an anterior character, similar to what was found with inductions by heterogeneous inductors. Holtfreter [38] and later Barth and Barth [39] showed that even in species that do not autoneuralize, transient exposure to low or high pH, or altered divalent cation concentrations can elicit a neural

response. Holtfreter proposed that a 'subcytolytic effect' was responsible for this autoneuralization, presumably releasing the proposed evocator from slightly damaged cells and permitting it to act as a neuralizing agent in ectodermal cells which would not normally utilize such a mechanism

As we will discuss in the following section, the latter findings represented a major setback in the analysis of neural induction, since they did not lead as expected to a clearcut biochemical mechanism that might explain induction in both normal and abnormal situations. The data imply that a neural response is very readily activated in gastrula ectoderm and, using recent terminology, that the response may be a 'default' state of this tissue. However, as will be discussed shortly, in light of more recent progress concerning mechanisms of induction and signal transduction in general, alternative explanations are possible for this outcome as well.

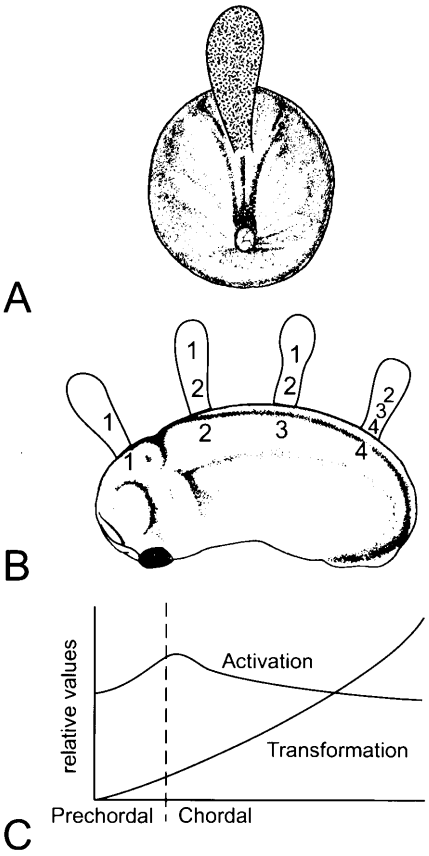

Figure 2. Nieuwkoop implant system and basis for his two-signal model of neural induction and patterning. (*A*) Heterotypic grafts were made by placing folds of neural-competent ectoderm perpendicularly into late gastrula or early neurula hosts. (*B*) A summary of Nieuwkoop's observations of these implants. Patterned neural tissue formed in these implants in predictable ways such that tissue at the base of the implant reflected the axial character of the host at the site of implantation, while more distal regions of the implant contained progressively more anterior structures. In this case the anterior is represented by axial character 1, and the posterior by level 4. These observations can be summarized by a simple rule that states that neural structures will be equal to or anterior to that of the host at the site of implantation. (*C*) The two-signal, or two-gradient model of neural induction and patterning along the A-P axis. Nieuwkoop observed that implants placed just posterior to the prechordal/chordal boundary contained quantitatively more neural tissue than implants placed elsewhere along the axis. He proposed that this is indicative of the level of a neural-activating principle present in the underlying dorsal mesoderm. A second principle was proposed to be responsible for transforming anterior neural tissue into more posterior structures, present in a posterior to anterior gradient leading to the smooth generation of positional values along the A-P axis. That this principle was highest in the posterior was suggested by implants placed far posteriorly, which often lacked anterior-most structures at their distal ends.

Building models for neural patterning

During the period when the search for neural-inducing factors was underway, other investigators were attempting to define the tissue interactions responsible for neural regionalization. Based upon reports that regionally specified dorsal mesoderm taken from neurulae often induced structures which were more anterior to the structures expected in neural-competent ectoderm [17] and because of studies suggesting the ability of the presumptive neuroectoderm to self-differentiate into anterior neural structures, Nieuwkoop devised a strategy to examine possible interactions within the neuroectoderm itself [40–42]. He felt that such intraectodermal signals may be involved in the fine tuning of the neural patterning signals received from the dorsal mesoderm. His strategy was to implant folds of competent ectoderm perpendicularly into the neuroectoderm of late gastrulae and early neurulae in order to observe the local activity of neural-inducing and regionalizing signals along the A-P axis of the host neuroectoderm (fig. 2A). Nieuwkoop found that implants grafted into the neuroectoderm were neuralized, and in addition, that they contained regionalized neural structures with the bases reflecting the axial character of the host at the level of implantation and more distal regions resembling more anterior structures in the host CNS (fig. 2B). Examination of the amount of neural tissue, and the A-P pattern present within the implants, led Nieuwkoop to propose a two-signal or two-gradient model of neural axis formation (fig. 2C). The first signal, which leads to neural activation, is derived in the dorsal mesoderm and is present along the entire A-P axis with a maximal value near the anterior end of the notochord. In the absence of any further signals neural tissue assumes an anterior character. A second mesodermally-derived signal was suggested to be responsible for transforming anterior neural tissue into more posterior neural structures. This factor, or activity, Nieuwkoop surmised, must be most active in the posterior and be effectively absent in the anterior with a gradient of values between these extremes setting up the range of A-P values present in the embryo. The proposed distribution of these two activities within the archenteron roof were later confirmed by Sala [18] in a series of

recombinant experiments supporting this two-gradient model.

Using a modified implant procedure, Eyal-Giladi [43] tested the principles of activation and transformation in the embryo. By removing transverse strips of presumptive neuroectoderm from the beginning of gastrulation through neurula stages and attaching these as implants to the ventral side of the same embryo she could observe the developmental fate of the ablated region of neuroectoderm. The A-P character of these implants served as a guide for defining what region of the neuroectoderm had been removed and as a reference point for assessing effects on dorsal axis formation. Interpretation of this paper is somewhat difficult because the removal of tissues from the embryo interfered with subsequent gastrulation movements, which could itself affect the A-P character of dorsal tissues, and because it is unclear whether implanted tissues differentiated with the A-P character they might have in vivo.

In young gastrulae Eyal-Giladi found that prosencephalic (midbrain and forebrain) structures were formed by neuroectoderm which had only recently been contacted by dorsal mesoderm (head mesoderm), and which would have been fated to become more posterior tissue based upon fate mapping of this region. In contrast, more caudal structures were present in implants made from neuroectoderm which had been in contact with dorsal mesoderm for longer periods. The duration of contact with dorsal mesoderm did not appear to influence the character of the neural tissue induced, since extended contact with the leading edge of the archenteron roof (caused by interference with gastrulation movements resulting from surgery) did not alter the prosencephalic differentiation of the presumptive anterior neuroectoderm. Therefore, neuroectoderm fated to lie caudally and form spinal cord will form prosencephalic structures when prevented from continued contact with the later involuting chordamesoderm. These data are consistent with the activation and transformation model in that they suggest that neural activation, the first step in neural induction, yields tissue of an anterior character which is only subsequently transformed into a more caudal fate during development by the transforming signal.

An important aspect of Nieuwkoop's observations is the ability of competent ectoderm to pass both the activating and transforming signals. In the recombinant experiments of Sala [18], and an earlier set of recombinants performed by Nieuwkoop and Nigtevecht [44], neural structures formed at a distance from the inducing tissue (dorsal mesoderm). This observation suggests that neural-inducing and transforming signals pass through the plane of the presumptive neuroectoderm. The presence of a graded set of neural structures within implants or in recombinants at progressively further distances from the inducer suggested to Nieuwkoop that these two signals may not pass with equal efficiency through the prospective neuroectoderm (see fig. 3 in the Nieuwkoop review, p. 305). The location of prosencephalic structures at the distal ends of implants and at the outside edges of the neuralized region within recombinants suggested that the activating signal passes further in competent ectoderm than does the transforming signal, since prosencephalic structures should represent neural tissue which has not been influenced by the transforming signal. These observations may explain the results of Ter Horst [17] mentioned previously, who found that recombinants of regionally specified dorsal mesoderm from neurula stage embryos and competent ectoderm often produced neural structures which were equal to or more anterior to those expected to be induced based upon fate maps of the mesoderm used in the recombinant.

A second model for neural regionalization has come from the work of Saxén and Toivonen who performed cell-mixing experiments in which heterogenous inductors of neuroectoderm and mesoderm were either implanted together or used to induce a population of cells which could then be dissociated and combined in various ratios [45, 46] In implants, or in cell culture, each inductor induced a subset of the structures present when the two inductors, or induced cell types, were present together. From these and other later experiments the authors proposed the presence of two principles which act in gradients (the two-gradient model), one of which (M) acts in a caudo-cephalic gradient to induce mesoderm and caudalize neural structures, and the other which exists in a slight cephalo-caudal gradient (N), which induces anterior neuroectoderm and placodal ectoderm. In various combinations these two principles induce the entire range of neural structures along the A-P axis. If one equates the effect of the mesodermalizing factor with that of the transforming signal then this two signal model has significant similarities to that of Nieuwkoop and colleagues. In both models the 'transforming' signal is responsible for inducing posterior neural character, and induced neural tissue forms anterior structures in the absence of these mesodermal signals. One difference between the two models is that the two-gradient model N principle induces anterior neural tissue as well as ectodermal placodes and is not viewed as a basal anterior neural state. In Nieuwkoop's original model the activating principle induces only anterior neural tissue whereas placodal material was thought to arise through secondary interactions with the induced neural tissue.

An additional two-signal model was also proposed by Yamada [47] based upon a series of recombinants between competent ectoderm and a heterologous inducer (protein extract from ox muscle treated in various ways with and without the inclusion of iron powder). By comparing the relative inductive frequency of certain neural and mesodermal derivatives with the overall

general inductive potency of the inducer, Yamada formulated an optimal value of induction for each structure. These data in combination with a modified version of Dalcq and Pasteels theory of morphogenic potential [48, 49] led Yamada to develop a model which involved two interacting potentials, the dorso-ventral potential (Pdv) and the cephalo-caudal potential (Pcc) mediated by the dorso-ventral and cephalo-caudal mediators (Mdv, Mcc), respectively. Mdv was thought to be a diffusible substance while Mcc was thought to be a mechanical action of cells undergoing convergent extension in the caudal regions of the embryo. Unlike the other models just mentioned, Yamada's double-potential model encompassed patterning in each of the three germ layers along both the A-P and D-V axes. According to this model anterior neural development would require high Mdv to yield the most dorsal structure, neural tissue, and low Mcc to yield anterior (the default pathway in this model) neural structures. More caudal neural structures such as spinal cord would require high levels of both Mdv and Mcc. If one equates Mdv to Nieuwkoop's activating principle, and Mcc to the transforming principle, then these two models have a number of similarities with regard to explaining neural axis formation. A major difference lies in the broader scope of the double-potential theory and in that the mediator of the cephalo-caudal potential is not a diffusible molecule as proposed for the transforming agent but a mechanical condition of stretching and shifting of cells present in a posterior to anterior gradient. Yamada distinguishes not only the amount of cell movement but also the direction of these movements, which change from a proximo-distal movement anteriorly to a more tangential, or medio-lateral, movement posteriorly. In addition, while the Yamada model introduces important concepts about morphogenetic changes that occur during gastrulation, it is not clear how these changes alone might cause formation of posterior neural types.

Combining the similarities of each of these proposals a consensus view emerges in which one factor, or set of factors, activates anterior neural induction, and a second factor, or factors, generates posterior neural cell types, though the details among the models differ. We will return to this issue later and discuss how more recent data obtained using molecular markers for neural differentiation and patterning fit with the concepts of the proposals discussed above.

The modern era: old embryological issues revisited

With the advent of molecular cloning techniques, highly sensitive procedures for detecting macromolecules, and sophisticated methods for purifying them, experimental embryologists can now resolve important questions which could not be addressed in earlier times. For example, we can now assess developmental events well before terminal differentiation occurs by monitoring levels of regulatory gene products during early stages of determination. This has permitted a much more subtle analysis of inductive interactions by providing assays for very early stages of induction and patterning, when the sources of signals and responses had previously been impossible to examine.

The availability of these new tools has rekindled interest in a number of unresolved issues in neural induction and patterning. The mechanisms of neural axis specification have been the subject of a number of recent studies which have clarified important properties about A-P axis formation. Another area of investigation which has been intensively examined in recent years is the relative roles that vertical and planar signals play in neural induction and patterning by dorsal mesoderm. Perhaps the most elusive of the long-standing questions regarding neural induction has been the isolation and characterization of putative neural-inducing molecules, a number of which have now been described. Advances in each of these areas will be discussed below.

Recent insights into neural patterning

Nieuwkoop suggested that neural activation, which gives rise to anterior neural development, precedes transformation into a more posterior neural character. This implies that cells destined to lie in the posterior neural tube might transiently express an anterior neural character prior to expressing characters associated with its future position. Cells located at the presumptive anterior of the developing CNS, however, would only express anterior neural character having no influence from the transforming signal. Sive et al. [50], in examining cement gland differentiation, have demonstrated that this anterior ectodermal structure is also transiently specified in regions of the presumptive neural plate during gastrulation. The authors conclude that presumptive neuroectoderm passes through a transient anterior character prior to its final specification as posterior neural tissue, reminiscent of the conclusions of Eyal-Giladi [43]. Similarly, a study by Sharpe and Gurdon [51], examining the regionalized inducing ability of dorsal mesoderm, found that both anterior and posterior dorsal mesoderm taken at late gastrula stages could induce a relatively anterior neural marker, *XIF3*, while only posterior mesoderm induced the posterior neural marker *XhoxB-9*. Dorsal mesoderm taken earlier at mid-gastrula stages could only induce *XIF3* expression, with anterior or posterior pieces unable to induce *XhoxB-9* in competent ectoderm. Although the pieces taken at the two stages did not necessarily correspond to the same groups of cells, the data suggest that dorsal mesoderm at the leading edge of involuting mesoderm induces somewhat more anterior neural markers while later involuting mesoderm induces posterior neural markers as well as more anterior ones.

Although the information gained through the use of regionalized neural markers in the studies above is consistent with the Nieuwkoop two-signal model, not all of the existing data can be readily reconciled with this view. For example, Saha and Grainger [52] have dissected presumptive dorsal ectoderm of *Xenopus* embryos into anterior, middle, and posterior thirds at the mid-gastrula stage (stage 11.5; all staging according to ref. 53) and neural plate stage (stage 14), and have found that at the earlier stage all three pieces will express both anterior and posterior neural markers. Only at the neural plate stage are the markers localized to the regions fated to express either anterior or posterior markers. The observation that each piece of the presumptive neural plate at the mid-gastrula stage could express both anterior and posterior neural markers does not appear to be consistent with the activation/transformation hypothesis. If the two signal model is correct one might not expect presumptive anterior neuroectoderm, which at this stage is not likely to have been exposed to a transforming signal, to express posterior neural markers. This result suggests an alternative view of early neural patterning in which A-P neural properties are transiently activated along the entire neural axis, and which only become restricted during neurulation. This 'broad activation' model therefore can be distinguished from the two signal model by the expression of posterior neural markers in presumptive anterior neuroectoderm. The activation of anterior neural properties in posterior regions is less informative since both models suggest that posterior regions will activate anterior properties during the early phases of neural regionalization.

The pattern of activation of a number of putative regulatory genes may provide a molecular basis for the transient activation of A-P properties along the entire neural axis. The homeobox gene *Otx-2* has been implicated in anterior neural determination [54]. In *Xenopus* it is expressed in anteriormost neural tissues during neurula stages, but is transiently expressed more broadly within presumptive neuroectoderm during gastrulation [55]. Another homeobox-containing gene, *Xhox-3*, thought to play a central role in determination of posterior mesoderm in *Xenopus* [56, 57], is transiently activated along the entire A-P axis during gastrula stages before becoming localized to the posterior at the end of gastrulation [52, 58]. The transient broad activation of a subset of genes involved in regional determination may be a general feature of developmental systems in which some plasticity is initially required to integrate spatial information in an orderly way [52].

Definitive tests of both the activation/transformation model and the broad activation models will require further experimentation to determine whether they are mutually exclusive or whether elements of each or both are correct. For example, the study of Saha and Grainger [52] does not test whether the most posterior fates in nervous system are activated transiently in anterior neural tissue. In this study the posterior marker, *XhoxB-9*, is expressed nearly throughout the spinal cord and has a sufficiently anterior boundary of expression that its activation in anterior neural tissue isolated at early stages does not completely test this point. Examination of more posterior markers will be required. In addition, the explantation required for these experiments might activate a regulative response which overrides mechanisms that normally occur in the embryo (e.g. a process more akin to activation/transformation). Thorough analysis of the A-P-inducing activities of putative neural-inducing molecules (described below), coupled with careful examination of their expression in vivo, should provide a more direct and definitive evaluation of the activation/transformation model.

New insights into early mesodermal regionalization

In order to understand neural A-P patterning it is of course important to understand the early A-P patterning of dorsal mesoderm. The report by Spemann [12] suggested that the organizer in the early gastrula embryo already contained significant A-P patterning information. As discussed earlier, a number of reports indicate that regionalization of the mesoderm is not fixed until neural plate stages. More recent work has provided strong evidence supporting the latter conclusion. The vitamin A derivative retinoic acid (RA) is thought to be important in A-P determination and certainly has potent teratogenic effects when applied to young *Xenopus* embryos: there is a striking loss of anterior structures in such cases. Resistance to RA treatment does not disappear until the end of gastrulation, again suggesting that A-P regionalization is not fixed until this stage; this effect is at least in part due to direct action of RA on mesodermal regionalization [59–61]. The study of Saha and Grainger [52] described above also indicates that A-P properties in dorsal mesoderm are not fixed until neural plate stages.

While there is still debate, as discussed below, about the relative contributions of vertical and planar signalling in neural patterning, there is universal agreement that dorsal mesoderm is patterned along the A-P axis, and that this information is imparted in some manner to the presumptive neuroectoderm. Such transcriptional regulators as *goosecoid* [62] and *Xbra* [63] are localized in anterior and posterior regions, respectively, of dorsal mesoderm, and could play a role in regional differences within the mesoderm. However, the system responsible for generating regionalization and the nature of regional signals remain unknown, with the possible exception of a signalling system involving RA or a related retinoid. Although suggested by the data described

above, a direct role for RA in regionalization in vivo has not been demonstrated. In contrast, significant progress in identifying molecules which are important in D-V patterning of mesoderm has been made in recent years [4].

Routes of neural induction

Vertical signalling. Many recent studies have focused on the route by which neural-inducing and patterning signals are passed within the embryo. Spemann, even after Holtfreter reported that exogastrulae lacked neural tissue, was not convinced that neural-inducing signals must pass only vertically from germ layer to germ layer during gastrulation [64]. Nonetheless, this view of neural induction has predominated for most of this century and has received support from several recent experiments involving the use of regionalized neural markers. Hemmati-Brivanlou et al. [65] recombined dorsal mesoderm from late *Xenopus* gastrulae (stage 12.5) with neurally competent ectoderm so that these tissues were in vertical contact. In this case the ectoderm was taken from UV-treated embryos which, as a result of this treatment, lack dorsal structures and therefore serve as a source of non-dorsalized ectoderm with which to assess the neural-inducing and patterning properties of dorsal mesoderm. Recombinants were aged and analyzed for the expression of *Engrailed-2* (*En-2*), a homeodomain protein expressed at the midbrain/hindbrain border. Anterior notochord (dorsal mesoderm) induced *En-2* expression in 81% of the cases while posterior notochord induced *En-2* in only 36% of the cases. Thus the mesoderm which normally would underlie the midbrain/hindbrain region in the embryo induces *En-2* expression in a higher percentage of cases, and to a greater extent, than does more posterior mesoderm. Similar results have been observed in the mouse embryo where anterior mesendoderm from headfold stage embryos, but not posterior mesendoderm, induced *En-1* and *En-2* expression in pre- to early-streak stage ectoderm [66]. The finding that posterior dorsal mesoderm taken from early *Xenopus* neurulae can induce even low levels of *En-2* expression competent ectoderm may suggest that at this stage the archenteron roof is not yet fixed with respect to its inducing ability. Alternatively, as discussed earlier, recombinants between posterior dorsal mesoderm and neural competent ectoderm may be expected to form neural structures which are equal to and more anterior than expected owing to the proposed greater diffusion of the activating versus the transforming factor or factors.

Further support for the importance of vertical signalling in A-P patterning comes from the studies described in the previous section concerning the timing of mesodermal regionalization [52] and of RA effects on A-P axis determination. All of these studies argue that this axis is not fixed until neural plate stages. Thus, during the preceding stages of gastrulation the presumptive neuroectoderm is exposed to vertical signals in dorsal mesoderm that are likely to have an important impact on A-P regionalization.

Planar signalling. Interest in the idea that neural-inducing signals are passed in a planar fashion from cell to cell along the border of the presumptive neuroectoderm and dorsal mesoderm (fig. 1A) has been revived in recent years. A report by Kintner and Melton [67] demonstrating the expression of neural cell adhesion molecule (NCAM), which serves as an early marker of neural differentiation, in exogastrulae of *Xenopus* embryos suggested that the question of vertical versus planar neural induction needed to be reexamined. NCAM-positive tissue was present at the border between the exogastrulated mesendodermal mass and the large epidermal sack. Since vertical contact between mesoderm and ectoderm was presumed to be absent in these embryos, it was inferred that neural tissue was induced in a planar fashion along the common border of these tissues.

Several potential differences between this study and the work of Holtfreter on exogastrulae [9] could explain these findings. First, Holtfreter relied upon histological criteria to identify neural tissue while Kintner and Melton used a neural marker expressed early in the neural differentiation program. It is possible then that the ectoderm of exogastrulae undergoes some of the early steps toward neural development but lacks the proper signals for complete neural differentiation. A subsequent report by Dixon and Kintner [68] suggests, however, that this may not be the case. These authors examined the expression of both NCAM and NF-3, a neurofilament-like protein which marks post-mitotic neurons, and found that both markers are expressed at near normal levels in *Xenopus* exogastrulae. A second potential explanation concerns the species used to create exogastrulae. Holtfreter used urodele embryos which contain a single layered marginal zone, and therefore the entire presumptive mesoderm must involute around the dorsal lip during gastrulation. The situation is more complicated in *Xenopus* where the marginal zone has two distinct layers. Head mesoderm and pharyngeal endoderm form from cells that delaminate from the deep layer at the site of the blastopore lip, and therefore do not involute. As such, the early movements of these cells may well be refractory to the treatments used to induce exogastrulation and may create vertical contact with the presumptive neuroectoderm prior to the majority of the cell movements characteristic of exogastrulae (for a further discussion of this issue see the article by Nieuwkoop, p. 305).

Additional evidence concerning the potential role planar signals in neural induction in *Xenopus* comes from the study of explants of dorsal mesoderm and presumptive neuroectoderm taken from early gastrula embryos.

These explants, referred to as 'Keller sandwiches', prevent vertical contact between mesoderm and ectoderm during culture and are made prior to the involution of dorsal mesoderm. Explants taken from early gastrula embryos (stage 10 +) develop histologically distinguishable neural tissue [69]. Recent work by Keller et al. [70] has however raised questions regarding the time at which one can make explants and still have assurance that only planar signals have passed. Explants made after stage 10− (first sign of bottle cell formation) may contain presumptive neuroectoderm which has been subjected to early vertical interactions with head mesoderm and pharyngeal endoderm, which undergoes early movements related to the vegetal extension of dorsal tissues and bottle cell formation [70, 71]. Therefore, data obtained with such explants must be viewed with caution.

However, in support of the view that early planar signals are acting on presumptive neuroectoderm before gastrulation is the report of Savage and Phillips [72]. They claim that a marker for epidermal differentiation is normally suppressed in dorsal ectoderm and that this marker is repressed when presumptive ventral ectoderm is combined with dorsal lips from early gastrulae. Ventral ectoderm is never underlain by dorsal mesoderm and therefore has never experienced vertical contact with this tissue. These observations provide support for an early signal from dorsal mesoderm that may be important as an initial step in the neural induction process for dorsal ectoderm. Consistent with this proposal are the results of Sharpe and Gurdon [73] which imply that dorsal animal cap ectoderm is biased toward a neural response. They show that dorsal ectoderm activates high levels of the neural marker *XhoxB-9* in response to a neural inducer, while ventral animal cap ectoderm does not. These results do not, however, test whether the bias is a result of a signal from dorsal mesoderm, though this remains a plausible hypothesis.

Planar neural-regionalizing signals

The presence of neural tissue in exogastrulae, or Keller sandwiches, suggests that planar signals may be sufficient to induce neural tissue adjacent to dorsal mesoderm, but does not reveal whether this neural tissue is regionalized with respect to the embryonic axis. Evidence for such patterning signals passing through the ectoderm comes from several sources. *Xhox-3*, a homeodomain protein expressed in neural tissue at the midbrain/hindbrain region of tadpole stage *Xenopus* embryos, is reported to be expressed in exogastrulae [74]. Furthermore *Xhox-3* expression is detected at some distance from the mesoderm/ectoderm boundary suggesting that planar signals have passed through the ectodermal sack to reach this distal point. However, expression of a single regionalized neural marker in these embryos does not definitively demonstrate neural regionalization. In order to demonstrate this, at least two such regionalized neural markers need to be analyzed in a single embryo, or explant.

Such coexpression has been demonstrated using Keller sandwiches [75, 76]. *En-2* (midbrain/hindbrain), *XKrox-20* (rhombomeres 3 and 5), and *XhoxB-9* (posterior two thirds of spinal cord) have all been localized in a single Keller explant. Not only are each of these markers expressed within these explants, but they are expressed in the proper A-P sequence found in the normal embryo. These results indicate that at least a partial A-P neural axis may be established through planar signals. The expression of the forebrain markers *distal-less 3* (*X-dll3*), and *XANF-2* in Keller sandwiches suggests that this induced axis can extend distally into the forebrain region [77, 78].

Limitations of planar signalling

Although extensive, neural axes induced by planar signals are not complete. Perhaps the most revealing aspect is the absence of a floor plate in either exogastrulae, or in Keller sandwiches. The floor plate forms from midline cells present in the early gastrula and comes to lie at the ventral-most region in the neural tube. This structure is thought to be induced by the underlying notochord, and in conjunction with the notochord is thought to play a role in D-V patterning of the neural tube [4, 5]. In addition to defects in D-V patterning, neural tissue induced by planar signals are also deficient in at least some aspects of A-P patterning. *XhoxB-1*, a homeobox gene expressed in rhombomere 4 of the hindbrain, is not regionalized properly in Keller sandwiches made from stage 10− embryos, while *XKrox-20*, expressed in adjacent rhombomeres 3 and 5, is expressed properly in these explants (Poznanski and Keller [78a]). A further example of the limit of planar signals is suggested by the absence of eye tissue in either exogastrulae or Keller sandwiches. Significantly, eyes are present in Keller explants which include anterior dorsal mesoderm in vertical apposition with the presumptive anterior neuroectoderm, suggesting that both vertical and planar signals are required for inducing eyes [68].

Cooperative signalling

Spemann (1927) preferred the idea that planar signals are the dominant signal in the early aspects of neural development, since prior to gastrulation no vertical interactions are yet possible between these two distinct germ layers which share a common border. At the outset of gastrulation in *Xenopus* the entire presumptive A-P axis of the spinal cord extends over a distance of only around 5–7 cell diameters [79, 80] along the dorsal midline. During gastrulation and early neurula stages,

prior to axial specification, powerful convergent extension movements bring lateral cells into the midline, extending this region 12-fold. If regional neural-inducing signals are passed prior to these extraordinary movements then it is possible that a gradient of a diffusible substance arising from the dorsal mesoderm in a planar fashion could set up the entire A-P neural axis. During later stages of gastrulation, and neurulation, Spemann suggested that vertical interactions with dorsal mesoderm predominate in determining the axial properties of the neuroectoderm. Considering that these two germ layers are both undergoing massive cell movements during gastrulation, it may not be surprising that definitive regionalization [51, 52] occurs at mid-neurula stages after the majority of these morphogenetic movements have ceased. By delaying definitive regionalization of the A-P axis of both tissues until mid-neurula stages this would help to ensure that the A-P properties of the neuroectoderm and mesoderm become properly aligned.

Neural-inducing signals

It was not until the advent of modern molecular biological techniques that widespread interest in identifying candidate neural-inducing molecules was revived after the frustrations of investigators in the 1940s and 1950s. Much of this interest was spurred by advances toward identifying mesoderm-inducing molecules. A number of molecules belonging to the fibroblast growth factor (FGF) family and transforming growth factor-β (TGF-β) superfamilies were identified as having mesoderm-inducing capabilities [81–83]. The techniques employed to identify mesoderm inducers could now, in large part, be borrowed for the search for neural inducers. In addition, *Xenopus laevis* had since replaced urodele species as the favoured embryonic system with which to study these early inductive events. Fortunately for those interested in neural inducers the neuroectoderm of *Xenopus* is more difficult to autoneuralize, and therefore serves as a more suitable system to identify potentially specific neural-inducing molecules. This recent work, like that which preceded it, has once again provided both candidate neural-inducing molecules, and data which questions widely-held views of neural induction.

Noggin. The first candidate neural-inducing molecule, noggin, was isolated through a novel expression screen for molecules which could rescue embryos ventralized by exposure to UV light prior to cortical rotation [84]. In addition to its dorsalizing activities noggin was found to induce neural tissue in *Xenopus* blastula stage animal caps in the absence of detectable dorsal mesoderm [85]. This remarkable finding suggests that noggin can induce neural tissue directly, and does not act secondarily through the induction of dorsal mesoderm. In order to fulfill the requirement of a neural-inducing molecule noggin should be expressed in known neural-inducing tissues, and this expression should include the period of ectodermal competence for neural induction (blastula and gastrula stages, stages 8–11). Appropriately, zygotic transcripts for noggin are detected in the presumptive dorsal mesoderm of the blastula and are present in the organizer region during gastrulation [84]. During neurulation expression continues in the derivatives of the organizer including head mesoderm and notochord, both of which have been shown to have neural-inducing abilities. Thus noggin appears to be expressed at the right time, and right place, for a neural-inducing molecule. Early reports suggested noggin may activate only the early steps of the neural induction pathway since noggin-treated animal caps failed to express detectable levels of markers for several subtypes of differentiated neurons [85]. A recent report, however, suggests that noggin does induce the expression of *sybII*, a synaptobrevin involved in vesicle trafficking and fusion in synaptic vesicles, and thus a marker for differentiated neurons [86].

Interestingly, in the absence of mesoderm, noggin reportedly induces neural tissue expressing only anterior neural markers such as *Xotx2* (forebrain-midbrain), while in the presence of dorsal mesoderm induces neural tissue with a more posterior character (spinal cord) [85], reminiscent of the activating and transforming signals proposed by Nieuwkoop. This work has been extended by Lamb and Harland [87] who have found that bFGF (a candidate neural inducer itself, see below; refs 87–89) can modify the neural structures formed in response to noggin treatment, shifting these structures toward more posterior values. This suggests that bFGF or a related molecule such as eFGF [90] could play a transforming role in the embryo (see below).

Although noggin induces rather broad expression of the anterior neural marker *Xotx2* in animal cap explants, similar treatment has recently been shown to induce more localized expression of *cpl-1*, and *etr-1*, markers of dorsal and ventral brain regions respectively. Interestingly, these genes are induced in animal caps exposed to noggin in non-overlapping domains of expression suggesting some level of D-V patterning in these explants [86]. This apparent regionalization does not appear to be dependent upon prior influence on the caps by dorsal mesoderm since similar results are observed in UV-treated embryos. The authors suggest that this regionalization could be due to uneven exposure of the cap to noggin combined with a graded response to noggin by these markers, a cryptic prepattern that is independent of cortical rotation disrupted by UV treatment, or the ability of induced tissue to self-organize a rudimentary D-V axis via cell-cell interactions [86]. Certainly, this intriguing problem will stimulate further investigation to determine which of these, or other explanations, are correct.

Follistatin. The search for mesoderm-inducing molecules, in addition to providing the framework for similar studies on neural induction, has provided unexpected insights into neural induction pathways. Activin, a member of the TGF-β superfamily of signalling molecules, has been implicated in mesoderm induction [91–93] and patterning along the D-V axis [94]. Experiments designed to inhibit signalling by activin, and thus mesoderm induction, have led to some surprising findings. Animal caps isolated from embryos injected with a construct expressing a dominant negative form of the activin type II receptor failed to form mesoderm in response to activin treatment, but expressed transcripts for NCAM, a pan-neural marker [92]. The absence of detectable mesodermal markers suggested that this induction was a direct response to the dominant negative receptor and not due to secondary induction by dorsal mesoderm induced in response to the activin treatment. It was suggested that by blocking an inhibitory signal from activin, the dominant negative activin receptor allowed animal cap ectoderm to express a latent tendency to form neural tissue [92]. Evidence to support this view came from the finding that follistatin, an activin-binding protein, directly induces neural tissue in animal caps [95]. In addition, unlike activin and noggin, follistatin does not induce detectable mesoderm in animal caps, and therefore the presence of induced neural tissue appears to be due to a direct induction. Again, this point is crucial since follistatin has recently been shown to dorsalize ventral mesoderm, paralleling the action of noggin [96].

Due to the high degree of homology among TGF-β superfamily members it has not been possible to state conclusively whether the dominant negative receptor is specifically inhibiting activin signalling, or affecting a closely related molecule such as BMP-4 or Vg-1 which are thought to interact with this receptor (see below and refs 97–99). In addition, as mentioned below, there is evidence to suggest that follistatin binds to other TGF-β superfamily members as well as to activin. Similar to what has been reported for noggin, neural tissue induced by follistatin is characteristically anterior in nature as determined by expression of anterior neural markers [95]. Follistatin transcripts are encoded maternally and are zygotically expressed in the organizer at the onset of gastrulation and later in the prechordal and anterior chordal mesoderm during gastrulation and neurulation [95], consistent with a role in neural induction or in dorsalization of the mesoderm.

Chordin. *Chordin*, the vertebrate homologue of the *Drosophila* gene *sog* (short gastrulation; ref. 100) has recently been identified as a gene whose product has strong dorsalizing properties [101], and has direct neural-inducing activity [96]. In *Drosophila*, *sog* acts to antagonize the activity of *dpp* which is required for dorsal development [100]. *Sog* and *cordin* (*chd*) encode a novel protein containing four repeats of a cysteine rich region which shares some homology to domains present in thrombospondin and procollagen [102]. In *Xenopus* embryos *chd* is expressed in the dorsal mesoderm during gastrulation in a pattern which mimics the fate map of the overlying presumptive neuroectoderm during these stages. Similar to what has been described for noggin and follistatin, chordin induces neural tissue with anterior character [96]. In a remarkable finding Sasai and colleagues have demonstrated that chordin interacts with BMP-4, the *Xenopus* homologue of Dpp [96]. Interestingly, while Dpp directs dorsal development in *Drosophila*, BMP-4 appears to be involved in ventral development in *Xenopus* suggesting that while the molecules involved in setting up D-V polarity have been conserved between fly and vertebrates, the axis may have been inverted [102, 103]. In addition to playing a role in D-V patterning Dpp is thought to define the boundary between neurogenic and ectodermal cell fates in *Drosophila*.

As a further parallel between signalling mechanisms in *Drosophila* and vertebrates (discussed further below), BMP signalling has been shown to be sufficient for epidermal development while antagonizing the neuralizing activities of noggin and follistatin in *Xenopus* embryos [96, 99]. Although it is unclear how BMP-4 blocks the action of noggin and follistatin, it would be of interest to know if either, or both, of these molecules can inhibit the neural-inhibitory action of BMP-4. The recent observation that follistatin-deficient mice show a wider variety of defects than do activin-deficient mice suggests that follistatin may bind molecules other than activin, perhaps members of the BMP family [104].

bFGF. A fourth factor implicated in neural induction is basic fibroblast growth factor (bFGF) whose ability to induce mesoderm has been well documented [105, 106; see also review by Isaacs, p. 350]. Kengaku and Okamoto [88] have tested a number of known inducing factors for neural-inducing activity using a dissociated animal cap assay system. They dissociated animal caps prior to treatment in order to ensure complete and even access of the factors tested to the responding cells, and found that of EGF, PDGF, TGF-β1, TGF-β2, aFGF, bFGF, and activin, only aFGF and bFGF consistently produced cultures with detectable neural tissue. The absence of a myocyte marker, Mu1 antigen, in these cultures suggests that the induction was direct, and the authors point out that the levels of bFGF used were well below that required to induce mesoderm in these same cells. The fact that these cells were dissociated and that other factors (activin and TGF-β2) induced at least some neural tissue makes this data less than convincing. *Xenopus* animal caps are known to autoneuralize after being dissociated for even an hour [107] raising the possibility that bFGF was having some effect other than as a neural inducer, or as a putative inducer but in

conjunction with a system activated by tissue dissociation.

In order to address these questions these authors have extended their previous work. In this most recent study they use a more sensitive approach, reverse transcription-polymerase chain reaction (RT-PCR), to identify the presence of neural and mesodermal tissue in their culture system. Surprisingly, they show that bFGF induces a range of neural markers expressed along the A-P axis, with higher doses inducing posterior markers such as *XhoxB-9*, and lower doses inducing more anterior genes such as *XeNK-2* (midbrain-forebrain region), and *En-2*. These inductions occur without the presence of detectable mesodermal markers such as *noggin*, *Xbra*, or *gsc*, and at 20-times lower concentrations of bFGF than are required to induce mesoderm consistently in these cultures [89]. Based upon these results they speculate that an FGF family member acts as a true morphogen in the embryo, patterning the A-P neural axis.

Lamb and Harland [87] have also recently examined the ability of bFGF to induce neural tissue directly. In this study animal caps were not dissociated, but were cultured in a low calcium and magnesium medium (LCM) to help keep the caps from healing which would prevent access of the inner layer cells to the added bFGF. Although bFGF did induce muscle actin expression in some explants it did not induce its expression in all explants, some of which expressed *nrp-1*, a general neural marker, suggesting a direct induction of neural tissue. This is supported by the ability of bFGF to induce the general neural marker, NCAM in animal caps that had been aged past the period of mesodermal competence. It should be noted however, that untreated caps cultured in LCM express *XAG-1*, a cement gland marker not normally expressed in this tissue. Significantly, this marker has been shown to be induced in ectoderm by anterior neural tissue [55, 108, 109]. However, Elinson [110] has demonstrated that the cement gland can form in the absence of other detectable dorso-anterior structures, thus the response seen may not be coupled to anterior neural development.

Interestingly, Lamb and Harland observed that unlike neural tissue induced by noggin, neural tissue induced by bFGF expressed posterior neural markers such as *XhoxB-9*, while not expressing anterior markers such as *Xotx2*. In addition, when they induced animal caps with both noggin and bFGF they obtained expression of *Xkrox-20*, a marker of rhombomeres 3 and 5, which neither molecule was able to induce alone. In addition these caps expressed *Xotx2* and *XhoxB-9* on opposite ends of the explants suggesting that there is some apparent A-P prepattern in these caps, similar to what has been reported for the apparent D-V pattern reported in noggin-treated caps [86]. Unlike the study by Kengaku and Okamoto [89], Lamb and Harland do not observe a shift in A-P character of the induced neural tissue with different doses of bFGF: rather, they correlate such changes with the age of the animal caps exposed to bFGF. Animal caps from early gastrulae (stage 10.25) treated with bFGF express the posterior marker *XhoxB-9* but do not express more anterior markers tested. However, caps aged until mid-gastrula (stage 11) express *En-2* (midbrain-hindbrain boundary), *Xkrox-20* and *XhoxB-9* when treated with the same concentration of bFGF. Caps aged until late gastrula (stage 12) prior to treatment express only *En-2*, while not expressing either *Xkrox-20* or *XhoxB-9*.

An important aspect of these recent studies is that if bFGF induces posterior neuroectoderm by itself then this finding is at odds with the activation/transformation model. According to the model a neural-inducing principle should only induce tissue of anterior character, while a transforming principle might not be expected to induce neural tissue but should influence its axial character, and at high concentrations induce mesoderm. Given the problems associated with cell dissociation and the expression of *XAG-1* in untreated caps cultured in LCM, it would be of interest to know if bFGF-coated beads could induce neural tissue in mid to late gastrula ectoderm cultured in physiological media. If the neural-inducing ability of bFGF is further substantiated then the two signal model would require modification to incorporate a dual role for a transforming molecule.

Cerberus. Recent studies have also identified a novel secreted molecule whose transcripts are enriched in the organizer and subsequently in the advancing anterior endo-mesoderm which will give rise to the foregut, liver and anterior midnut. *Cerberus* named after the mythological guardian dog which has multiple heads, induces ectopic head-like structures when injected into ventral-vegetal blastomeres. These ectopic structures develop in mirror image to the normal anteroposterior axis, lack trunk and tail structures including somites and notochord, contain a single eye, suggesting the absence of a pre-chordal plate, and develop a secondary liver and heart [110a]. Strinkingly, *cerberus* has the ability to induce anterior nerural tissue characterized by *Otx2* and *N-CAM* expression in animal cap explants [110a]. No expression of the axial mesodermal markers, *collagen II*, *α-actin* or *α-globin* could be detected in these explants, however, markers for presumptive heart mesoderm and a pan-endodermal marker are detected. Therefore, from these data one cannot conclude that *cerberus* can induce neural tissue directly, but may do so secondarily through induced endoderm or mesoderm.

Bouwmeester and coworkers [110a] also asked whether BMP-4 had the ability to counteract the neural-inducing activity of *cerberus* in animal caps by coinjection experiments. Indeed they demonstrate the BMP-4 is able to suppress the neuralizing effect of injected *cerberus* mRNA. These data, together with the later zygotic

expression of *cerberus* (compared to *chordin*), support the authors view that *cerberus* may function downstream of *chordin* in inducing neural tissue.

HGF/SF. An additional neural-inducing molecule has been identified in the chick embryo, hepatocyte growth factor/scatter factor (HGF/SF) [11]. HGF/SF has been shown to be expressed in Hensen's node in the chick (the equivalent of the dorsal lip in amphibian embryos) during the primitive streak stage, suggesting proper temporal and spatial localization for a neural-inducing molecule [111]. HGF/SF induces epiblast of the area opaca to express L5, an early marker of neural differentiation, when cultured in collagen gels. As with other putative neural-inducing molecules HGF/SF does not induce the expression of several mesodermal markers including *brachyury* and *goosecoid*, suggesting that the induction of neural tissue is direct [111]. Recently an HGF homologue from *Xenopus* has been cloned [112] and found to be expressed predominantly in ventral mesoderm of late gastrula stage embryos, suggesting a role for this molecule in the development of this tissue. It remains to be seen if other HGF members may be expressed earlier in *Xenopus* development in a manner consistent with an involvement in neural induction.

Neural default state

Given these recent advances in identifying potential neural-inducing molecules, how can we account for the ability of ectoderm from certain amphibian species to autoneuralize? The ability of newt and salamander animal cap ectoderm to form neural tissue in response to either non-specific or no signals suggests that epidermal differentiation and not neural differentiation requires inductive signals. Although *Xenopus* ectoderm does not easily autoneuralize, it has been shown that by dissociating animal cap cells during their period of neural competence, and keeping them separate for extended periods, they will undergo neural differentiation [107, 113]. At present it might appear more unlikely that the act of dissociating these cells produces a neural-inducing signal as proposed by Holtfreter [38], but instead that dissociation has led to a release of an inhibitory signal present in the intact tissue. Perhaps cell-cell contacts are responsible for this inhibition, or alternatively the method used for dissociation is sufficient to release a surface-bound repressor, such as BMP-4 or activin, from the cell surface. If the latter is true, one would expect dissociation of animal cap cells from embryos injected with an RNA encoding the BMP-4 gene would yield less neural tissue than would non-injected animal caps.

Parallels to neurogenesis in *Drosophila*

As we learn more about the molecular pathways that lead to neurogenesis in vertebrates many parallels are emerging between signalling pathways and those which control neurogenesis in *Drosophila*. In both vertebrates and flies ectodermal cells have a cell fate choice between becoming either epidermal or neural. Genetic analysis has established that the default pathway for *Drosophila* ventral ectoderm is to become neural. Epidermal differentiation on the other hand requires neural inhibitory signals passed laterally from neural progenitors [114]. As we have already pointed out above there are striking parallels which exist between the Dpp/sog pathway in *Drosophila* and the BMP/chd pathway in vertebrates both in controlling D-V patterning as well as in the choice between epidermal and neural cell fates. In addition to these extracellular molecules there are two major groups of transcription factors in *Drosophila* which have been identified as involved in controlling this cell fate choice. These are the proneural genes, which include those of the *Achaete-scute* complex (AS-C), and the genes of the *Enhancer of Split* complex (E(SPL)-C), which control the epidermal developmental pathway. The proneural genes of the AS-C are so named because of their ability to confer neural character on ectodermal cells, and on their requirement for the development of particular neural progenitor cell types [114–116]. Three AS-C homologues have been identified in vertebrates, two of which appear to play the role of proneural genes in the systems examined [117–121]. These genes encode basic helix-loop-helix (bHLH) proteins which bind DNA and activate transcription as heterodimers with ubiquitous E proteins, which are a family of bHLH proteins which share homology with daughterless which plays an analogous role in *Drosophila*. One of these genes, ASH1 (*achaete-scute* homologne 1) is expressed in anterior CNS progenitors during later embryogenesis in both the mouse and in *Xenopus*. Guillemot et al. [119] have succeeded in knocking out this gene in the mouse and have observed that mice null for this gene lack certain neural progenitors including those for the olfactory neurons and for the autonomic nervous system. Therefore, neither the expression pattern of ASH1, nor its null phenotype, are consistent with a role in the initial cell fate choice between an ectodermal or neural cell fate in the gastrula stage embryo in either species. In contrast, the expression pattern of ASH3 in *Xenopus* embryos is consistent with a role in determining whether an ectodermal cell will adopt a neural or epidermal cell fate [118, 121]. In addition, misexpression of ASH3 in *Xenopus* embryos, either alone or in conjunction with a binding partner E12, appears to lead to an expansion of the developing CNS at the expense of surrounding placodal and neural crest derivatives, consistent with a role as a vertebrate proneural gene. Interestingly, this effect is restricted to tissues which are normally exposed to neural-inducing signals in the embryo, affecting dorsal ectoderm but not ventral ectoderm. Ferreiro and coworkers also demonstrate that

while overexpression of XASH3 leads to the expression of several neural-specific genes, this expression is transient and dependent on continued overexpression of XASH3 in these cells. Therefore they conclude that XASH3 is acting downstream of initial neural induction, promoting neuronal differentiation in these cells [118].

Recently, a new bHLH factor has been described which appears to act earlier in the neurogenic pathway. This factor, *NeuroD* is expressed in differentiating neurons of both the peripheral and central nervous systems of mice and *Xenopus* embryos [121a]. Unlike XASH3, however, *neuroD* has the ability to convert ventral and lateral ectoderm of the *Xenopus* embryo into neural tissue assayed by *N-CAM* expression [121a]. Strikingly, Lee and coworkers also demonstrate that animal caps isolated at the mid-blastula stage from neuroD RNA injected embryos develop *N-CAM* positive tissue. From the temporal expression pattern of *neuroD* it is clear that it is not a determinative factor for neural development, but is rather a differentiation factor which when misexpressed has the ability to promote the program of neural differentiation. Perhaps additional studies will uncover bHLH factors which act upstream of *neuroD*, *ASH1* and *ASH3* to control neural determination under the influence of neural-inducing molecules discussed above.

In addition to these two groups of genes, the products of the genes *Notch*(N) and *Delta*(Dl), have also been implicated in controlling this cell fate choice. The products of these two genes are thought to represent a receptor-ligand pair involved in cell-cell communication among neural and non-neural cells whereby the neural cell inhibits others from becoming neural. These molecules do not appear to impart information controlling cell identity, since mutations affecting these genes have both neural and non-neural phenotypes. Several vertebrate homologues of N have been reported [122–130]. *X-Notch-1* (formerly *Xotch*) is expressed at the boundary of the presumptive neuroectoderm and epidermis in developing *Xenopus* embryos [122]. Expression of constitutively active forms of *Notch-1* in cultured cells [131, 132] or in *Xenopus* embryos [133, 134] leads to an inhibition of cellular differentiation although there is no clear relation between epidermal and neural differentiation. There is however an increased amount of neural tissue in such embryos and the period of neural competence appears to be extended, suggesting a possible delay in developmental timing mechanisms. Recent work has also revealed the existence of a vertebrate *Dl* homologue in *Xenopus* which shares a similar domain of expression to *X-Notch-1*, and expression of an active form of *X-Delta-1* leads to a similar phenotype as observed with *X-Notch-1*, suggesting that these molecules may well be acting in the same pathway in vertebrate embryos as in *Drosophila* [135].

Unresolved issues

While there has been remarkable progress in the field of neural induction in recent years, the next decade promises to be even more revealing as a number of classic questions come closer to being given definitive answers. Perhaps in some sense the broadest issue which will be addressed is the evolutionary conservation of neural determination mechanisms. Will the process of neural induction and early neural patterning in vertebrates share the same striking conservation of genetic circuitry with evolutionarily distant organisms, such as *Drosophila*, as has been seen in such processes as eye determination and later stages of A-P regionalization? The initial comparisons discussed above suggest that there will be such similarities, but too little is known at present for a definitive answer. Because in some cases there are gene families in vertebrates with very high sequence identity to single *Drosophila* gene products the correspondences may represent a combination of conservation and divergence that will themselves be very informative about evolutionary processes as the story unfolds further

While advances in technology have permitted a more careful analysis of the tissue interactions responsible for neural induction and patterning, there is still no clear resolution of a number of long-standing questions. The debate still continues, for example, regarding the relative roles of vertical and planar signals in neural induction. For all its many advantages, *Xenopus laevis* may simply not be the organism of choice for settling this question because of the complexities associated with gastrulation in this organism. It will be most revealing to study this issue in amphibians in which gastrulation involves involution of a single layer, thus potentially obviating the controversies about transient vertical contacts in exogastrulae that are such a concern to those studying *Xenopus*. In addition, this should simplify related approaches, e.g. preparation of Keller explants, used currently to examine this question in *Xenopus*. Another approach to this issue that should be productive is to examine the pre-gastrula tissue interactions responsible for determining D-V differences in neural responsiveness in animal cap ectoderm. As more gene products expressed in particular regions of the early gastrula are discovered this should become a more tractable means for investigating early planar signalling.

At present it seems difficult to decide which of the models for neural regionalization discussed earlier is likely to be correct. That is changing, however, as can be seen by recent experiments that have begun to define putative anterior and posterior neural-inducing signals. This work has begun to yield a picture of a tightly linked neural A-P and mesodermal D-V axis (fig. 3). Three of the putative neural inducers, noggin, follistatin, and chordin, dorsalize induced mesoderm and

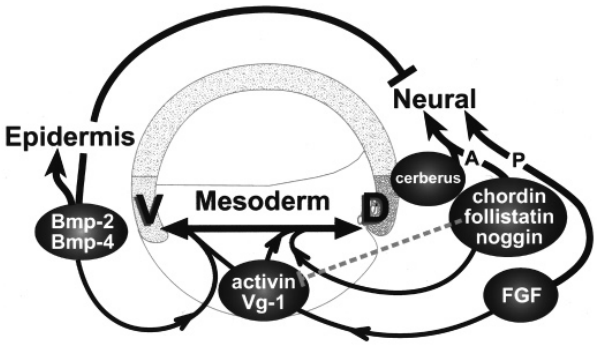

Figure 3. Diagram of proposed neural-inducing and patterning signals and their potential interactions. Since neural tissue is induced and patterned by dorsal mesoderm it holds that factors which induce and pattern mesoderm will greatly influence the future neural axis. Recent studies have, however, suggested that this interaction between factors responsible for mesodermal patterning, and formation of the CNS, may have a more direct effect than previously thought. Of the five molecules implicated in neural induction in amphibians, three (chordin, follistain, and noggin) dorsalize induced mesoderm and directly induce anterior neural tissue. FGF has been shown to induce ventral mesodermal cell types, and to induce directly posterior neural tissue. Members of the BMP family of signaling molecules ventralize induced mesoderm and inhibit neural differentiation by promoting epidermal differentiation in ectoderm. Similarly, activin which can induce both dorsal and ventral mesodermal cell types depending upon its concentration, may also act to inhibit neural differentiation by switching ectodermal cells toward a mesodermal pathway rather than a neural pathway. This activity is inhibited by the action of follistatin, an activin-binding protein (dashed line). The pattern that is emerging is striking. Factors which dorsalize mesoderm, induce anterior type neural tissue, while those that lead to ventral mesoderm either repress neural development or induce posterior neural tissue. This leads to a model in which mesodermal D-V patterning and neural A-P patterning are tightly linked, reminiscent of the interaction suggested by Yamada [47].

directly induce anterior neural tissue. The fourth factor, bFGF, induces ventral mesoderm and appears to induce posterior neural tissue. In addition BMP-4, which ventralizes induced mesoderm, inhibits neural differentiation by promoting epidermal differentiation in ectoderm. The pattern that begins to emerge is striking (fig. 3): factors which dorsalize mesoderm induce anterior neural tissue while those that induce, or lead to, ventral mesoderm development either repress neural development or induce posterior neural tissue. The idea that A-P and D-V axes are intimately connected is certainly not a new idea [47], but until now we have not had the proper reagents to examine this possibility so precisely. The identification of additional factors involved in these patterning events, and learning how they may interact with the known signalling molecules outlined above, should help determine how tightly these two axes are linked in early development.

Underlying the neural regionalization question is the step which precedes it: what is responsible for regionalization of dorsal mesoderm? While there has been striking progress in understanding the mechanisms of mesoderm induction and regionalization of mesoderm along the D-V axis, much less is known about the tissue interactions and molecules underlying A-P regionalization. There is no apparent reason for the lack of progress on this particular issue, e.g. no insurmountable technical problems, and one might thus anticipate significant insights into this problem in the near future.

One particular question that remains almost a total mystery is what regulates competence changes in the embryo. The regulation of neural competence plays a critical role in the extent of neural induction yet there is little information about what governs this intriguing process [136]. The suggestion that *Notch* family members might play a role in regulating developmental timing is intriguing, but it is not clear whether this gene is important in the normal timing of responsiveness of ectoderm to neural-inducing signals. Because so little is known about the signal transduction mechanisms leading to a neural response it is perhaps no surprise that little is known about what controls competence. As receptors and early downstream responses to neural inducing signals are characterized further, however, this should become an important and productive area of investigation.

Much of the recent progress concerning signal transduction mechanisms leading to neural induction and patterning has been due to the development of novel assays for identifying gene products [83] and the perturbation of gene activities in embryos (by misexpression or through the use of dominant negative constructs). The latter approaches have often been used in rather simple ways, for example, determining effects on overall development of embryos or on isolated animal caps from embryos injected with particular constructs. One way of focusing the effects of such perturbations that should become increasingly helpful will be to make mosaic embryos in which only a small region of the embryo contains the perturbed gene activity. This is readily accomplished in amphibian embryos and should allow investigators to test subtle features of patterning models, for example.

A challenge that remains for the future will be how to design more refined means for modifying gene activities in vertebrate embryos. The techniques currently in use in amphibians have been very productive, but will not lead to the wealth of information that has been so helpful in untangling genetic hierarchies in *Drosophila* and other genetically tractable organisms. Clearly it is the hope of investigators studying zebrafish development that this organism will be the solution to this particular problem, but whether these embryos will permit the embryological manipulations that are also required remains to be determined. Other organisms, e.g. the mouse and amphibians more genetically manipulable than *Xenopus laevis*, may be very helpful in addressing this problem, which is one that must be resolved before definitive genetic hierarchies involved in neural induction and patterning can be determined.

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