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Short historical survey of pattern formation in the endo-mesoderm and the neural anlage in the vertebrates: the role of vertical and planar inductive actions

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Abstract. After some introductory remarks about *vertical* versus *horizontal* inductive interactions and about *planar* versus *homoiogenetic* induction, the author discusses: a) the historical development of the more recently studied endo-mesoderm induction in the Urodeles and in the anuran *Xenopus laevis*, b) the possible causal relationship between endo-mesoderm induction and the initiation of the gastrulation process, and c) the older history of the regional neural induction as initially studied in the Urodeles and only recently analysed in the anuran *Xenopus laevis*. The essential *vertical* interaction in the neural induction process both in urodelian and in anuran amphibians is emphasized.

Key words. Historical survey; endo-mesoderm induction; initiation of gastrulation; regional neural induction; Urodeles and *Xenopus laevis*.

Some introductory remarks

A few remarks about the history of embryological research

The development of a particular field of science is often characterized by an irregular frontier, which may at any time be markedly altered by local breakthroughs, leaving adjacent areas far behind.

Our insight into the successive events which characterize embryonic development has often followed a reversed order, the later events being analysed and understood long before earlier events are studied. Neural induction was discovered long before endo-mesoderm induction was considered. This was partly due to the wide acceptance of the nineteenth century's germ layer concept, which stated that from the very beginning of development the vertebrate embryo should consist of three concentric germ layers or should already contain their presumptive anlagen. Only after the germ layer concept began to weaken, due to experimental observations which did not support a strict germ layer concept, was a more objective approach possible. Thus, several studies on pattern formation inside the mesodermal and endodermal layers were performed before the induction of the endo- and mesoderm was recognized as an important step in the development of the vertebrate embryo. This present survey, which is chiefly based on amphibian and bird development, therefore represents only a picture of our current knowledge of the early developmental events which seem to characterize vertebrate development.

The nature of inductive interactions

Inductive signals are biochemical factors which are transferred from the inducer, which produces these factors, to the reacting cells which have developed a special responsiveness or competence for them. The period of competence of the reacting cells is usually *much shorter* than the period during which the signal(s) is/are produced by the inducing cells. Moreover, the responsiveness or competence of the reacting cells is *much more specific* than the inducing signal(s). The latter can easily be replaced by artificial signals, as so-called heterogeneous inducers, leading to the same or a closely similar result.

A short theoretical consideration of the various types of inductive interactions

Interaction is in principle a *reciprocal* process, passing between two cell layers endowed with different properties. These reciprocal inductive actions may occur simultaneously or at different times.

A *vertical inductive interaction* represents an interaction between two different cell layers. One can only expect vertical interaction to occur after the embryo has formed at least two different cell layers, which usually occurs after the beginning of the gastrulation process. Since all layers may be polarized and may contain several inductive signals in different spatial patterns, *any pattern* may actually be transferred between the two cell layers in *vertical* interaction.

Horizontal inductive interactions fall into two different categories, namely *planar* inductions and *homoiogenetic* inductions. Planar inductions may occur between different cell groups in a continuous cell layer. The stimulus can only spread with decrement into the adjacent cells, and only a strong signal may have any appreciable effect. Planar induction is always a 'down-hill' process. Homoiogenetic induction represents the spreading in a

homogeneous cell layer of a self-propagating process in which each induced cell first produces the inducing signal before transferring it to its neighbour. This rather slow and time consuming process will continue until competence of the reacting cells disappears. It therefore usually leads to the formation of a sharp boundary of the induced structure. We shall meet both these aspects in the following sections.

Finally, it must be noted that this paper restricts itself to the morphogenetic analysis of early vertebrate development, leaving the related biochemical and genetical analyses to other authors.

Stepwise achievement of the final pattern in the induced endo-mesoderm during cleavage and gastrulation in the Urodeles and in the anuran, *Xenopus laevis*

Before entering the realm of endo-mesoderm induction we must first discuss some important studies on the regional segregation and determination of the endo-mesodermal mantle of the urodele neurula. Yamada [1] studied the dorso-ventral segregation of the mesodermal mantle by combining separate ventral to dorsal regions with the mid-dorsal notochordal anlage, observing a shift in differentiation capacity in the dorsal direction. He deduced the existence of a gradient in the mesoderm with its highest value in the mid-dorsal notochord and successive lower values in the somitic mesoderm, the nephrogenic anlagen and the lowest value in the mid-ventral blood islands, the ultimate pattern being determined during the neurulation process.

Okada and Hama [2, 3] and Hama [4] observed fundamental changes in the antero-posterior segregation of the endo-mesoderm during the gastrulation process. The future anterior archenteron roof, which is going to lie under the forebrain, does not differentiate into prechordal endo-mesoderm when isolated before invagination, but forms notochord and somites and induces hindbrain and spinal cord in competent ectoderm. However, the same material, isolated after passing the dorsal blastoporal lip, forms prechordal endo- and mesoderm and induces forebrain in competent ectoderm, thus changing from an initial trunk inducer into the definitive head inducer. This change can, however, also be achieved by cultivating the presumptive prechordal mesoderm for some time in a tissue culture medium, or combining it with non-competent ectoderm, thus imitating the potential change in developmental fate. These observations were fully confirmed by Hoessels in her 1971 PhD thesis [5].

Much later studies of Kanéda and Hama [6] and Kanéda [7, 8] have brought further insight into this remodelling process. They observed that at the early gastrula stage only the anterior half of the future marginal zone is mesodermized[1], the posterior half still representing uninduced ectoderm. During invagination the anterior part of the presumptive marginal zone comes into direct contact with the still uninduced posterior part, leading to a reciprocal *vertical* interaction, changing the initial trunk inducer into head inducer and establishing the definitive trunk inducer in the posterior portion of the marginal zone. Kanéda [8] called attention to the simultaneously occurring further extension of the primary mesodermalizing action from the 'dorsal-mesoderm inducing centre', now acting around the blastoporal lip.

There is still another important feature in the so-called middle layer of the vertebrate embryo, namely that it not only forms mesodermal structures, but also endodermal ones. The archenteron roof segregates craniocaudally into pharynx endoderm, prechordal head mesoderm and posterior head, trunk and tail chordamesoderm. The tail somites actually form from the most caudal portion of the neural plate (Bijtel [9–11] and Nakamura [12, 13]). Spofford [14–16] showed that the most caudal portion of the archenteron roof is acting as a special tail inducer. See also the section on neural induction on p. 310.

In the older literature there are a number of unrecognized cases of actual mesoderm induction. The oldest is Chuang [17] describing the formation of trunk mesodermal and neural structures when studying the effect of so-called heterogeneous inducers, representing factors liberated from adult animal tissues and acting upon competent gastrula ectoderm. These studies were further extended by Toivonen [18, 19] who distinguished between heterogeneous head and trunk inducers. Then in 1953 Toivonen [20] described guinea pig bone marrow as a nearly pure mesoderm inducer. All these inductive factors were, however, classified as artificial inducers, the actions of which had no direct relationship to normal development, which was still considered to be based upon the old germ layer concept. Here I refer to the further work of Toivonen and Saxén on the mesoderm inducing capacity of heterogeneous inducers, described in the section on regional neural induction (see p. 311). The Finnish school was primarily interested in the role of inducers in the patterning of the CNS and was not directly concerned with the significance of mesoderm formation in normal development.

Since I was primarly interested in the events occurring in normal development, in the late 1960s I started a series of recombination experiments, using successive animal-vegetal regions of the urodele blastula/early gastrula, eliminating the mesodermal marginal zone region. The formation of nearly complete embryos out of recombinants of animal 'ectodermal' caps and vegetal yolk mass endoderm (see fig. 1A,B) demonstrated for the first time that mesoderm does not arise from one of the initial moieties of the early embryo, but is actually formed de novo via an interaction between the vegetal

[1] In the meantime, the induction of the endo-mesoderm had been elucidated.

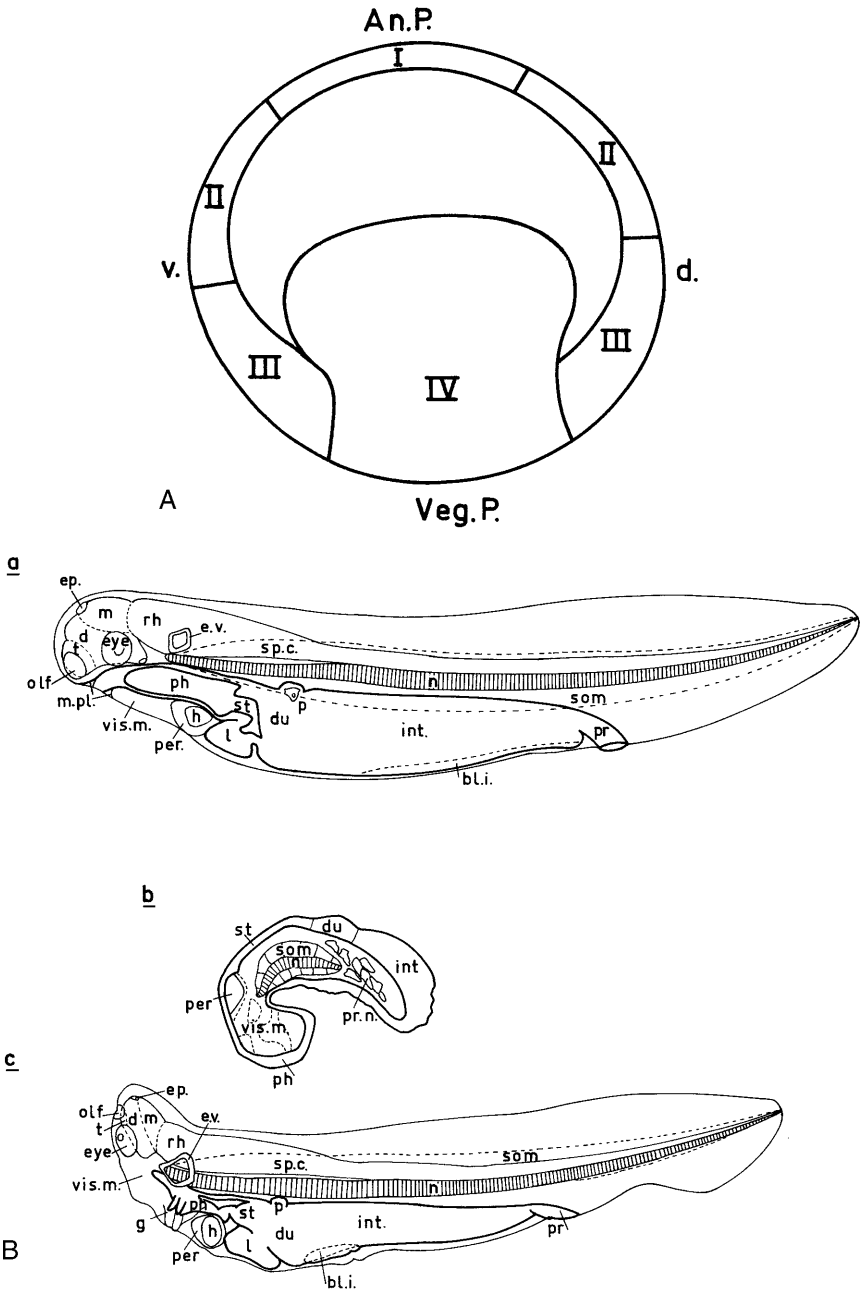

Figure 1. (*A*) Scheme of isolates and recombinates of successive animal-vegetal regions of the urodele blastula/early gastrula. Isolates of regions I–II form only atypical ectoderm; isolates of region IV only atypical endoderm. An.p. = animal pole; d = dorsal; v = ventral; Veg.p. = vegetal pole. (*B*) Isolates of region III form a scala of endo- and mesodermal structures (b). Recombinates of regions I, II and IV form a complete, though slightly smaller embryo (c). See corresponding control embryo (a).
bl.i. = blood islands; d. = diencephalon; du- = duodenum; ep. = epiphysis; e.v. = ear vesicle; eye = eye anlage; g. = gills; h. = heart; int. = intestine; l. = liver; m. = mesencephalon; m.pl. = mouth plate; n. = notochord; olf. = olfactory placode; p. = pancreas; per. = pericard ph. = pharynx; pr. = proctodaeum; rh. = rhombencephalon; som. = somites; sp.c. = spinal cord; st. = stomach; t. = telecephalon; vis.m. = visceral skeleton and musculature.

yolk mass endoderm and the animal 'ectodermal' cap material [21]. Marking experiments showed that the mesoderm developed out of the animal 'ectodermal' moiety under an inductive influence emanating from the vegetal 'endodermal' yolk mass. Translocation experiments of the animal cap with respect to the yolk mass showed that, in the blastula, the dorso-ventral polarity resides in the yolk mass endoderm and not or no longer in the animal cap [22]. Using recombinants of different urodelian species or ^{3}H-marked and -unmarked moieties, it was shown that not only the entire mesoderm, but also the pharyngeal and dorsal intestinal endoderm were newly formed. It became evident that the entire ring-shaped marginal zone which surrounds the vegetal

yolk mass and is demarcated by the dorso-ventrally expanding blastoporal groove, represents the newly formed third, endo-mesodermal moiety of the amphibian embryo [23]. Testing the inductive capacity of dorsal, L and R lateral and ventral parts of the vegetal yolk mass, it became evident that there is a *dorsal meso-endoderm-inducing centre*[2] within the yolk mass, which is responsible for the induction of the dorsal axial endo-mesoderm, while lateral and ventral parts induce only ventral endo-mesodermal structures [24]. It is characteristic of the vitality of the germ layer concept that even at this stage of analysis Nakamura and Takasaki [25, 26] and others still considered experimental mesoderm induction as an artificial process which plays no significant role in normal development (see also Nakamura [27]).

The action of the 'dorsal meso-endoderm-inducing centre' has the character of a *planar* induction process, spreading in the spherical blastula with decrement from the yolk mass endoderm in the direction of the animal pole. Its highest expression induces the pharyngeal (and dorsal intestinal) endoderm, leading subsequently to the formation of the trunk notochordal and somite mesoderm (the initial trunk inducer, see p. 306) and further to the axial tail mesoderm. It thus gives rise to the formation of the antero-posterior axis of the embryo [24, 28]. As already described on p. 306, a subsequent vertical interaction between the invaginating and still uninduced posterior presumptive marginal zone leads ultimately to transformation of the former into head inducer as well as to development of the latter into the definitive trunk inducer. The lateral and ventral endo-mesoderm also shows a cranio-caudal and dorso-ventral segregation, forming a three-dimensional pattern. Here, a dorso-ventrally spreading *planar* influence from the notochord plays an important role [29, 30].

Mesoderm formation in the anuran *Xenopus laevis* differs in several respects from that of the urodele amphibians. The South African clawed toad forms a rather extreme case among the anuran amphibians. Though most anuran species show a partially internally located marginal zone [31], the *Xenopus* blastula/gastrula is completely double-layered, consisting of an outer epithelial layer and an inner sensorial layer. Quantitative analysis of recombinates and embryos with ^{3}H-marked and -unmarked animal caps and vegetal yolk masses demonstrated that in *Xenopus* all mesodermal structures are formed from the sensorial layer of the animal 'ectodermal' cap under an inductive influence from the vegetal yolk mass. Recombinates of separate outer and inner layers of the animal cap of *Xenopus* blastulae with yolk mass endoderm showed that the inner layer formed exclusively mesodermal structures, while the outer layer yielded predominantly endodermal ones. Under experimental conditions the outer layer can, however, also form some mesodermal derivatives [32]. In normal *Xenopus* development, mesoderm formation is entirely restricted to the inner, sensorial layer, while endoderm formation seems to be restricted to the outer epithelial layer. The *Xenopus* blastula/early gastrula is moreover characterized by very early involution of the future anterior mesoderm, an involution which proceeds nearly independently of endodermal archenteron formation [33].

When testing the inductive capacity of the already involuted mesoderm at stage 10 (sharp) in comparison with stage 10+ (see the *Xenopus* Normal Table by Nieuwkoop and Faber, 1956, 1967, 1975 and 1994 [34]), the relatively high number of cases with differentiating notochordal and somitic mesoderm points, in my opinion, in the direction of the initial trunk character of the most anterior mesoderm, changing into head inducer immediately after involution [33]. We must therefore conclude that in *Xenopus* the formation of the mesoderm, which initially occurs by *planar* induction, may be completed by an additional *vertical* interaction of anterior and more posterior presumptive marginal zone material, as in the Urodeles. The main difference between *Xenopus* and the Urodeles is the *very early* mesoderm involution, which starts apparently independently of archenteron invagination [35]. This has important consequences for the neural induction process (see p. 312). The separate endoderm induction in the outer layer of the animal 'ectodermal' cap is directly related to the entirely endodermal nature of the anuran archenteron.

Although I highly value Keller's accurate analysis of morphogenetic movements in the early embryo, which will be discussed on pages 312, 313, I have considerable objections to some of the terminology which he introduced. The term 'marginal zone', used by Vogt in 1929 [36], refers to the equatorial area of the blastula/early gastrula, which represents the future *invaginating* endo-mesoderm. Keller's terms 'invaginating' and 'non-invaginating' marginal zone are confusing; 'invaginating' marginal zone is simply superfluous, because that includes the primary definition, and 'non-invaginating' marginal zone is a 'contradiction in terms'. The so-called non-invaginating marginal zone is no marginal zone, but represents the later induced neural anlage.

The possible causal relationship between endo-mesoderm induction and gastrulation in amphibians

The process of gastrulation has been extensively studied by Vogt [36] in the Urodeles as well as in some Anurans, using vital dye marking. The complex tissue movements of the invaginating meso- and endoderm were carefully described. After Holtfreter [37] studied the phenomenon

[2] Unfortunately, this dorsal meso-endoderm-inducing centre has been unscientifically named the 'Nieuwkoop centre'.

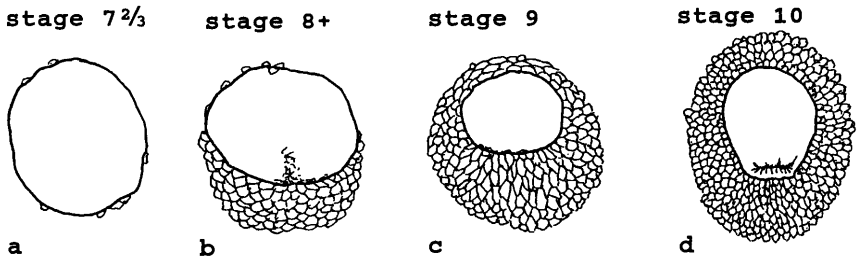

Figure 2. (*a–d*) Hand drawings of vegetal yolk masses of axolotl embryos (zone IV of fig. 1A), isolated from successive stages (stages $7\frac{2}{3}$, 8+, 9 and 10), showing increasing 'programmed' flask cell formation (seen from vegetal side).

of exo-gastrulation, he analysed [38, 39] the morphogenetic behavior of flask cells, which initiates the gastrulation process. From isolation and recombination experiments, Nieuwkoop [40] observed that the presumptive mesoderm of the urodeles is characterized by a change in cell surface properties, which is expressed in the tendency to reduce the area of the outer surface facing the medium, and to become surrounded either by ectoderm, as in normal development, or by endoderm, as for example in exo-gastrulae. This phenomenon is compensated for by the spreading of the animal ectodermal cap with original egg surface, called 'surface coat' by Holtfreter [41]. It is the mesoderm which apparently loses these surface properties. Disappearance from the outer surface by some form of invagination or involution is therefore an essential feature of mesoderm formation.

The first indication of the gastrulation process is the formation of so-called bottle or flask cells along the periphery of the vegetal yolk mass. Flask cell formation is characterized by an inward displacement of the main cell body and a maximal reduction of the outer surface. This usually leads to a marked concentration of pigment granules, predominantly localized in or beneath the surface coat. Flask cell formation starts on the dorsal side and progresses along the lateral towards the ventral side of the endodermal yolk mass. After invagination these flask cells change again into cuboid cells, and may subsequently flatten into large sheet-like cells during the formation of the archenteron, with marked extension of the anterior pharyngeal portion. This latter observation is actually at variance with Holtfreter [38, 41] who thought that flask cells were pinched off and then degenerated.

Hardly any research has been done on the possible causal relationship between endo-mesoderm induction and gastrulation, the latter being initiated by flask cell formation at the periphery of the endodermal yolk mass. There is, however, one observation which, in my opinion, points towards a causal relationship, first referred to by Nieuwkoop [43]. When one isolates the entire yolk mass from an early urodele gastrula, flask cell formation starts in the isolate on its dorsal side and progresses via the lateral towards the ventral side, just like in normal development, flask cell formation being, however, much more pronounced on the dorsal than on the ventral side. After a certain time the flask cells regress and the yolk mass rounds up again. When one isolates the yolk mass endoderm at a slightly earlier stage, flask cell formation starts after a certain time-lapse on the dorsal side, but is less extensive and does not reach the ventral side. Isolation at a still earlier stage leads to a still more restricted flask cell formation along the dorsal and dorso-lateral side of the yolk mass only. Still earlier isolation reduces flask cell formation to a very local phenomenon at the dorsal side of the yolk mass. Isolation at or before stage $7\frac{2}{3}$ fully prevents flask cell formation [44] (see fig. 2). Nakamura and Takasaki [25] and Nakamura et al. [45] showed by isolation experiments that in *Cynops phyrrogaster* mesoderm formation starts at the 32/64 cell stage, so that mesoderm induction must already have spread to some extent when flask cell formation is initiated around stage $7\frac{2}{3}$. From these observations it must be concluded that some influence from adjacent parts of the blastula must play a role in flask cell initiation. The most likely candidate is, in my opinion, the induced adjacent endomesoderm. Although Doucet-de Bruïne [44] observed a flask cell-stimulating influence in recombinates of unprogrammed vegetal yolk mass from stage $7\frac{2}{3}$ with dorsal marginal zone mesoderm, she could not show a direct correlation between the amount of induced mesoderm and the extent of adjacent flask cell formation. The ventral yolk mass being non-responsive, she deduced a more autonomous nature of flask cell formation in the vegetal yolk mass endoderm. I still feel that the stage-dependent character of flask cell formation in isolated yolk mass endoderm argues against autonomous flask cell formation and actually suggests the existence of a causal relationship. Since recombination experiments with yolk-rich axolotl material are rather difficult to perform at these early stages, they are, in my opinion, not fully conclusive and ought to be repeated on a different urodele species.

In *Xenopus laevis*, which has a fully internally located marginal zone, mesoderm involution seems to be initiated before and is independent of archenteron invagination [35]. The latter, though starting much later,

subsequently catches up with mesoderm involution. This more independent behaviour must be due to the double-layered nature of the blastula/gastrula and with the separate endo- and mesoderm induction in respectively the outer epithelial and the inner sensorial layers of the ectoderm [33].

The causal analysis of induction and spatial patterning of the neural plate during gastrulation and neurulation in amphibians

The notion of neural induction is much older than that of endo-mesoderm induction and goes back to the classical work of Spemann and Mangold [46] on the organization centre of the amphibian embryo. The development of an additional neural plate in the ventral ectoderm of a *Triturus* embryo, in which an extra dorsal blastoporal lip was grafted ventrally, led to the concept of neural induction as an interaction between the invaginated axial mesoderm and the overlying ectoderm. Subsequently Spemann [47] tested the inductive capacity of dorsal blastoporal lip material taken from successive stages of gastrulation, demonstrating a more or less regionally specific induction of forebrain, hindbrain and spinal cord by successively older dorsal blastoporal lips, and suggesting the transfer of an overall pattern from the archenteron roof onto the overlying ectoderm. These observations were essentially confirmed by Sala [48], who tested the inductive capacity of successive antero-posterior portions of the archenteron roof of early neurulae acting upon competent early gastrula ectoderm. The cultured explants showed, however, a more complex regional differentiation of neural structures than that which would correspond strictly to the patterning of the archenteron roof. We shall return to this work later. Spemann's classical work led to a large number of studies on the possible nature of the inducing agent, which will, however, not be discussed here.

Nieuwkoop and others [49] showed that the pattern formation in the neural plate is of a more complex nature. The fold implantation experiments led to a number of very interesting conclusions. First, locally attached folds of competent ectoderm showed the propagation of the neuralizing signal over a considerable distance (see fig. 3A, B, C), a length roughly corresponding to half the width of the neural plate at the cranio-caudal level of implantation. This propagating action typically represents what we now call a homoiogenetic induction process, extending in the proximo-distal direction in the attached fold. Implantation at different cranio-caudal levels showed an unexpected regional segregation along the length of the neuralized section of the fold. Implantation in the prechordal region of the gastrula or neurula led to an additional forebrain formation, while implantation in the caudal trunk region gave rise only to the formation of a small spinal cord. Implantation into the anterior notochordal region, however, gave rise to a complex neural formation, consisting proximally of hindbrain tissue followed distally by midbrain and more distally by forebrain structures. The tendency for the formation of more anterior neural structures also existed in slightly more posterior implants. This clearly did not correspond to the transfer of a single overall pattern from the archenteron roof onto the overlying ectoderm. The only satisfactory explanation was the notion of a two-step induction process, the first, 'activation', leading to neuralization and ultimately to forebrain development and the second, 'transformation', being responsible for the subsequent transformation of presumptive forebrain into hindbrain and spinal cord, depending upon the intensity of the inductive action. This actually represents a double-gradient hypothesis for neural induction.

After participation in the first team work, which led to the publication of Nieuwkoop and others [49], Sala [48] concluded from his recombination of different cranio-caudal regions of the archenteron roof with competent gastrula ectoderm, that the two inductive actions have a different spatial distribution in the archenteron roof: the activating action, being strong in the anterior part and falling off in the caudal direction to nearly zero, and the transforming action, which is absent in the anterior part and increases in intensity in more posterior regions, reaching its maximum in the most caudal region of the archenteron roof.

Folds of 'virgin' ectoderm taken from successively older stages and implanted at the same anterior notochordal level, showed that the competence of the ectoderm for activation falls off rapidly at a midgastrula stage, whereas the competence for transformation remains unaffected [50]. Much more recent experiments of Nieuwkoop and Albers [51], in which forebrain tissue of successively older gastrula and neurula stages was grafted into various cranio-caudal regions of a host neural plate, showed that the competence for transformation actually begins at a midgastrula stage and only falls off at the open neural plate stage. This implies that activation and transformation have completely separate periods of competence, due to the fact that they represent successive steps in the induction process.

Albers [52] could show that the medio-lateral spreading of the neuralization process is a homoiogenetic induction process which propagates slowly in the aging ectodermal layer, ending abruptly when neural competence runs out. It leads temporarily to placodal ectoderm formation before neural competence completely disappears [53].

The transformation process, spreading likewise from the dorsal midline in the lateral direction, also has the character of a homoiogenetic induction process. The falling off of the competence of the neurectoderm for transformation leads temporarily to neural crest forma-

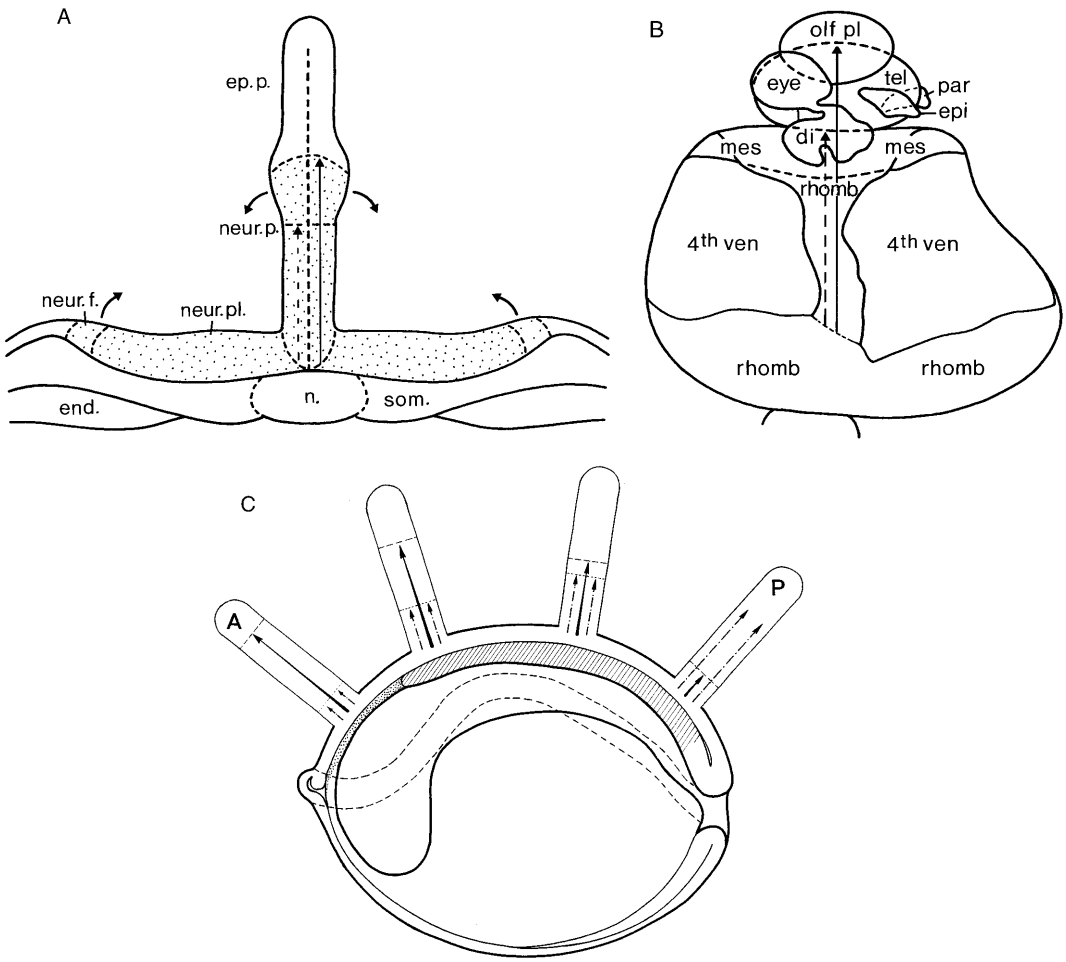

Figure 3. (*A*) Transverse section through anterior hindbrain region of urodele host neural plate and longitudinal section through implanted fold of competent ectoderm, showing proximo-distal extension of activating inductive influence (⟶) and of subsequent transforming influence (– – –›), spreading through ectodermal fold. ep.p. = epidermal portion of fold; n. = notochord; neur.f. = neural fold; neur.p. = neural portion of fold; neur.pl. = host neural plate; som. = somitic mesoderm. (*B*) Regional differentiation of ectodermal fold implanted in anterior hindbrain region of host embryo. di. = diencephalon; epi. = epiphysis; eye. = eye anlage; mes. = mesencephalon; olf.pl. = olfactory placode; rhomb. = rhombencephalon; tel. = telencephalon; 4th ven. = fourth ventricle. (*C*) Spreading of activating (⟶) and transforming (– – –›) inductive influences, in folds of competent ectoderm, implanted respectively in prechordal, anterior, middle and posterior notochordal regions of the host neural plate. A. = anterior; P. = posterior.

tion in the most lateral, outer region of the neural anlage [54]. Different cranio-caudal levels of the neural anlage show different intensities of the transformation process, being more pronounced in the more caudal regions. This seems theoretically at variance with a purely homoiogenetic propagation of the inducing signal. This pattern also holds for the activation process, whose initial intensity likewise determines the ultimate extent of neuralization. The intensity of the inductive action may in some unknown way influence the speed of signal propagation and/or the intensity of the transferred inductive signal, since the aging of the reacting cells seems unaffected.

We must return to the historical survey of the analysis of the neural induction process. Here I will discuss in particular the work of Toivonen and Saxén, who studied the inductions provoked by heterogeneous inducers acting upon competent gastrula ectoderm. They regularly observed the formation of both mesodermal and neural structures and formulated a double-gradient hypothesis, based upon a mesodermal and a neural inductive agent (Toivonen et al. [55] and Saxén and Toivonen [56]). Around the same time Yamada proposed [30] a third double-gradient model, which has much in common with my own activation-transformation hypothesis. He called the formation of anterior neural structures an expression of 'dorsalization' of the embryo (recall Yamada in ref. [1]) and the subsequent formation of more posterior neural structures 'caudalization'. Whereas dorsalization might be caused by a biochemical factor, caudalization should be the consequence of a mechanical factor leading to longitudinal stretching and convergence. We shall return to this at the end of this section. The controversy between the Finnish and the Dutch schools led to a large number of publications by Toivonen and Saxén [56, 57] defending

the existence of only two inductive agents, whereas I advocated the presence of three successive inductive actions in early development, namely a mesodermizing, a neuralizing and a transforming action, the first dealing with endo-mesoderm formation and second and third being concerned with neural development. These inductions should be based upon different inductive signals and should be acting during different periods of competence of the totipotent animal, 'ectodermal' moiety.

I will only mention here one of the elegant experiments performed by Toivonen and Saxén [57], in which they introduced a mesoderm-inducing heterogeneous inducer (bone marrow) and a neural-inducing heterogeneous inducer (liver) side by side in one and the same sandwich of competent gastrula ectoderm, and obtained a complete axial system with all its mesodermal and neural cranio-caudal structures. It is evident what their conclusion was, though it was not the correct one.

Interestingly, it was Toivonen and Saxén [58] who finally resolved the controversy between their and our hypothesis by combining disaggregated forebrain tissue from an early neurula with increasing amounts of chordamesoderm and actually observing the transformation of potential forebrain tissue into hindbrain and spinal cord. This proved the existence of the third, transforming inductive action.

Although midbrain, hindbrain and spinal cord represent transformed neural structures, transformation actually concerns the turning of potential forebrain into hindbrain and spinal cord, which have essentially the same basic regional structure. It could be demonstrated in extirpation experiments that midbrain actually develops due to a tertiary interaction between the fore- and hindbrain domains [59].

Holtfreter [37], studying axolotl exo-gastrulae, observed convergence-extension in the segregating caudal ectoderm bordering the caudal chordamesoderm. Keller et al. [60] showed that convergence-extension is actually based upon a *planar* inductive signal spreading into the ectoderm as well as into the neural tissue. I think we have to investigate further the active, mechanical role of convergence-extension in the neural transformation process, as already proposed by Yamada [30; see also 61].

Although the origin of the cranio-caudal pattern of the central nervous system is essentially based upon a *vertical* transfer of both the activating and the transforming signals from the endo-mesodermal archenteron roof into the overlying midline of the ectoderm, both signals propagate *homoiogenetically* during the subsequent medio-lateral extension of the neural anlage, a process which likewise occurs in attached ectodermal folds. Although these folds give the impression of a planar spreading of an antero-posterior pattern, it actually represents a spatial overlap of two successive homoiogenetic induction processes.

Tail somite formation from the most caudal region of the neural plate under the influence of the most caudal region of the mesodermal archenteron roof (see refs. 14–16) may actually represent the cumulative effect of a *vertical* as well as *planar* transforming action, the latter acting around the dorsal blastoporal lip.

Xenopus laevis, the South African clawed toad, is at present the universally used representative of the anuran Amphibia. It must be emphasized that *Xenopus* shows a rather extreme form of gastrulation and neurulation among the anuran Amphibia (see the Normal Table of *Xenopus laevis* by Nieuwkoop and Faber, 1956, 1967, 1975 and 1994 [34] and the corresponding Atlas by Hausen and Riebesell, 1990 [62], as well as ref. 35).

Nieuwkoop and Koster [33] have reinvestigated the regional neural induction process in *Xenopus laevis*, which according to Doniach et al. [63], Doniach [64] and Ruiz i Altaba [65–68] should fully or at least largely be based upon a *planar* spreading of inductive signals from the chordamesoderm into the adjacent ectoderm. There are, in my opinion, three serious objections to such a notion:

1) Neural induction in the Urodeles is essentially due to *vertical* induction between the invaginating archenteron roof and the overlying competent ectoderm, is well documented.

2) *Xenopus* exo-gastrulae as well as Keller explants and sandwiches show opposing cranio-caudal patterns in the neural structure found in the ectodermal moiety and in the evaginated endo- and mesoderm. This is incompatible with *planar* signalling, which always runs 'down hill'. With *planar* induction the cranio-caudal pattern of the neural structures ought to be continuous with that of the chordamesoderm.

3) Total exo-gastrulae of axolotl [37] show a complete segregation of the ectoderm from the endo- and mesoderm without the formation of any neural structures in the ectodermal moiety, pleading convincingly against a *planar* spreading of the neuralizing signal. The explanation is actually that in the double-layered *Xenopus* gastrula, mesoderm formation is restricted to the inner, sensorial layer [35, 69, 70]. *Xenopus* has a fully *internal* marginal zone. Moreover, mesoderm involution takes place independently of archenteron invagination and starts very early in development. It has already markedly advanced at an early gastrula stage and has reached the bordering ectoderm, inducing neural structures by *vertical* interaction, before any archenteron invagination has taken place [33]. This fully explains the cranio-caudal patterning of the neural structures found in Keller explants and sandwiches (see diagrams in Keller, ref. 70). In exo-gastrulae a similar process occurs. The prechordal mesoderm migrates normally into the ectodermal moiety before the evagination of the endo- and mesoderm starts. The mesoderm actually becomes sub-

divided into the involuting prechordal mesoderm and the evaginating chordamesoderm and endoderm, which leads to opposing cranio-caudal patterns in the neural and endo-mesodermal structures [33].

The *Xenopus* exo-gastrulae and Keller explants and sandwiches show an interesting additional feature. The neural structures of forebrain character, formed in the ectodermal moiety, are partially transformed into hindbrain and spinal cord in the absence of any notochordal or somitic structures. This can only be due to a *planar* spreading of the transforming signal from the adjacent chordamesoderm. This is to be expected since the transforming signal is maximal in the caudal chordamesoderm, where an activating action is nearly absent [48]. It must be emphasized that such a *planar* spreading of the transforming signal does not, however, play any appreciable role in *normal* development, since involution of the prechordal mesoderm is directly followed by the involution of the notochordal mesoderm, leading to a *vertical* transfer of the transforming signal. The final conclusion therefore is that in *Xenopus laevis* the cranio-caudal patterning of the neural anlage is essentially based upon *vertical* signalling, like in the Urodeles (see the remark on page 306 on tail somite formation).

In the case of neural induction we have had a 'devil's advocate' in the person of M. Jacobson (Jacobson and Horose [71] and Jacobson [72–75]), who completely denied neural induction and advocated self-differentiation of preformed cells in particular cell-lineages, simply ignoring the overwhelming evidence for epigenetic development of the CNS. He had, however, to withdraw his statements [76].

Endo-mesoderm and neural induction in other vertebrate groups

Among the other vertebrates, mesoderm induction has particularly been studied in birds. Here my former pupil Mrs. Eyal-Giladi and her coworkers have done excellent work, carefully summarized in her 1991 review [77].

Although some interesting work has been done on the development of fishes, which belong to the Anamnia, no real analysis of the underlying induction processes has so far been undertaken. Here, I only wish to refer to the Russian work on sturgeons [42, 78], in which early, holoblastic development strongly resembles that of urodelian amphibians. There is moreover the careful analysis of germ layer formation in teleosts by Ballard [79–81], suggesting in situ formation of the mesoderm by delamination instead of invagination. The role of ingression along the periphery of the teleost blastoderm is, however, stressed by Trinkaus [82, 83] and others (see also Pasteels, ref. 84). It must be emphasized that the extension of the so-called gastrulation process with the overgrowth of the entire yolk mass is rather misleading, since the regional organisation of the axial mesoderm and of the nervous system is already completed at an earlier stage.

Experimental work on the early development of the reptiles was particularly hampered by the restricted availability of early developmental stages. I would like, however, to mention the work of Pasteels [85–87] on reptilian gastrulation.

Amniote development is characterized by primitive streak formation, which is essentially comparable with the gastrulation process in the Anamnia. The first adequate Normal Table of the chick was made by Hamburger and Hamilton in 1951 [88], covering the development from primitive streak formation up to hatching. It has been the merit of Eyal-Giladi and Kochav [89] and Kochav et al. [90] to analyse the very early development of the chick embryo and to divide the development from fertilization to primitive streak formation into XIV stages, covering the first 20 hours of intra-uterine development.

I think it is desirable to describe briefly early avian development in order to emphasize the strong parallels and the differences with amphibian development. Fertilization of the chick egg takes place in the infundibulum. During descent through the oviduct the huge egg, which shows a pronounced animal-vegetal polarity by the uneven distribution of the yolk, becomes surrounded by successive layers of albumen and subsequently by the shell membranes during their rotation along the egg's long axis in the oviduct [91]. The small, plasma-rich blastoderm, situated on top of the huge yolk, shows perpendicular cleavage during the first 5–6 cell divisions. After 2 hours a subgerminal cavity appears between the blastoderm and the yolk mass, while tangential cleavages also begin to occur. After 11 hours, the opaque blastodermal disc has become 5–6 cells thick. Cleavage is characterized by the accumulation of glycogen and by the formation of pre-nucleoli at stage IV and of mature nucleoli, producing rRNA, at stage VI. Determination of bilateral symmetry occurs between the 14th and the 16th hour of egg rotation [92]. Due to the presence of the chalazae the egg is slightly turned upon its side when shell and albumen are rotated around the stationary yolk mass, as a consequence of gravity acting upon the strongly polarized egg. The oblique position of the blastoderm leads to formation of the embryonic antero-posterior axis perpendicular to the longitudinal axis of the egg with its posterior pole at the uppermost end of the blastoderm [92, 93].

The formation of the area pellucida starts at stage VII by the shedding of deep cells into the subgerminal cavity. As a consequence the area pellucida thins to a one cell thick layer. It is surrounded by a thick, opaque area opaca [94].

The formation of the hypoblast occurs partly by polyingression of cells from the area pellucida epiblast and partly by postero-anterior migration of cells from its

thickened posterior edge, called 'Koller's sickle'. Vakaet [95] called the cellular blastodermal wall the 'germ wall' and Eyal-Giladi [96] unfortunately called it the 'marginal zone'.[3] Whereas Eyal-Giladi uses the term 'primary' hypoblast for both phenomena, Canning and Stern [97] and Stern and Canning [98] distinguish between 'primary' hypoblast formation by polyingression and 'secondary' hypoblast formation from Koller's sickle (see also Vakaet, ref. 99).

There is still another point of controversy between Stern and Eyal-Giladi. The stage XIII chick blastoderm consists of an upper epiblast and a lower hypoblast layer, separated by a narrow slit, called 'blastocoel' by Eyal-Giladi. Unfortunately, Stern et al. [100] considers the space between the blastoderm and the yolk mass equivalent to the amphibian 'blastocoel', which Kochav and Eyal-Giladi [93], in my opinion correctly, call the 'subgerminal cavity'. Eyal-Giladi et al. [101] could demonstrate that the stage XIII blastoderm actually consists of two separate layers with clearly different cellular properties.

We now come to the main amniote characteristic, namely 'primitive streak formation'. We must give Waddington the honour of having performed the first hypoblast translocation experiments [102, 103], which pointed to a possible role of the hypoblast in primitive streak formation. New [104] developed the in vitro technique for the chick embryo. Spratt and Haas [105, 106] showed that blocking the fountain-like cell movements in the hypoblast by cultivation of the isolated blastoderm upon a solid substrate actually interferes with primitive streak formation. According to [107–110], the mesodermal, postero-anteriorly extending primitive streak is caused by an inductive action of the expanding hypoblast upon the overlying totipotent epiblast. Cells from the germ wall are added to both layers, particularly at the posterior Koller's sickle side. These conclusions are based upon a large number of recombination and translocation experiments, which cannot be separately discussed in this review, but which are summarized in Eyal-Giladi's excellent review [77].

The axis-inducing capacity of the hypoblast, which starts at stage XIII, holds essentially for the entire hypoblast boundary, where hypoblast and epiblast are in direct contact with each other, although showing a strong dominance by its posterior region. The conclusion is that primitive streak formation actually represents the formation of the axial mesoderm [107, 111–113].

Azar and Eyal-Giladi [114] repeated Waddington's experiments [102, 103] by combining hypoblast and epiblast from different stages in different mutual orientations. They found that both the inductive capacity of the hypoblast ànd the corresponding competence of the epiblast are maximal at stage XIII. They observed a more rapid fall-off of the competence of the epiblast than of the inductive capacity of the hypoblast.

Mitrani and Eyal-Giladi also studied the intrinsic polarity of epi- and hypoblast by combining intact epi- and hypoblast with dis- and reaggregated hypo- or epiblasts, thus abolishing the intrinsic polarity in the latter [112, 115]. They could show that both layers have an intrinsic antero-posterior polarity, but that that of the epiblast is the most essential. Contrary to the situation in the urodelian amphibians, where no polarity could be demonstrated in the epidermal layer, the anuran *Xenopus* shows dorso-ventral differences in both endo-mesodermal and neural competences.

We must call attention to the fact that primitive streak formation not only represents mesoderm formation, but also involves the formation of the entire embryonic endoderm. This so-called 'definitive endoderm' ingresses through the primitive streak and enters the hypoblast, as demonstrated by a large number of authors [95, 116–126]. As with the amphibians we must therefore speak of *endo-mesoderm* induction.

We must briefly discuss the contrary opinion of Stern and Canning [97, 98] acting as devil's advocate by denying endo-mesoderm induction on the basis of observations using monoclonal HNK-1 antibody and complement for marking and ablation. They advocate mesoderm formation from preformed cellular elements, namely from the polyingressing cells of the epiblast. Besides the fact that several other explanations can be proposed for their observations, they ignore the overwhelming experimental evidence provided by Eyal-Giladi and coworkers supporting endo-mesoderm induction in the chick embryo (see above).

The conclusion must therefore be that, though endo-mesoderm formation in the primitive streak of the avian embryo differs markedly from endo-mesoderm formation in the amphibians, the essential character of the process is that it is due to an inductive action from the underlying endoderm (hypoblast) upon the totipotent overlying ectoderm (epiblast) which holds for both Anamnia and Amniotes. It is, moreover, highly improbable that mesoderm formation could be preformistic in the Amniotes, which have doubtless evolved via the reptiles from the anamnian amphibians, where epigenetic endo-mesoderm formation seems to be firmly documented.

Though the ingression of endoderm and mesoderm through the primitive streak is well documented (see above), the regional and temporal aspects of the subsequent neural induction process are barely studied. The prechordal mesoderm, which ingresses after the anterior endoderm through Henson's node and migrates anteriorly, is thought to be responsible for forebrain induc-

[3] The 'marginal zone' represents the inner rim of the blastodermal wall, but is not equivalent to the presumptive endo-mesodermal marginal zone of the amphibian gastrula.

tion in the overlying epiblast. The induction of mid- and hindbrain and spinal cord probably occurs during the antero-posterior regression of the primitive streak, so that the successive antero-posterior regions of both the axial mesoderm and the neural anlage are laid down successively in an antero-posterior sequence. One gets the impression that the sequentially induced neuroderm follows the antero-posterior pattern of the underlying mesoderm, as suggested in the classical experiments of Spemann [47] for the amphibian embryo. Hara [128] made the first pioneering experiment on the regional induction of the CNS in the chick. In recombinates with competent ectoderm he observed the induction of more anterior structures than corresponded with the region of tested axial mesoderm. These results could be satisfactorily explained with Nieuwkoop's activation-transformation hypothesis. Gallera [129] studied the regression of the inductive power of the mesoderm of successive stages (see also ref. 130). Storey et al. [127] concluded recently that the induction of the head region of the chick CNS may be radically different from that of the trunk region. Fold implantation experiments by Nieuwkoop et al. [49] demonstrated clearly the existence of two inducing signals, acting successively and showing a different antero-posterior distribution along the embryonic axis. There is, in my opinion, no valid argument that the same principle does not likewise hold for the avian embryo and for all the Amniota.

Severe technical restrictions hampered the analysis of early mammalian development, due to the early implantation into the uterine endometrium. In vitro culture techniques of pre-implantation stages have been developed by several authors, but these techniques allow only a short culture period. Experimentation with the very small, yolk-poor mammalian embryo is moreover very demanding. I would like to mention here the painstaking marking experiments by Lawson et al. [130a, 131] and Lawson and Pederson [132, 133], demonstrating early cellular migration in the U-shaped blastoderm before and during primitive streak formation.

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