

Retinoic acid and the late phase of neural induction

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Summary

Regional neural gene expression in *Xenopus* is the result of a number of processes that continue well beyond the end of gastrulation. By considering two of the basic features of neural induction, the duration of contact between mesoderm and ectoderm and the timing of neural competence, it has been possible to distinguish two phases in neural tissue formation. The late phase includes the period following gastrulation.

A factor in determining regional neural gene expression is the difference in inducing ability of the mesoderm that develops during gastrulation along the anterior-posterior axis. The resulting ability to express regional neural genes is subsequently refined during the

late phase by a signal that progresses from the posterior part of the embryo. Using a dorsal explant system, it is shown that this progressive signal can be mimicked by the addition of retinoic acid (RA). However, the observation that regions along the anterior-posterior axis respond in different ways to the addition of RA suggests that additional factors are also important in defining regional neural gene expression. One possibility is that the expression of retinoic acid receptors along the axis may demarcate regions that respond to RA in particular ways.

Key words: *Xenopus*, neural induction, retinoic acid.

Mechanisms of neural induction

The *Xenopus* neural tube forms as a result of cell interactions during the first 24 hours of embryonic development. The key event during this period is gastrulation when the mesoderm involutes and the anterior-posterior axis is formed. At this stage, signals from the mesoderm divert competent ectodermal cells from an epidermal to a neural pathway of development (Gurdon, 1987). It has recently become clear that at least two mechanisms generate these signals, one involves an interaction between the involuted mesoderm and the overlying ectoderm (reviewed by Hamburger, 1988; Sharpe and Gurdon, 1990) whilst the second appears to be independent of involution, occurring along a tangential interface of the two cell types (Dixon and Kintner, 1989; Ruiz i Altaba, 1990). In vitro, both mechanisms can be shown to cause the formation of a regionally distinct neural tube. In the first case, using the 'sandwich technique' of wrapping the mesoderm in competent ectoderm, regions of post-gastrulation mesoderm isolated from along the anterior-posterior axis induce the expression of different regional neural markers (Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1990). In the second case, planar induction is assayed in 'Keller sandwiches' (Keller and Danilchik, 1988) and the expression of regional neural markers can be followed by whole-mount in situ hybridisation (Doniach et al., 1992).

Little is known about either mechanism at the molecular level. However, they could well be manifestations of the same short-range (up to a few cell diameters) signals. In

both cases, gastrulation plays an important role in generating the anterior-posterior axis; regional differences in the inducing ability of the mesoderm become more apparent following the involution and extension of the mesoderm, whilst groups of responding cells each expressing a regional neural marker become widely distributed along the axis following convergence-extension movements (Doniach et al., 1992, and this volume).

The timing of interactions between mesoderm and neurectoderm define two phases of neural induction

A feature common to all embryonic inductions is the involvement of two different cell types, one that produces an inducing signal and a second capable of responding to that signal over a window of time known as the period of competence. One of the first steps that we have taken to characterise neural induction involved analysing the timing of these interactions between mesoderm and ectoderm that give rise to neural tissue (Fig. 1; Sharpe and Gurdon, 1990).

The duration of the inducing signal was measured by removing the neurectoderm from the embryo during gastrulation and neurulation and culturing it in isolation to a time point equivalent to the tailbud stage then determining the levels of expression of a panel of neural markers. The neural markers included *XlHbox6*, a homeobox gene that is expressed predominantly in posterior neural tissue (Sharpe et al., 1987), *XlF6*, the *Xenopus* mid-sized neurofilament

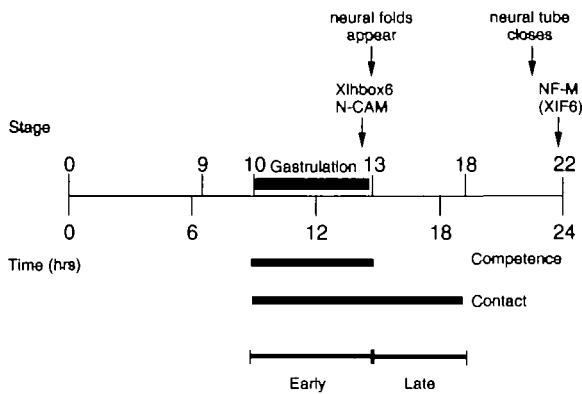


Fig. 1. Key features of neural tissue formation during the first 24 hours of development. Over the first 24 hours, equivalent to embryos reaching stage 22 of development at 23°C, all the events leading to neural tube closure have occurred. Neural induction probably starts with the onset of gastrulation though the first externally visible result is the formation of the neural folds at the end of gastrulation. Neural marker genes such as N-CAM (Kintner and Melton, 1987) and *XIHbox6* (Sharpe et al., 1987) can be detected at a slightly earlier stage. The early and late phases of neural tissue formation are defined by the duration of neural ectoderm competence and the period of contact between mesoderm and ectoderm required for the complete expression of neural markers in isolated neurectoderm.

gene (Sharpe, 1988), and *XIF3*, encoding a peripherin-like intermediate filament expressed mainly in anterior neural tissue in the embryo (Sharpe et al., 1989). Neural induction was said to be complete when the dissected neurectoderm was observed to express maximal levels of the neural markers and hence the responding tissue had received all the signals from the mesoderm that are required for independent neural differentiation. In addition to signals that convert ectoderm cells to the neural lineage, these may also include signals that maintain cells in the newly induced state. The experiment showed that the neurectoderm required signals from the embryo until the mid to late neurula stage in order to complete neural induction. Furthermore, this seemed to be the same for all the neural markers tested, there was no difference in the duration of contact required for full expression between the marker of anterior neural tissue (*XIF3*) and posterior neural tissue (*XIHbox6*).

In order to measure the *competence to respond*, ectoderm was removed from the early gastrula embryo and cultured in isolation for a period of time before recombination with a piece of inducing mesoderm. The end of competence was measured as the loss of ability of these conjugates to express a panel of neural markers when grown on to a fixed stage (equivalent to the tailbud stage). In this assay, competence is lost towards the end of gastrulation. This contrasts with the much longer period required for mesoderm-ectoderm contact. Using a transplantation technique, Servetnick and Grainger (1991) have similarly shown that competence to form neural tissue is lost at the end of gastrulation; furthermore, they observe that it is rapidly replaced by competence to form secondary neural structures such as the eye and otic vesicles.

Thus, from the comparison of the duration of contact and

the time of loss of competence, it appears that neural tissue formation can be divided into two phases. The early phase is defined by the ability of the neurectoderm to respond to signals from the mesoderm and the late phase by the loss of competence and the time that neurectoderm can differentiate independently of mesodermal signals. So, what is happening in the late phase? One possibility is that this phase is required in order that the neural phenotype is maintained, suggesting that the first stages of neural induction could be reversible. Little is currently known about the stability of recently induced tissues. An additional possibility is that the late phase may be required for the patterning of regional neural gene expression along the anterior-posterior axis, and this is explored in the next section.

Regional neural gene expression in the late phase

Classical experimental embryology has outlined two possible mechanisms by which regional neural induction may occur. In the first, the anterior-posterior character of the inducing mesoderm that unfolds during gastrulation is imparted to the overlying neurectoderm (reviewed in Hamburger, 1988). This requires several inducers, which in the extreme will equal the number of regions that can be defined along the anterior-posterior axis of the neural plate. In support of this theory, it has been observed that some homeobox-containing genes are expressed in both mesoderm and neurectoderm and that each gene has its own particular boundary that is initially conserved between the two germ layers. It has been suggested that this reflects the ability of the mesoderm to impart its regional character on to the overlying neurectoderm, in a form of positional homeogenetic induction (De Robertis et al., 1989).

The second model suggests that neural induction first generates anterior neural tissue on to which a range of anterior-posterior characteristics are subsequently imposed in response to a gradient of a second signal derived from the posterior of the embryo. This 'gradient' model reduces the number of inducers to two and suggests that regional neural gene expression depends on the ability of neurectoderm cells to interpret the graded signal (reviewed by Saxen, 1989).

Recent experiments and observations have shown that neither model explains neural induction completely. For example, although anterior mesoderm taken at the end of gastrulation differs from posterior mesoderm in its ability to induce the panel of regional neural markers (Sharpe and Gurdon, 1990), these differences do not correlate with the final defined pattern of neural gene expression along the anterior-posterior axis. This is illustrated by the observation that only posterior mesoderm induces the neurofilament gene *XIF6* and yet this gene is ultimately expressed along the length of the neural tube. In another example, the ability of post-gastrula axial mesoderm (notochord) to induce expression of the *engrailed* gene has been examined. Again there is a difference in inducing ability, with the region underlying the eventual position of expression being the strongest inducer, but this ability is also found at more posterior positions along the anterior-posterior axis (Hemmati-

Brivanlou et al., 1990). In both cases, although particularly marked, the differences in the inducing ability of the mesoderm do not provide the complete solution to the problem of regional neural gene expression.

In terms of the 'gradient' model, it might be predicted that the ability to express neural markers is found progressively from the posterior end, whilst the ability to express anterior markers is initially found throughout the neural plate. This can be examined by isolating the dorsal tissues of the embryo that constitute the neurectoderm and underlying mesoderm (a dissection that may also contain a few dorsal endodermal cells). This explant can then be divided along the anterior-posterior axis and the ability of each piece to express neural markers determined. Repetition of this process at different time points during the late phase of neural tissue formation should make it possible to identify the progress of a signal that is propagated from the posterior end of the embryo. For example, the ability to express the neurofilament gene *XIF6* is initially confined to posterior regions but is progressively found in middle and anterior regions. *XIHbox6* shows an ability to be expressed progressively in the mid-region of the neural plate (Fig. 2; Sharpe, 1991).

By marking cells with Nile Blue sulphate, it is possible to follow their fate during subsequent development. Marked cells in the mid region at the end of gastrulation give rise predominantly to spinal cord (Fig. 3 and unpublished data). Although there is extensive distortion of the mark, this seems to be displaced both to the anterior and to the posterior. Marks placed at the lateral boundary of the mid region at the end of gastrulation converge towards the mid line and also extend both to the anterior and posterior. A much more extensive analysis of the fate map of both superficial and deep tissues in this region at these stages has been carried out by Keller (1975). Although the results are essentially the same, the observations reported here clarify the fates of the particular explants used in the above experiment. These results suggest that the observed differences in the ability of regions to express *XIF6* and *XIHbox6* are not due to sampling errors that result in different regions being examined at each stage. It seems more likely that the mid-region at the end of gastrulation is normally fated to give rise to spinal cord but does not have the ability to express *XIHbox6* because it has not received the required signal. The evidence that RA might be involved in this signal and the involvement of cell migration is considered in the next section.

Retinoic acid can mimic the signals that result in regional neural gene expression in the embryo

Retinoic acid (RA) was first shown to affect *Xenopus* development by Durston and colleagues (1989) who noted that treatment of embryos resulted in altered differentiation of the neural tube. These studies have been extended (Papalopulu et al., 1991; Sive et al., 1990; Sive and Cheng, 1991; Ruiz i Altaba and Jessel, 1991a, b) to show effects both on neural patterning and mesoderm formation. Retinoic acid also affects neural gene expression and can act in combination with growth factors to alter the patterns of gene

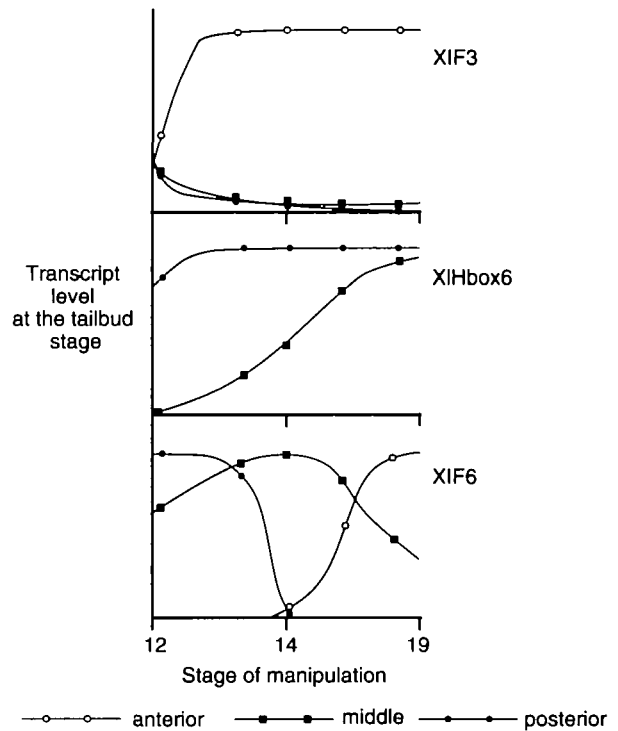


Fig. 2. The ability to express regional neural markers varies along the anterior-posterior axis in the late phase of neural tissue formation. The pattern of expression of *XIF3*, an anterior neural marker, remains stable in the anterior region. However, expression of *XIHbox6* in the mid region increases progressively during the late phase. Likewise there is a trend for the ability to express *XIF6* (NF-M) to be found in more anterior regions. The figure is a qualitative representation of the levels of expression of the marker genes in explants isolated at the stage shown on the x-axis and grown on to the tailbud stage determined by RNAase protection assay. A representative analysis is described in Sharpe (1991).

expression in isolated ectodermal tissue (Cho and De Robertis, 1990). In addition, RA has an effect on a wide range of developmental and regenerative systems (reviewed by Brockes, 1989).

It is possible to isolate the dorsal explants described above at the end of gastrulation and ask whether RA results in an altered ability to express the panel of neural markers. The observation is that physiological concentrations of RA can confer the ability to express *XIF6* onto the anterior piece and *XIHbox6* onto the mid piece (Sharpe, 1991). This shows that RA can mimic the endogenous signals that result in regional neural gene expression in the embryo. In addition, the transformation in ability takes place in isolated explants, suggesting that recruitment of cells by movement from one region to another along the anterior-posterior axis is not required. Unlike assays in which development is perturbed by RA, in this approach RA is seen to restore normal patterns of gene expression. The identification of RA and related retinoids in the *Xenopus* embryo at this stage (Durston et al., 1989) strengthens the argument that they may also be the endogenous signals. The 'gradient' model implied that the imposition of posterior character was dependent on the gradient of a signal derived from the posterior end of the embryo (reviewed by

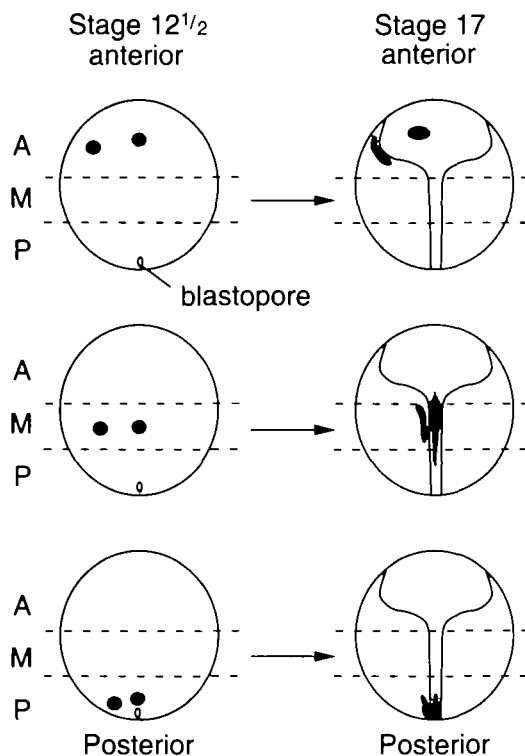


Fig. 3. Fate mapping the neural plate at the beginning of the late phase. Nile Blue sulphate marks were used to follow the fates of regions of cells along the anterior-posterior axis of the dorsal side of the embryo. One mark was applied to each embryo, the figure shows representative examples of marks placed centrally in each of the three regions that were removed as explants, with one mark on the dorsal mid-line and one displaced laterally.

Saxen, 1989). Whilst this idea remains attractive, there is no direct evidence that a gradient of any factor is involved.

Retinoic acid has been shown to activate the expression of homeobox genes expressed in an embryonal carcinoma cell line in a dose-dependent fashion (Simeone et al., 1990). Those genes at the 3' end of the homeobox gene cluster are activated in response to low doses of RA whilst those at the 5' end require higher concentrations (Simeone et al., 1990). This also reflects the order of expression of these genes along the axis of the embryo with the genes located at the 5' end of the cluster being expressed at the posterior end of the embryo (Wilkinson et al., 1989; Hunt et al., 1991). It is therefore interesting to ask whether the availability of RA in the *Xenopus* embryo may play a part in determining the boundaries of expression of homeobox-containing genes. The explant experiments showed that RA can stimulate the expression of *XIHbox6* in the mid region suggesting an involvement in determining the anterior boundary of expression of this gene. However, when applied to the anterior explant, RA did not cause the expression of *XIHbox6* (Sharpe, 1991). This is a little surprising since cells in this region are capable of responding to RA as shown by their altered ability to express *XIF6*. This suggests that the availability of RA is not the sole factor that controls the boundaries of expression of genes such as *XIHbox6*. An explanation might be that the anterior-pos-

terior axis is divided into a number of regions and that RA acts independently within each region. For example, the level of RA may affect the boundary of expression of *XIHbox6* within the mid region but be unable to affect *XIHbox6* expression in the anterior region at all. One way in which these hypothetical regions may be demarcated is by the distribution of the retinoic acid receptors that provide the molecular machinery for the interpretation of endogenous signals.

Future directions

The retinoic acid receptors are ligand-dependent transcription factors and fall into two main classes. The first to be discovered were the RARs which can be subdivided into α , β and γ subclasses (Benbrook et al., 1988; Brand et al., 1989; Giguere et al., 1987). A second group, the retinoid X receptors (RXRs) whose ligand is likely to be the 9-*cis* retinoic acid isomer can also be divided into three subgroups (Mangelsdorf et al., 1990, 1992). RARs and RXRs can act as heterodimers to modulate the expression of target genes (Kliwer et al., 1992). The proposition is that expression of the receptors will be restricted along the anterior-posterior axis and that the combination of different receptors will be used to demarcate regions that respond to RA in a particular way. In this respect, it has already been shown that *Xenopus* RAR γ is restricted in its expression to particular regions along the anterior-posterior axis (Ellinger-Ziegelbauer and Dreyer, 1991). Restricted patterns of expression of RAR have also been documented during the development of other species (for example, Dolle et al., 1989; Ruberte et al., 1990). Although the number of receptors available is large, and includes isoforms generated by alternative splicing (Kastner et al., 1990; Leroy et al., 1991; Zelent et al., 1991), the initial patterns of RAR expression in *Xenopus* may be quite simple if only a small number of regions need to be demarcated.

In conclusion, it is likely that differences in the inducing ability of the mesoderm and a progressive posterior signal acting during the late phase will be involved in regional neural induction. RA is able to mimic the progressive signal and this or a closely related molecule may play a role in vivo. The different abilities of regions along the anterior-posterior axis to respond to RA may reflect the domains of expression of RAR.

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