SPECIAL REVIEW

Neural induction*

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* Dedicated to Sulo Toivonen, esteemed teacher, collaborator and friend, on the occasion of his 80th birthday.

Introduction

Neural induction and the research on its nature form an exceptional chapter in the history of modern biology. After Hans Spemann and his school made their pioneering experiments and observations in the twenties and opened an entirely new avenue for developmental biologists, practically every leading embryologist tackled this problem in the thirties and forties. Many devoted their entire scientific careers to this problem. Yet we are still seeking anwers to most of the central questions related to neural induction: how does the target tissue, the presumptive neuroectoderm, gain its responsiveness, and how does the organizer create its inductive capacity? Are the inductive signals carried by specific signal molecules, and if so, where are they synthesized and how are they transmitted to their target? And finally, what are these molecules, what is their exact target site and mode of action while converting undifferentiated ectodermal cells into neuroblasts and ultimately into organized structures of the different regions of the central nervous system?

In recent years, a great variety of biological interactions have been elucidated in detail at the cellular and molecular levels, whereas neural induction has remained an enigma. In 1962 we expressed the naive wish that the early sixties would mark the introduction of a new period of research in embryonic induction (Saxén and Toivonen, 1962). But sixteen years later we still had to ask, «Why do the scientists investigating embryonic induction lag behind their brilliant colleagues in many other fields of biology, in which the sixties and the seventies have witnessed many great victories» (Saxén *et al.*, 1978). This question has also remained unanswered. Consequently, a review of the topic is still merely a list of findings and observations, many of which have been made by scientists who use the most sophisticated modern technology, but fail to construct a unifying concept of the process of neural induction. To understand this failure and to appreciate the technical and intellectual difficulties in the field, the reader is here offered a rather extensive historical background to the problem. For details of the past history and development in the seventies, the reader is referred to the following monographs and reviews: (Spemann, 1936; Holtfreter and Hamburger, 1955; Saxén and Toivonen, 1962, 1986; Nakamura and Toivonen, 1978; Witkowski, 1985).

A review of the history of neural induction and of the development of the classic concepts seems proper in 1989, when a new era in the research in this field can be foreseen. Today's molecular technology has provided developmental biologists with many probes for examining the early postinductive events and for following gene activation in the induced cells. This line of research will most probably soon unravel many of the molecular events behind neural induction, a mystery of the past seven decades. For these recent developments, the reader is referred to the excellent review by Gurdon (1987).

Historical Background

In the 19th century, embryology, like many other fields of biomedicine, was characterized by a descriptive approach, and the normal events of ontogeny were mapped out in painstaking detail. Not until the end of the century did scientists become aware of the importance of asking questions related to the *control of development.* «Why» instead of «how». Perhaps the most influential of these early theories of determination was that proposed by Weismann in 1892 (Weismann, 1892) and known as the germ line theory. According to this theory, ontogeny was already determined in the early zygote —a modification of the classic preformation theory— and after this stage every cell followed its presumptive fate. Though incorrect in its basic doctrine, this hypothesis seemed to be a stimulus for the first experimental approach to the problem of determination, the process which «initiates a specific pathway of development by singling it out from various possibilities for which the cellular system is competent» (Hadorn, 1965).

In the early 20th century three milestones marked the development of new ideas and basic concepts: results in embryo-dividing experiments, detection of the organizer, and demonstration of the regional nature of the latter.

Totipotency and determination

According to Weismann's doctrine, separation of the blastomers at the two-cell stage should lead to the formation of two half-embryos. A simple experiment, first devised by Endres in 1895 (Endres, 1895) and still included in many elementary courses on embryology, involves the separation of these two cells with a gradually tightening hair-loop. As a consequence, two identical embryos develop showing that the two cells are toti- or equipotent, i.e., they both possess the regulative capacity to form entire organisms. The same experiment can also be performed successfully during later blastula and gastrula stages, provided that certain regulatory parts of the embryo are evenly distributed between the halves (Spemann, 1903). The conclusion is apparent: cells retain all their developmental options throughout early embryonic stages and remain *totipotent.*

The first definite demonstration of irreversible determination of an embryonic cell population came from Spemann's laboratory in 1916-1918 (Spemann, 1916, 1918). The experiment is illustrated in Fig. 1: when a fragment from the presumptive neural plate of an early gastrula is dissected and transplanted on the belly side of another gastrula of the same stage, the transplant will develop according to its new environment and contribute to the belly epidermis. It is thus still flexible, and its development can be regulated. However, when an identical transplantation is performed at an advanced gastrula stage, a secondary neural plate is formed from the transplant which has now been

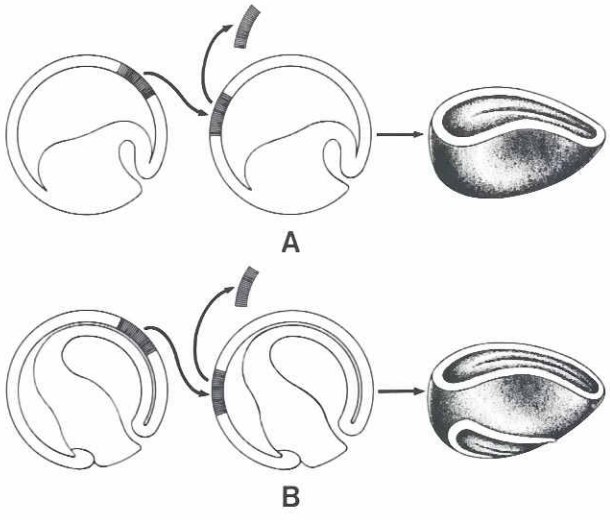

Fig. 1. *Schema of Spemann's transplantation experiment (1916,1918) demonstrating the determination of the presumptive neuroectoderm during gastrulation.*

irreversibly determined and cannot be regulated by its new ventral milieu. This determination can thus be timed precisely as having taken place during the mid-gastrula stage. The mechanisms of the determination, however, were not understood until the experiment described below was carried out.

Detection of the organizer

The key experiment performed by Spemann's student Hilde Mangold can be dated to May 8, 1921, but it was published much later, in 1924 (Spemann and Mangold, 1924) (Fig. 2). A part of the invaginating blastoporal lip from an unpigmented gastrula of *Triturus cristatus* was transplanted into the ventral ectoderm of a pigmented *Triturus vulgaris* gastrula. As a result, a secondary neural plate developed on the belly side of the host, but it was mostly pigmented, indicating that it must have been of host origin (most other secondary structures in the host as well as a small central part of the neural plate proved to be of donor origin). The apparent conclusion was that the transplant, the blastoporal lip, was responsible for the determination (induction) of the multipotent host ectoderm. The observations also explain the results of the earlier transplantation experiment (Fig. 1) demonstrating the totipotency of the early ectoderm and the determination of the neural plate after invagination by the inductively active blastoporal lip.

Sixty years later this pioneering experiment became the object of criticism, and its validity was disputed by Jacobson (Jacobson, 1982), who had traced the neural cells back to a blastula stage. Accordingly, the ectodermal cells converted into neural tube cells might have already been predetermined or cells with a neurogenic bias might have migrated into the secondary structures. After this criticism was published in 1982 the problem was discussed at a conference in May 1983, where we marshalled the «historic» evidence to refute Jacobson's views (Saxén and Toivonen, 1986). Very soon direct experimental evidence based on modern technology invalidated the criticism of Jacobson and gave additional support to the original explanation of Spemann (Gimlich and Cooke, 1983; Smith, 1983; Jacobson, 1984; Slack *et al.*, 1984; Smith *et al.*, 1985). We will present this evidence in a later context.

Demonstration of the regional nature of the organizer

After the development of the implantation technique (below), it became relatively easy to map out the inductor tissue. The important discovery was made that there is a difference between young and old gastrulas in the inductive action of the blastopore lip (Fig. 3). Hence, there were regional, qualitative differences in the inductive action of the invaginating blastopore tissue. A more detailed analysis of these regional features was performed by O. Mangold in 1933 (Mangold, 1933). He dissected the archenteron roof of a young neurula into four transversal segments and tested the inductive capacity of each by his newly developed implantation method (Fig. 11). The fragments were implanted into the blastocoele of a young gastrula where, during subsequent gastrulation, they were pressed against the ventral ectoderm, the target. The results summarized in Fig. 4 illustrate the macroscopic appearance of the secondary structures obtained by the different territories of the inductor. Microscopic analysis showed that the secondary (neural) structures constituted a series from the most cranial parts of the CNS to the spinal cord induced by the most caudal quarter of the inductor tissue. This regional specificity of the inductor has since been repeatedly demonstrated by basically the same experimental schema (Ter Horst, 1948; Sala, 1955; Leussink, 1970).

The confused thirties

The three landmarks reviewed above laid the framework and basis for the future analysis of the induction process. These

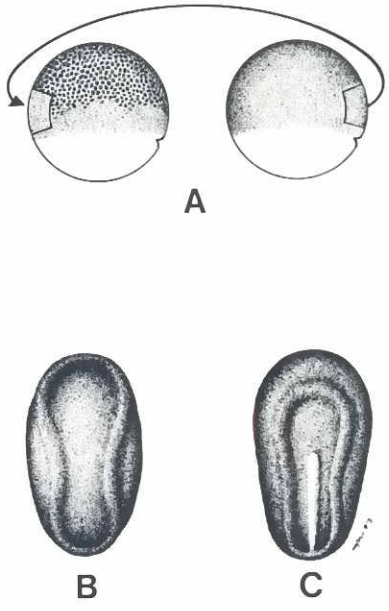

Fig. 2. *Schema of the heteroplastic transplantation experiment by Hilde Mangold (Spemann and Mangold, 1924). The dorsal lip of a pale* Triturus cristatus *gastrula is transplanted into the ventral ectoderm of a pigmented* Triturus vulgaris *gastrula. The secondary neural plate on the belly side of the host consists mainly of pigmented cells induced by the transplant (Saxén and Toivonen, 1986).*

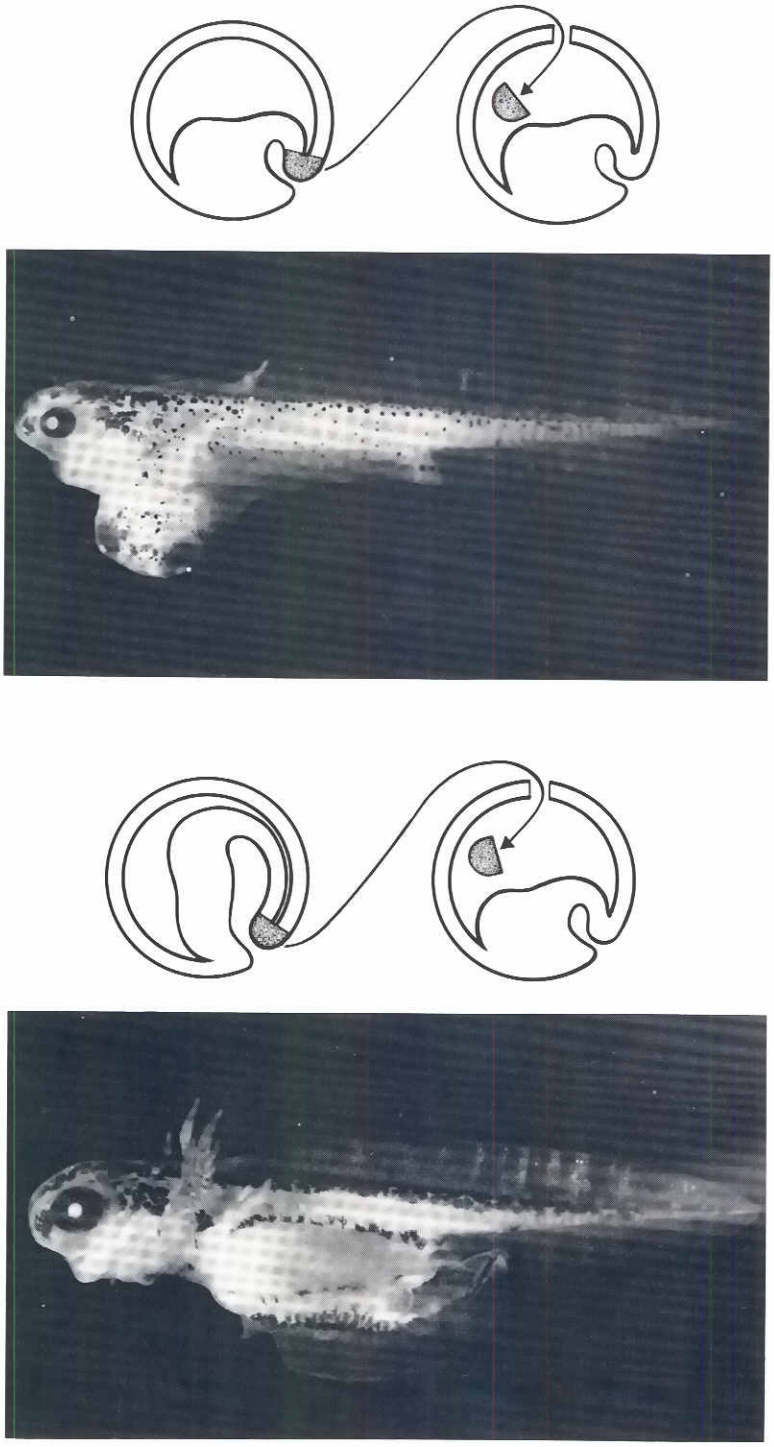

Fig. 3. *The inducing action of the dorsal lip of a young gastrula (above) and of the same region dissected from an advanced gastrula stage («head» and «tail» inductions).*

experimental results introduced the concepts of totipotency and regulative development, and the concept of an inductive interaction between the two tissues. The primary organization center with qualitatively different regional capacity had also been detected. Unfortunately, this solid work and the great discoveries also laid the basis for much confusion, contradictions and misconceptions.

In the thirties, developmental biologists concentrated on two obvious problems, the nature of the inductor(s) and the mode of establishing the regionality of the inductor (and, consequently, of the neural plate).

Search for chemically specified inductors

The final landmark in the pioneering work of Spemann's school was the demonstration of the chemical nature of induction. In 1932 Bautzmann, Holtfreter, Spemann and Mangold

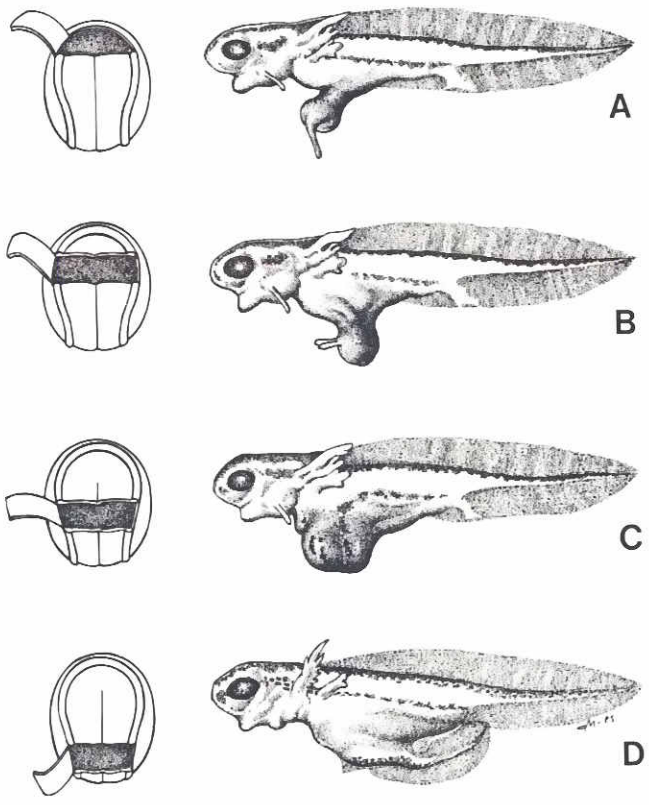

Fig.4. *Schema and results of Otto Mangold's (1933) experiment demonstrating the varying effect (regionality) of different territories of the inductor tissue (Saxén and Toivonen, 1986).*

(Bautzmann *et al.*, 1932) performed implantation experiments by using inductors isolated from an embryo but pretreated by various devitalizing means: crushing, freezing, heating, and treatment with various devitalizing chemicals. None of these treatments seemed to inactivate the inductive capacity of the implanted fragments. Their inductive capacity, however, had changed. Instead of inducing more or less harmonious tissue complexes, their action now led to chaotic formations. In this connection, Holtfreter (Bautzmann *et al.*, 1932) showed that in addition to the embryonic tissues, both living and devitalized adult tissues showed inductive activity. Moreover, the inducing factor(s) could be extracted from the tissues. As shown by Mangold (Bautzmann *et al.*, 1932), if an inductively active fragment of the embryo was incubated on a piece of agar and then completely removed, the implanted agar exhibited an inductive action.

The above results led the authors to two fundamental conclusions: induction is transmitted by chemical compounds, and the compounds are diffusible. As we shall see, both conclusions were basically correct, but required several subsequent specifications and additions. They led, however, to a decades-long, somewhat unfortunate period in the search for specific compounds responsible for induction. This era has been repeatedly summarized (Brachet, 1950; Needham, 1950; Saxén and Toivonen, 1962), and only some of the most important features will be listed below.

A German and a British school of embryologists were involved in this research, and both followed the same strategy: purified factors to be tested were either implanted in pellet form into the blastocoele or the competent ectoderm was exposed to these compounds in «sandwich»-type experiments (Fig. 11). From the very beginning, the two schools were able to report positive results, but the compounds yielding induction varied greatly. The following incomplete list gives a general idea of these «inductors»: sterols isolated from amphibian neurulae (Needham *et al.*, 1934), several organic acids (Wehmeier, 1934), inorganic, inert compounds like kaolin and silicon (Needham, 1950), and some carcinogenic hydrocarbons (Waddington, 1938). At the same time it became evident that not only living devitalized embryonic tissues could act as inductors, but also a great variety of tissues from adult amphibians and those from other species led to induction when brought into contact with the amphibian competent ectoderm (Bautzmann, 1932; Holtfreter, 1933, 1934).

The wide distribution of inductive agents in the animal (and plant) kingdoms and, especially, the wide range of active compounds that had no detectable common denominators, naturally led to confusion, frustration and disbelief in the entire basic phenomenon. Many of these results can be better understood in retrospect, and many can be considered experimental artifacts caused by intrinsic developmental capacities of the ectoderm. It seems clear today that these confusing results can mostly be explained through traumatization of the ectodermal cells. A variety of treatments without any «specificity» would lead to this: heat shock, high pH, hypotonic medium, treatment with ammonia, mechanical damage, etc. (Holtfreter, 1944, 1945; Holtfreter and Hamburger, 1955; Saxén and Toivonen, 1962). The mechanism of this phenomenon has been extensively discussed but not fully clarified. In modern terms, these data may show that the presumptive neuroectoderm has, during its prehistory, created a neurogenic bias which can be released by damage to the cell. In fact, John and collaborators (John *et al.*, 1984) have demonstrated the presence of a neural inducing factor in the gastrula ectoderm. Their fractionation results suggest that it is present in a masked, complex form from which it can be released by various treatments like homogenization and ethanol exposure. The importance of this «autoneuralization» in normal development is questionable, but experimentalists should be aware of it in order not to repeat the «chemical Odyssey of the 1930's» (Hermann, 1960). From the standpoint of science history, the epoch is interesting, but its unfortunate consequence was that many prominent schools of embryologists lost interest in the problem and dropped it (Saxén and Toivonen, 1986).

The first fractionation experiments, the other line of research, were started much later by Toivonen and Kuusi (1948), who were soon followed by many others. Especially faithful to this approach has been the group of Tiedemann, and most of our present knowledge of the chemistry of inductors and their mode of action is based on his work over the past 25 years (Tiedemann 1976, 1978, 1981, 1982, 1984). But before summarizing Tiedemann's findings, we must learn more about the biology of «primary induction», the early determination of the gastrula ectoderm. This brings us back to the confused thirties.

Quantitative versus qualitative hypothesis

Autoneuralization leads to neural differentiation of the presumptive neural ectoderm, but only to the formation of cranial neural structures and their derivatives, forebrain, eyes, etc.

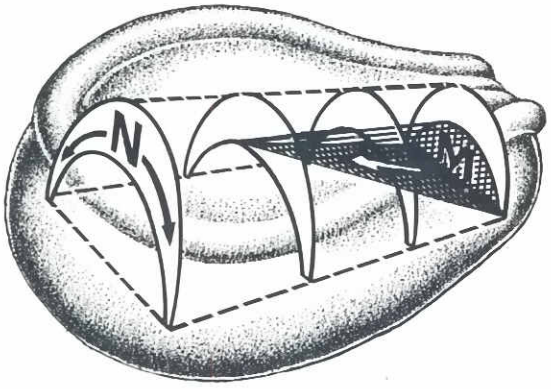

Fig. 5. *The «double-gradient theory» of Toivonen and Saxén (1955) as illustrated by Ede (1978).*

(«archencephalic» structures). However, both the normal inductor, the blastoporal lip, and many heterogenous inductors can convert the ectodermal target tissue into practically any ectodermal, mesodermal or endodermal derivatives, and frequently a whole, well-organized secondary embryo can be induced from this tissue (Fig. 3). This regionality and its physiological basis became the other main issue of the thirties and soon two seemingly opposite theories involved.

The quantitative theory of the Belgian school postulated the existence of two initial gradients in the embryo, a dorsoventral and a caudocranial gradient. This double gradient determined the production of the actual morphogenetic factor, the «organicine». As a consequence of the double gradient control of synthesis, the distribution of organicine in the embryo was uneven and formed, in fact, a third gradient. This concentraton of the organicine and the different threshold values of the totipotent target cells then determined the ultimate fate of the latter (Dalcq and Pasteels, 1937, 1938).

Meanwhile, extensive implantation experiments with heterogenous inductors led Chuang (1938, 1940) and Toivonen (1938, 1940) independently to the qualitative theory postulating several active compounds exerting different inductive actions. These were later defined by Lehmann (1945) as «archencephalic», «deuterencephalic» and «spinocaudal», depending on the craniocaudal territory of the induced structures. With elements from both basic hypotheses, Lehmann (1950) constructed his model according to which the regional characteristics of the CNS were determined by two inductive principles, the archencephalic and the spinal. These two could act either separately or jointly in different ratios.

It became possible to test this hypothesis after Toivonen (1953) had found a heterogeneous tissue (guinea pig bone marrow) expressing an almost pure mesodermal inductive action. According to the rephrased hypothesis, the regional nature of the inductor would be determined by two active principles: one leading to mesodermalization of the ectoderm and the other which would result in the induction of cranial neural structures only. The CNS derivatives of the hindbrain region and of the spinal cord would, consequently, be determined by a combined action of these two inductors. The first combination experiments seemed to support this hypothesis (Toivonen and Saxén, 1955). When a piece of guinea pig liver tissue (neuralizing inductor) and a fragment of bone marrow from the same source (mesodermalizing inductor) were implanted together into the blastocoele of a gastrula, the secondary structures showed a new spectrum. In addition to some cranial neural structures and mesodermal derivatives, caudal neural structures were regularly created. Based on these results, we then formulated a «double-gradient theory» illustrated in Fig. 5. According to the theory, the neuralizing factor forms a medio-lateral gradient while the mesodermalizing factor is distributed in the form of a cranio-caudal gradient. Consequently, all ratios of the two hypothetic factors could be obtained within the inductor tissue.

This hypothesis incorporated elements not only from the classic gradient ideology dating back to the 19th century but

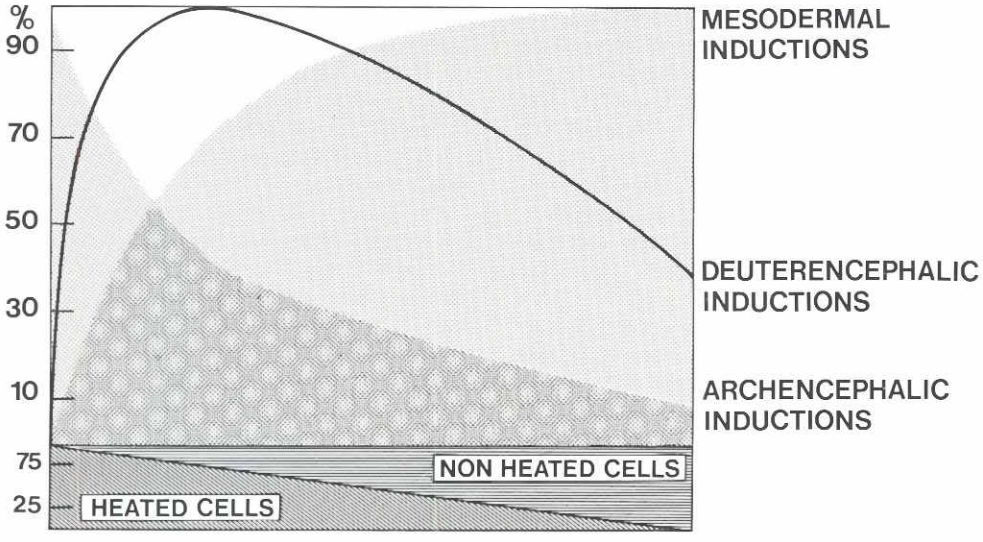

Fig. 6. *Results of implantation experiments in which the inductor consisted of various ratios of heated and non-heated HeLa cells. Different types of induction given as percentages of total (Saxén and Toivonen, 1961).*

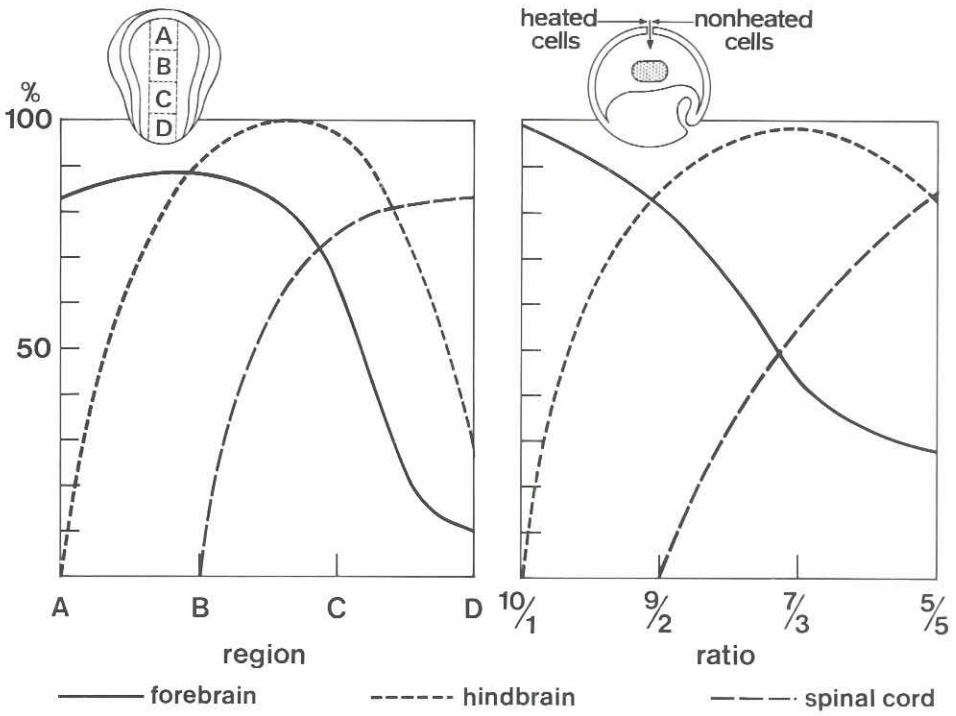

Fig. 7. *Comparison of the results illustrated in Fig. 6 with those obtained by Sala (1955) who examined the inductive effect of the different territories of the archenteron roof.*

also from the more recent ideas of Dalcq and Pasteels (1937, 1938) as well as those of Lehmann (1950). As long as the actual signal substances carrying the inductive capacity were unknown, only an indirect experiment to test the double gradient hypothesis could be devised. HeLa cells, a continuous cell line derived from a neoplastic tissue, had been shown to be potent inductors of the mixed type (central and caudal regions of the CNS plus mesodermal derivatives), but after short-term heat treatment they only retained the capacity to induce forebrain structures (Saxén and Toivonen, 1958). It was now possible to use these two cell preparations in various ratios to mimic the postulated effect of various ratios of neural/mesodermal inductive factors. The results illustrated in Fig. 6 were as expected, and the regional specific normal inductor explored by Mangold (1933) had thus been artificially reconstituted (Saxén and Toivonen, 1961). In fact, when these results were superimposed onto the more accurate mapping results of Sala (1955), a striking similarity was observed (Fig. 7).

Meanwhile Nieuwkoop and his group (1952) had performed extensive, well-designed experiments by using folds of the competent ectoderm as test material (Fig. 8). Briefly, when such folds were inserted at various cranio-caudal levels of the host, the proximal parts of the fold showed structures corresponding to the level of insertion, while the distal part of the extra ectoderm developed into neural structures of a more cranial level than that of the insertion. Similar gradients of secondary structures were also obtained in flattened explants of ectoderm where an inductor was placed in the center (Nieuwkoop and Nigtevecht, 1954). Based on such repeated observations, Nieuwkoop formulated his «activation-transformation» theory (Nieuwkoop *et al.*, 1955, 1958). Accordingly the two-step process initiated by the inductor first leads to activation of the presumptive neuroectoderm. This is expressed as a formation of forebrain derivatives, if not followed by a wave of «transformation» which is «a quantitative process through which, with increasing strength of action, progressively more caudal differentiative tendencies appear» (Nieuwkoop, 1955).

These two theories of the 1950s, the activation-transformation hypothesis of Nieuwkoop and our double-gradient hypothesis, were, in fact, not very different. Subsequent experiments were to combine the essential elements from both to form the present model. As this is still considered the biological basis of present experimentation, let us close our historical overview by presenting two sets of experiments concluding the long series of investigations started in the thirties.

Biological framework for neural induction

To examine the time factor as related to the double gradient theory, the following experiment was performed (Saxén *et al.*, 1964) (Fig. 9): ectodermal sandwiches were exposed either to neuralizing inductor or to a spinocaudal, predominantly mesodermalizing inductor. With the knowledge that both inductions should be completed within 24 h (Toivonen, 1958; Johnen, 1964; Ohara, 1981), the inductors were then removed. The ectoderm —which had now lost its competence (see below)— was disaggregated into a single-cell suspension and subcultured after reaggregation to follow its differentiation. Three types of subcultures were monitored (those of the two above-mentioned cell suspensions separately and those where the two were combined and thoroughly mixed at 24h). Should the third type of disaggregated/reaggregated cultures show a spectrum of structures differing from the two unmixed aggregates, the

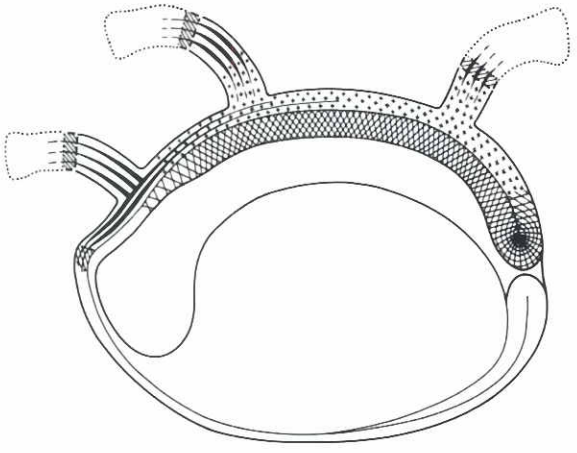

Fig. 8. *Summary of the results obtained by Nieuwkoop* et al. *(1952) with the ectodermal fold technique: folds of competent ectoderm were implanted at different levels of a young neurula. The distal region of the folds remains undifferentiated, the intermediate region (hatched) shows mesectodermal differentiation and the proximal part neural structures with hindbrain derivatives. The hypothesis postulates an initial activation (thick lines) followed by a wave of transformation leading to the more caudal neural structures (crosses) (Saxén and Toivonen, 1962).*

conclusion would be that they had been determined during a second step of interaction between the two types of induced cells. The expected result was obtained (Fig. 9), and it was now possible to rephrase the double-gradient hypothesis.

During an initial step of induction, the multipotent ectoderm is determined either in the neural or the mesodermal direction. The regionality of the CNS is then determined by a second- step interaction between the neuralized and mesodermalized cells.

This concept and its supporting evidence were brought closer to the *in vivo* situation by dissecting the interactants from normal neurula-stage embryos instead of using artificially induced tissues (Toivonen and Saxén, 1968). A piece of the anterior neural plate (prospective forebrain) was combined to a piece of caudal axial mesoderm, which were both in a disaggregated state. The method allowed a pseudoquantitative approach as different numbers of these fragments could be disaggregated and combined.

As predicted, the anterior neural tissue cultured alone gave rise to forebrain derivatives only, but when increasing amounts of mesodermal cells were added, the neural structures gradually became more caudal, and the series produced an entire array of different regions of the CNS (Fig. 10). The results indicate that neuralized cells at the early neurula stage, though already committed to becoming neuroblasts, are still competent to contribute to the various regions of the CNS. Their ultimate fate is thus regulated by an interaction with the cells of the axial mesoderm. This is a quantitative process in which the amount of mesoderm defines the regionality of the overlaying neural plate.

This theory postulating a double gradient and a two-step process includes elements from many previous hypotheses and is not far from Nieuwkoop's activation-transformation hypothesis. Japanese scientists have repeatedly emphasized the role of mesoderm in determining the shape, size and regionality of the neural plate (Takaya, 1956a, b, 1959, 1977, 1978). Compared with our original hypothesis illustrated in Fig. 5, the two-step concept has undergone an important modification: the model of two active principles acting jointly apparently explains our results, but might not adequately reflect the *in vivo* conditions. In the experimental model-system, we examine artificially induced (neuralized and mesodermalized) ectodermal cells after their second-step interactions, while *in vivo*, these interactants represent different cell lineages determined prior to and during gastrulation. Thus, the chain of interactive events ultimately leading to the regional differentiation of the CNS can be fractionated into at least three stages: (1) predetermination of the presumptive neuroectoderm and the determination of the inductor (mesoderm) prior to gastrulation, (2) actual «neural induction»

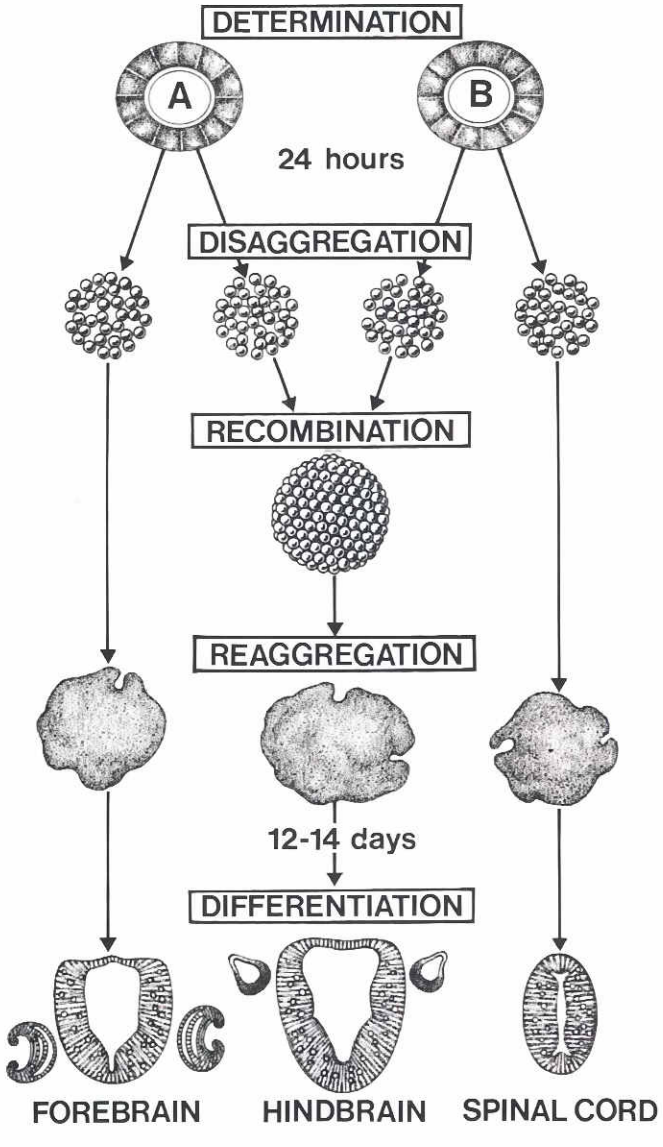

Fig. 9. *Schema of the experiment of Saxén* et al. *(1964) demonstrating the two-step mode of induction. After an initial 24-h induction with a neuralizing or spinocaudal inductor, the inductors were removed, and the disaggregated ectodermal cells were subcultured either separately or after complete mixing. The latter show a new spectrum of differentiating structures determined by a second step interaction between the cells induced at the initial stage.*

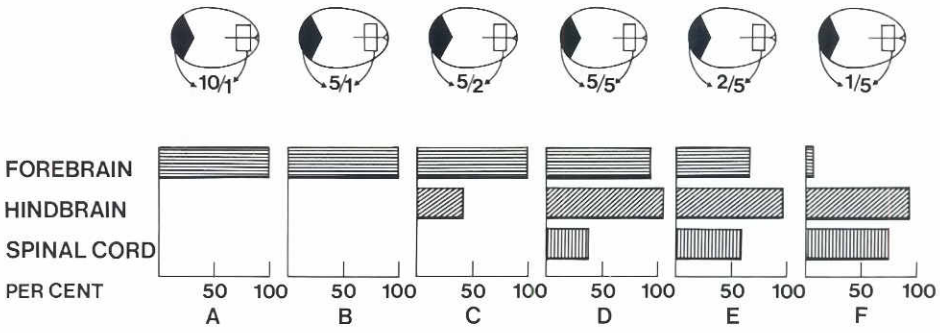

Fig. 10. *Schema and results of the experiment by Toivonen and Saxén (1968) in which the cells from the anterior neural plate were mixed with those from the axial mesoderm. Different mixing ratios yielded a varying spectrum of CNS structures, as the amount of mesoderm determined the final regional character of the neural structures.*

during gastrulation, and (3) the subsequent neural/mesodermal interaction responsible for the regional segregation of the CNS (See Fig. 22).

Model-systems

The entire background presented above was based on experimental work on amphibians. The same restriction is made for the rest of this overview, because the vast majority of experimentation is still performed on this favorite model-system. It should be pointed out, however, that since the classic experiments on chick embryos which Waddington performed in the early 1930's (1930, 1932, 1933), much work has been done on avian embryos, and many interactive events have been unravelled. This important fund of knowledge, omitted here for practical reasons, has been summarized by Deuchar (1969), Eyal-Giladi (1969, 1970), Gallera (1971), and Hara (1978). More recently, the attention of developmental biologists has been focused on prochordate embryos, and the existence of inductive systems has been confirmed there as well (summarized by Takata and Hama, 1978). In recent years, the early development and cell interactions of mammalian embryos have been elucidated, but the neural induction has so far remained unexplored.

Fig. 11 illustrates the major ways of exposing the competent ectoderm to an inductor. Two other methods are illustrated in Figs. 16 and 17.

The basic strategy is more or less the same: the competent gastrular ectoderm, either *in vivo* or isolated *in vitro*, is exposed to the solid or soluble inductor, and the response is monitored as initiation of morphogenesis. In addition, a variety of biochemical, metabolic, and ultrastructural parameters have been used to evaluate the response of the ectodermal cells. The popularity of this model-system is due to the large, readily available and easily-handled cells and their growth, differentiation, and organization in relatively simple culture conditions. One example is illustrated in Fig. 12.

Early Determinative Events

As shown in the classic embryo-dividing experiment, at the two-cell stage both blastomeres are *totipotent and uncommitted*. Their subsequent determination and developmental options are only partially known, and some contradictory views have been presented (Løvtrup *et al.*, 1978; Løvtrup, 1983). By the eight-cell stage the four animal and four vegetal blastomeres have created some self-differentiating capacities and show different modes of development when cultivated in isolation (Gallera, 1952). The animal part develops into differentiated, partly ciliated epithelium (Fig. 13), while the vegetal blastomeres give rise to abnormal, mainly headless embryos. These two poles become distinguishable in most species at an early morula stage, and soon the mesoderm, the prospective inductor, becomes determined. It is localized at the dorsal marginal zone between the animal (ectodermal) and the vegetal (endodermal) poles. Its determination can be monitored as its self-differentiating capacities when cultivated in isolation, and the appearance of its inductive properties can be similarly followed when combined to reactive gastrula ectoderm. The self-differentiative capacity is acquired at the morula stage and the inductive capacity somewhat later, at the midblastula stage (Nakamura and Matsuzawa, 1967; Nakamura and Takasaki, 1970; Nakamura *et al.*, 1971; Kaneda and Suzuki, 1983).

Determination of the mesoderm

The mechanism of determination of the mesoderm and its precise source are still matters of some controversy. Nieuwkoop and his group conclude that the mesoderm is derived from the ectodermal half, and that the determination is brought about by an inductive interaction between the ectodermal and the endodermal halves of the embryo (Nieuwkoop *et al.*, 1973). Nakamura and his team, however, believe that both ectodermal and endodermal cells contribute to the mesoderm at this stage, and that the latter is determined by an animal/vegetal gradient of a vegetalizing factor (Nakamura *et al.*, 1971).

Two sets of recent experiments give support to the above view of Nieuwkoop (1973). The strategy of Gimlich and Gerhart (Gimlich and Gerhart, 1984; Gimlich, 1986) was to avoid dramatic surgical manipulations that frequently lead to poor development of the dissected embryos. Instead they used ultraviolet irradiation prior to the first cleavage in *Xenopus* eggs, a treatment known to block subsequent differentiation of axial mesodermal and neural structures (Grant and Wacaster, 1972; Malacinski *et al.*, 1977; Scharf and Gerhart, 1980). When the irradiated embryos had reached the 64-cell stage, varying numbers of the eight vegetal-most cells were replaced by the corresponding cells from untreated embryos. Two such cells

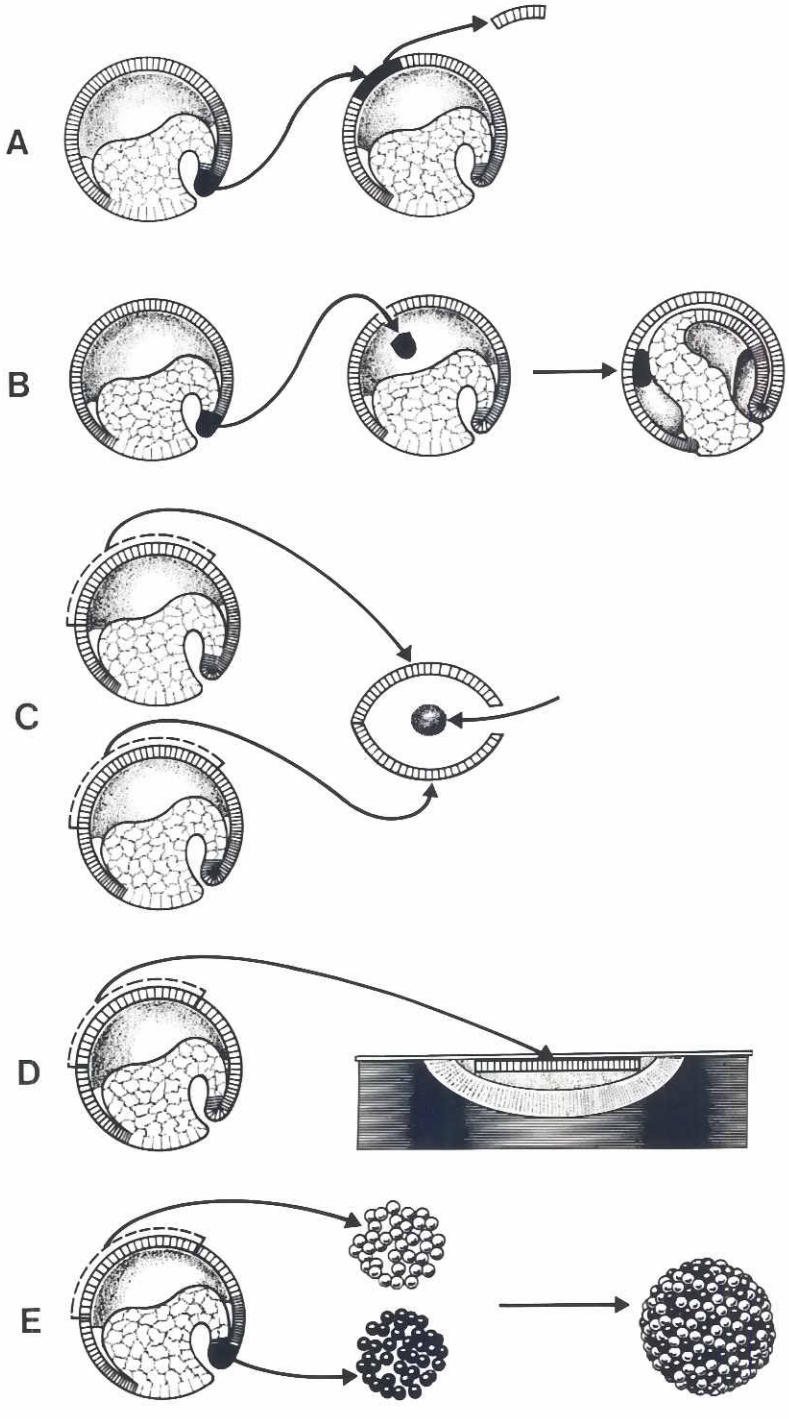

Fig. 11. *Schema of the commonly used experimental methods to analyze primary induction.*
A. The transplantation method by Spemann (1921)
B. The implantation method by Mangold (1933)
C. The explantation («sandwich») method by Holtfreter (1933).
D. The cultivation of flattened ectoderm (Becker, 1961).
E. The disaggregation-reaggregation method (Townes and Holfreter, 1955; Saxén and Toivonen, 1965; Gualandris and Duprat, 1981).

could rescue the axial structures of the recipient, and cell-labeling studies demonstrated that these structures were entirely of host origin without a contribution of the transplanted vegetal cells.

Slack, Smith and Dale (Slack *et al.*, 1984; Smith *et al.*, 1985) approached the problem with the classic isolation/transplantation technique and with the use of cell lineage labels. Labeled animal pole cells from early and midblastula stages were combined to unlabeled vegetal cells. The labeled animal pole cells responded by forming mesodermal structures like muscle and notochord identified by specific antibodies. This interaction between the two halves of the embryo is in a sense directive as the dorsovegetal material would induce dorsal type mesoderm (the organizer) and the ventrovegetal cells, ventral type mesoderm (Slack and Forman, 1980; Slack, 1983; Smith, 1983; Smith *et al.*, 1985). Further interactions between the organizer and the ventral mesoderm lead to the subdivision of the ventral mesoderm into various territories (Fig. 22).

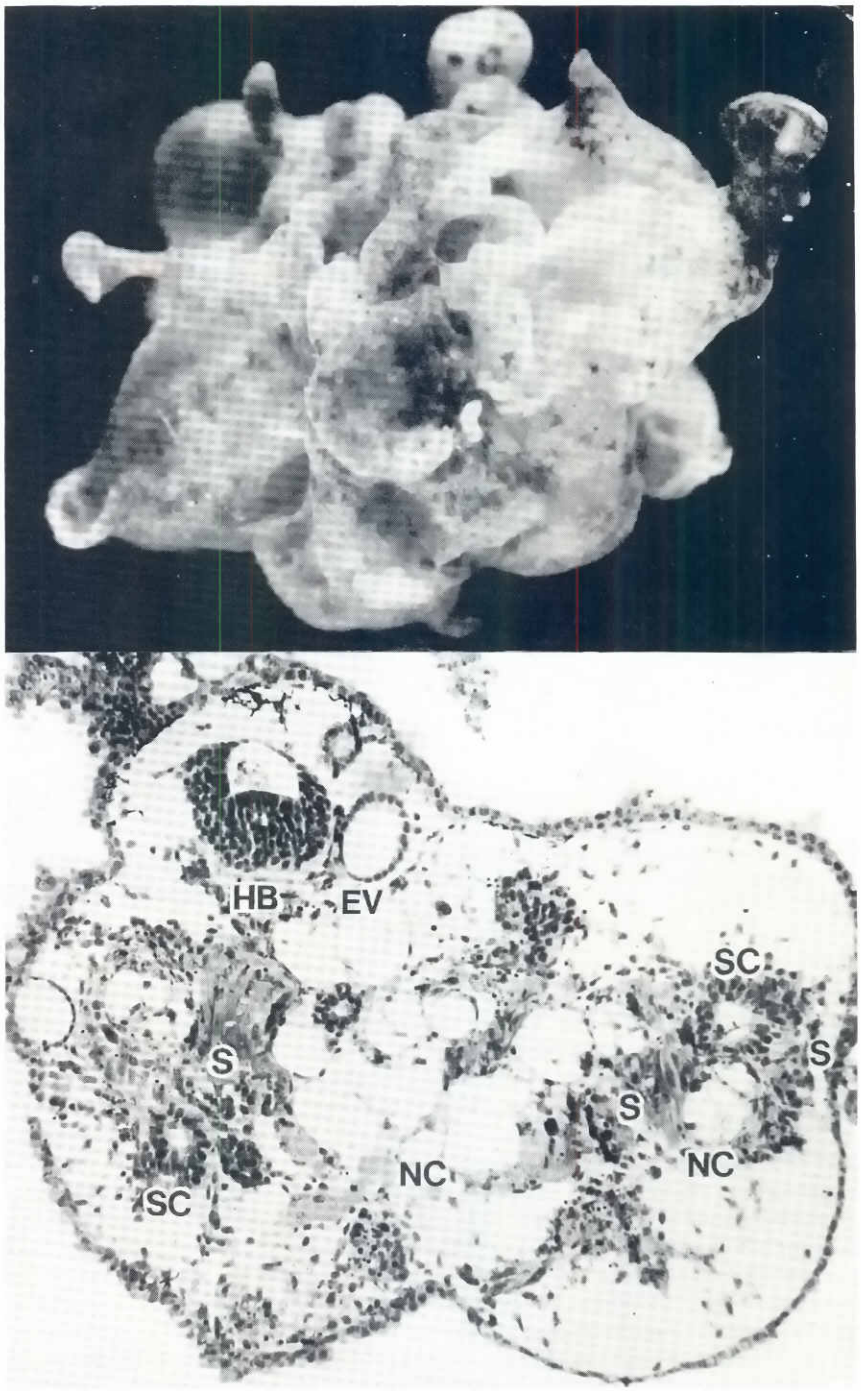

Fig. 12. *A reaggregate obtained from induced, disaggregated ectodermal cells and subcultured for 12 days. HB, hindbrain; SC, spinal cord; EV, ear vesicle; NC, notochord; S, somites.*

The inductive interactions between the animal and the vegetal cells have been studied in a series of experiments by Gurdon and his group (Gurdon *et al.*, 1985a-d). Since muscle cells are among the first to differentiate during amphibian embryogenesis, the authors recorded the determination of the mesoderm by its expression of muscle actin genes either through the use of specific mRNA probes or through immunohistology with antibodies recognizing alpha actinin (Gurdon *et al.*, 1984, 1985b; Mohun *et al.*, 1984) (Fig. 14). The muscle actin genes are not transcribed during oogenesis, but become activated towards the end of gastrulation after which some 5% of the embryonic cells express alpha-actinin. The activation can be experimentally induced by a short contact between the responding animal and the inducing vegetal cells. The muscle genes are expressed within 7 h of the initiated contact (Gurdon *et al.*, 1985a).

The decisive role of this inductive cell interaction in normal development has, however, been questioned by the same group. An alternative mechanism for the spatial and temporal control of activation of the muscle genes would be the existence of mesoderm/muscle determinants already in the fertilized egg

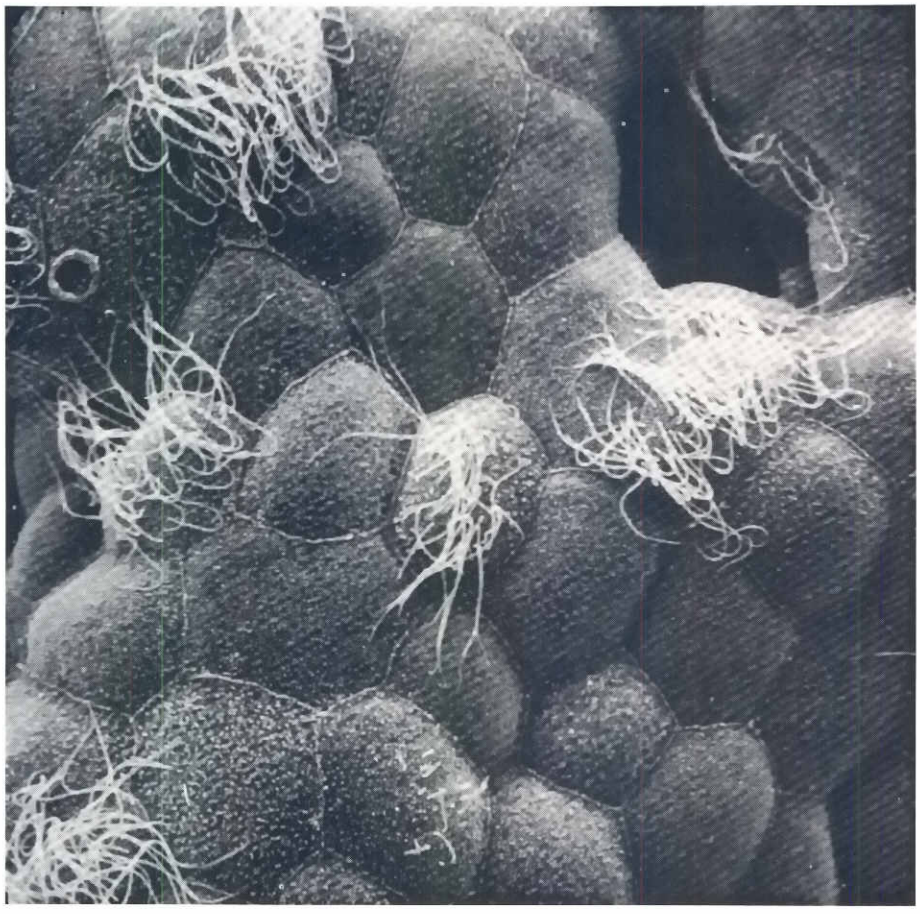

Fig. 13. *Ciliated epidermal cells developed from the animal blastomeres of an eight-cell* Triturus alpestris *embryo (Grunz, 1977) (Courtesy of Dr. Horst Grunz).*

where they are strictly localized and become activated after a certain number of cleavages. To test this hypothesis, the spatial relationships (contacts) between the animal and vegetal cells were disrupted in different ways (ligation, blastomere separation, disaggregation), and then the gene activation was monitored by the probes. Muscle gene activation was not prevented, and the authors concluded that «all components which are necessary for muscle gene activation are localized in the subequatorial region of a fertilized egg» (Gurdon *et al.*, 1985d). Apparently the strictly timed and localized determination of the muscle cells is implemented by a dual control mechanism that consists of both inductive tissue interactions and the distribution of localized egg components (Gurdon *et al.*, 1985a).

At the cellular level, rapid and profound changes have been described after mesodermal induction of the animal pole cells. When isolated cells from the animal hemisphere were exposed to a soluble mesoderm inductor, changes in cell shape and motility were recorded within a few hours. This transformation bears close resemblance to changes observed during gastrulation in intact embryos and hence provides a good model-system to study mesoderm induction (Cooke *et al.* , 1987; Symes and Smith, 1987).

Determination of the presumptive neuroectoderm

Early determination of the presumptive neuroectoderm is poorly understood. As shown by experiments related to autoneuralization, there seems to be a neurogenic bias already at the early gastrula stage, as a variety of non-informative, purely traumatizing exposures can bring the ectodermal cells to express neural differentiation. It is not known whether this expression reflects a selection of a predetermined subpopulation of cells or whether the prospective neuroectoderm is still a homogenous cell lineage with identical developmental options. Electrophoretic measurements have suggested the presence of several subpopulations of cells in the ectoderm with differing surface charge densities (Ave *et al.*, 1968), but whether this is an indication of actual biological (potential) differences in the cells has yet to be determined.

At the beginning of gastrulation, the presumptive neuroectoderm is omnipotent as far as can be judged from experiments in which these cells have been converted into neural, mesodermal or endodermal structures, respectively. During gastrulation or during *in vitro* cultivation this reactivity gradually weakens and is finally lost (Gallera, 1952; Nieuwkoop, 1955; Leikola, 1963), but the neuralized cells can still be transformed. When brought into contact with the axial mesoderm, their regional features can be altered as shown in the previous paragraph (Toivonen, 1967; Toivonen and Saxén, 1968; Kurrat, 1977, 1978). This flexibility is lost in *Triturus* neurulae at stage 15 (Toivonen, 1967) (Fig. 15).

The conventional view of a uniform neural potency, or competence, throughout the gastrula ectoderm should, however, be somewhat revised in the light of recent observations by Sharpe and others (1987). As a marker for early neural differentiation,

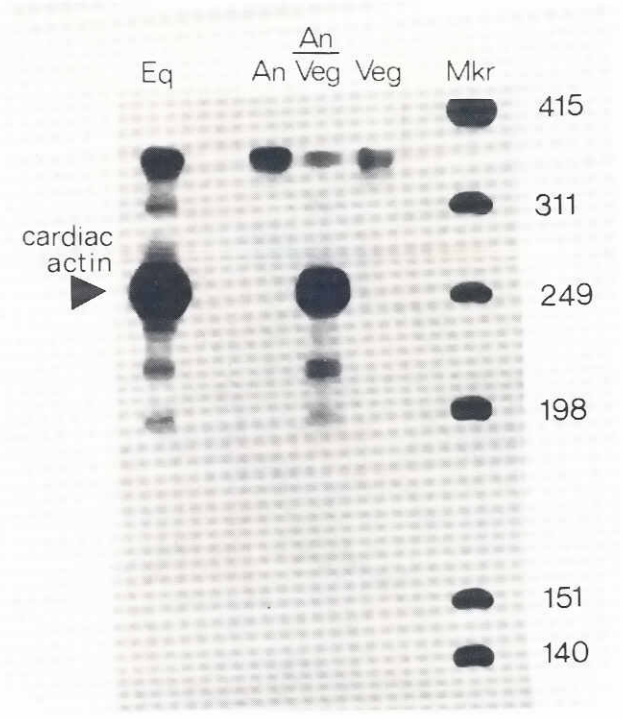

Fig. 14. *Demonstration of the transcription of the muscle-specific actin genes in the equatorial region (Eq) of a stage 8* Xenopus *embryo, and in animal/vegetal conjugates (An/Veg) subcultivated to stage 20 of the untreated controls. Separated animal (An) and vegetal (Veg) fragments did not transcribe the genes. (S1 nuclease protection analysis, using an M13 DNA probe specific for* Xenopus laevis *actin RNA). (From Gurdon* et al., *1985a) (Courtesy of Dr. John B. Gurdon).*

the authors used a *Xenopus laevis* cDNA clone isolated via homeobox homology and called XIHbox 6. This homeobox gene is expressed early during neural development *in vivo*, at stage 13 in *Xenopus,* and is restricted to the middle and posterior part of the neural axis. Experimentally, XIHbox 6 can be induced in the ectoderm after an animal/vegetal recombination, but is not expressed in the two fragments cultivated separately.

The expression of XIHbox 6 in the different portions of the neural-differentiated gastrula ectoderm was explored in combination experiments as illustrated in Fig. 16. The inductor was dissected from a stage 11 gastrula and wrapped in ectodermal fragments from either the dorsal or the ventral portion. Quantitative estimation of the expression of the «neural» gene XIHbox 6 indicated that the dorsal ectoderm was more regularly induced and the expression was stronger than in the similarly combined ventral fragments. Similar results were obtained when another early marker for neural differentiation was used; the neural cell adhesion molecule N-CAM is expressed during early neurogenesis throughout the neural axis (Jacobson and Rutishauser, 1986; Kintner and Melton, 1987), but showed marked quantitative differences in the dorsal and ventral ectoderms exposed to the inductor *in vitro* according to the technique in Fig. 16.

The obvious conclusion from the above series of experiments is that there are regional differences in the competence of the gastrula ectoderm predisposed towards neural direction before induction had commenced.

As so little is known about the mechanism of neural induction and stimulating factors, it is difficult to speculate what creates the reactivity or competence of the ectodermal cells. If, as will be discussed later, neural inductors act via receptors of the cell membrane, their development prior to gastrulation should be a prerequisite for a response. But whether the loss of competence can be attributed to changes in these receptors remains an open question. The loss can be prevented in experimental conditions by inhibition of the protein synthesis of the ectodermal cells (Grunz, 1970). It has also been suggested that the reactivity is associated with the cell generation cycle. Under the experimental conditions of Suzuki and Ikeda (1979), the ectodermal cells of stage 12 gastrula lost their competence within 18 h both *in vivo* and *in vitro*. It was calculated that each cell had divided once during this period. It might be speculated that the gradual appearance of the neural competence before gastrulation (above) could reflect the number of cells that enter the sensitive phase of a certain cell cycle.

Considering the origin and determination of the cells of the CNS, a new view has recently been put forward by Jacobson

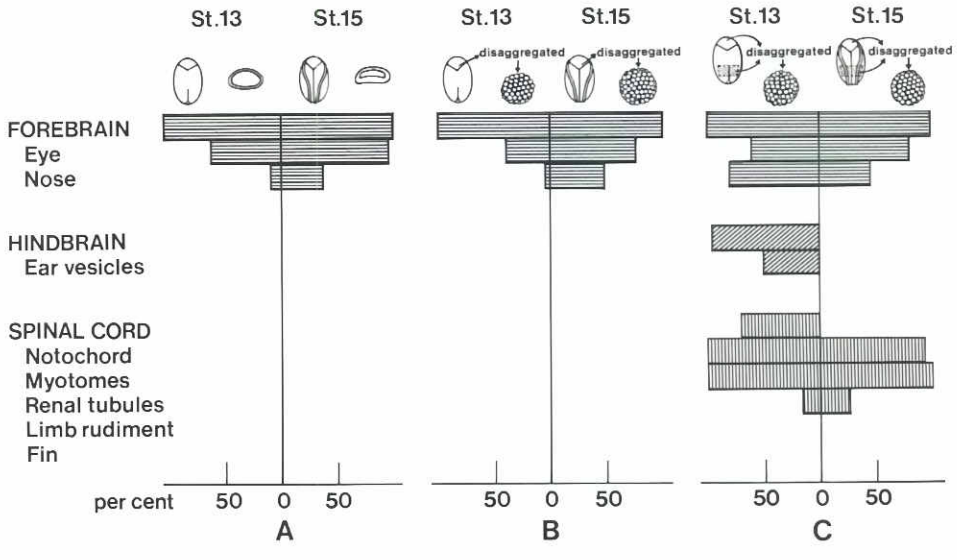

Fig. 15. Loss of the transforming capacity of the neural plate cells during gastrulation. *At stages 13 and 15 both isolated pieces of the anterior neural plate and their disaggregated cells develop into forebrain derivatives during prolonged* in vitro *cultivation (percent distribution indicated in histograms A and B). When combined to fragments of the caudal axial mesoderm (C) the stage 13 neural material becomes caudalized, whereas the material from stage 15 exclusively expresses cranial neural structures according to its prospective fate (Toivonen, 1958).*

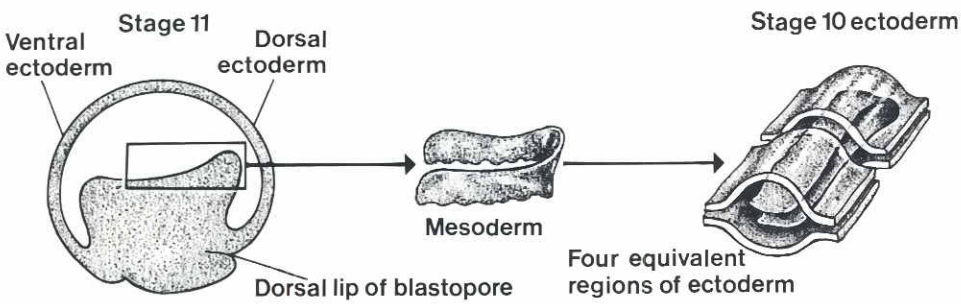

Fig. 16. *Schema of the experimental protocol to test the neural competence of various regions of the gastrula ectoderm. (Sharpe* et al., *1987).*

(1982). According to this view, the cells can be traced back to the 512-cell stage when compartments are formed from small numbers of «founder cells». Their fate can be followed in the embryo by reliable marking techniques, and, indeed, cells from the same area of the blastula (the compartment) subsequently populate a certain area of the CNS.

Having demonstrated this early compartmentalization of the cells of the CNS, Jacobson (1982) disputes the validity of the classic transplantation experiments that have been thought to prove the inductive action of the blastoporal lip (chordamesoderm). He emphasizes two apparent defects in the device and conclusions of the Spemann-Mangold-type experiments: (1) the cells of the secondary neural structures might have been previously determined (or such cells might migrate to the secondary structures), and (2) the previous authors making such transplants did not convincingly demonstrate the genuine neuroid character of the cells in the secondary «neural tube» —these might equally well be epidermal cells forming tube-like structures. Direct counter-evidence for these views has since been put forward by several authors, including Jacobson himself (1984).

At the 32-cell stage, Gimlich and Cooke (1983) labeled *Xenopus* embryo blastomeres, the progeny of which populate domains just above the dorsal or ventral marginal zone at gastrulation. The embryos then served as host for the transplanted blastoporal lip at an early gastrula age. Analysis of the labeled cells in the secondary CNS structures and in the uninduced control larvae showed convincingly that the former were made up of labeled cells that do not normally contribute to the CNS but form belly and tail epidermis. Essentially the same experiment was subsequently conducted by Jacobson (1984), Slack *et al.*, (1984) and Smith *et al.*, (1985), and the results were identical to those reported above. Jacobson's conclusion was that the results «may be regarded as a vindication of Spemann's theory of the organizer» (1984).

The second argument, the genuine neuroepithelial nature of the «neuroid» cells of epidermal origin, remained however to be explored. An indirect way of showing a profound change in the epidermal cells converted into «neuroids» was to follow an epidermal marker, the peanut lectin known to bind to epidermal cells (Smith 1985). It was shown that ectodermal cells converted into secondary «neuroid» structures in transplantation/induction experiments lose their epidermal phenotype recorded as a loss of affinity for the lectin (Slack, 1985; Smith *et al.*, 1985). Similarly, loss of an epidermal antigen following induction was reported by Akers *et al.* (1986).

A more straight-forward approach was chosen by Duprat and her collaborators (1984, 1985a-c, 1986, 1987) who used two specific neuronal markers, the presence of tetanus-toxin binding sites and the expression of neurofilaments. Immunofluorescence examination demonstrated these markers in cells of the neural plate of young neurulas after a few days of cultivation *in vitro* without the chordamesoderm. At the beginning of culture, the markers were not detectable, but the determined cells acquired them during *in vitro* differentiation. An even more important experiment was done by inducing competent ectodermal cells of an early gastrula by a short-term (4-h) co-cultivation with cells from the chordamesoderm (Duprat *et al.*, 1982). The ectodermal cells exposed to the inductor tissue showed outgrowth of small neuronal processes, and, after some 3 to 4 days, immunohistologically recognizable neurofilaments appeared in these cells. A similar association was reported between the expression of the neuronal phenotype and the acquisition of tetanus-toxin binding sites (Duprat *et al.*, 1986).

In conclusion, the two sets of experiments reviewed above seem to be in good agreement with the classic concept of «neural induction» by the invaginating blastopore lip.

Transmission of Inductive Signals

The data reviewed above should warrant the conclusion that from the early blastula stage on, through gastrulation and early neurulation, morphogenetically significant dialogues occur between two cell lineages in close apposition to each other. As first shown by Spemann (1901) and repeatedly demonstrated in recent years, such morphogenetic or inductive alterations are not restricted to the early stages of embryogenesis but can be demonstrated throughout organogenesis in a variety of tissues (reviews: Grobstein, 1967; Saxén *et al.*, 1976a, 1980; Wessells, 1977). In none of these situations are the hypothetic signal substances carrying the morphometric messages known (see, however, next paragraph). As long as the chemical nature of the «inductors» and their mode of action on the target cell are unknown, exploration of their transmission mechanism remains meaningful and might provide indirect information on the nature, synthesis and localization of these molecules.

Three modes of transmission have been outlined (Grobstein, 1956a; Saxén, 1972, 1980; Saxén *et al.*, 1972, 1980; Weiss and Nir, 1979; Table 1): (1) transmission mediated by actual cell contacts (membrane association or short-range diffusion); (2) transmission in the extracellular matrix (between the matrix molecules or between the matrix and the cell surface); and finally (3) transmission by long range diffusion.

Interestingly, all these alternative transmission mechanisms have been demonstrated in one inductive situation or another: cell contacts mediate kidney tubule induction (Wartiovaara *et al.*, 1974; Lehtonen, 1976; Saxén *et al.*, 1976b), matrix interac-

tions are involved in tooth morphogenesis (Thesleff *et al.*, 1977, 1978), and the classic lens induction operates apparently via diffusible factors (Karkinen-Jääskeläinen, 1978). The mechanisms involved in primary neural induction and in the segregation of the CNS are still somewhat unclear, but certain conclusions can be drawn.

The agar-transplantation experiments by Bautzman *et al.*, (1932) and the fact that induction can be brought about by soluble factors (next paragraph) led originally to views favoring the diffusion idea, but the possible significance of actual cell contacts was also stressed by Paul Weiss (1949). A direct test to distinguish between these alternatives would be the separation of the interactants by various means, and this has become the main technique especially after Grobstein (1956b) developed the basic method.

TABLE 1

ALTERNATIVE MODES OF TRANSMISSION OF INDUCTIVE SIGNALS (Saxén, 1980)

Long-range transmission (50,000 nm)
1. Free diffusion
2. Matrix interaction
Short-range transmission (5 nm)
3. Short-range diffusion
4. Interactions of surface-associated molecules
5. Transfer of molecules through intercellular channels

Transfilter experiments

In the first separation experiments by Holtfreter (1933), an interposed vitelline membrane prevented neural induction, and similar results were obtained by Brahma (1958) with porous filter membranes. Because of the nature of the interposed material in these investigations and because of their design, it was difficult to draw conclusions. Therefore the Grobstein-type technique was modified for primary induction by Saxén (1961).

The method (Fig. 17) allows the separation of the interacting components by any type of filter and subcultivation of the tissues. The first series of experiments with this technique and both heterogeneous inductors and normal dorsal lip tissue yielded positive results in a high percentage of cases (Saxén, 1961, 1963). Electron microscopic examination of the interposed Millipore filters with an average pore diameter of 0.45 microns and thickness of 25 microns seemed to exclude cytoplasmic penetration into the filter and, thus, actual cell contacts (Nyholm *et al.*, 1962). The induced structures were exclusively neural and, with few exceptions, belonged to the forebrain region.

After the development of a new filter-type, the polycarbon Nuclepore® filters with better specifications than the spongy filters used before, the matter was reopened by Toivonen and his group (Toivonen *et al.*, 1975, 1976; Toivonen and Wartiovaara, 1976; Toivonen, 1979). The results of these studies can be briefly summarized as follows (Fig. 18):

Nuclepore filters with pore diameters down to 0.05 microns do not prevent neural induction. The induced structures are of forebrain type when small pore-size filters are used and when the time of exposure is relatively short, 18 to 22 h. Filters with larger pores (0.5 microns and larger) allow the second-step transformation of the neuralized cells after prolonged transfilter contact with the inductor. Caudal neural structures are then accompanied by mesodermal derivatives.

These experiments now incline us to conclude that the relatively small neuralizing inductor is transmitted by diffusion and does not require actual contacts between the interactive cells. In experimental conditions the caudalization of the neuralized cells brought about by an interaction with mesodermal cells seems to require cell contacts established through the large pores of the filter during prolonged culture.

These observations are in agreement with those of Minuth (1978) who used basically the same experimental setup as we did and a living normal inductor. However, Kawakami *et al.*, (1978) have reached different conclusions. By using alcohol-killed heterogeneous inductor, they obtained mesodermal inductions regularly after a short (3-h) transfilter exposure of the ectoderm. Only a shallow ingrowth of ectodermal cell processes was seen in the Nuclepore filters of varying pore sizes. The authors conclude that their findings do not support the view of contact-mediated mesodermal induction (and subsequent caudalization of the CNS). We would emphasize, however, that a heterogeneous, killed inductor is known to release soluble inducing factors in experimental conditions, while the situation during normal *in vivo* development might be different.

A dual mechanism might likewise operate during the early determination of the inductor tissue, the mesoderm. If the vegetal and the animal hemispheres are separated by a Nuclepore filter with an average pore diameter of 0.4 um, direct cytoplasmic contacts are prevented, but the mesoderm-inducing signal will traverse the filter (Grunz and Tackle, 1986). Similar results were obtained when the activation of the actin gene is used as a marker for mesoderm induction; dissociation of the interactive cells and cultivating them in close proximity but without intimate contacts allowed induction (Gurdon *et al.*, 1984; Sargent *et al.*, 1986). Furthermore, direct blocking of communication of the cells via gap junctions (by antibodies against the gap junction protein) did not affect the inductive interaction leading to the gene activation (Warner and Gurdon, 1987).

The above observations on the primary mesodermalization of the animal pole cells are not necessarily applicable to the subsequent stage of development, the dorsalization of the mesoderm. The interaction was prevented when the ventral and dorsal marginal zones were separated by a Millipore filter (thickness 25 um, nominal pore) (Slack and Forman, 1980). Hence the possibility should again be considered that these two steps of mesoderm induction are implemented by different active molecules.

Ectodermal/mesodermal interphase

With the spatial and temporal distribution of the ectodermal-mesodermal interactions in mind, direct observations on the interface should prove useful in evaluating the mode of transmission of the signal substances. In the early days of electron microscopy, Eakin and Lehmann (1957) already reported actual cytoplasmic bridges between the interacting ectodermal and mesodermal cells. The contact surface mostly showed an intact membrane, but in places direct intercellular anastamoses were visualized. The authors were inclined to conclude that these channels might be traversed by the inductor molecules. These observations do not fully concord with the more recent findings of Grunz and Staubach (1979b), who also describe

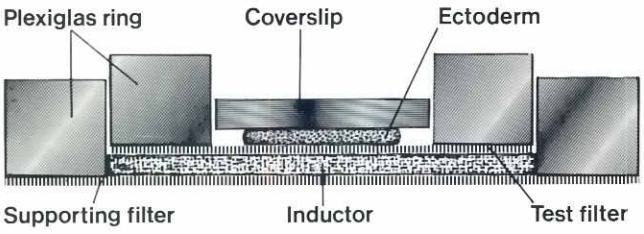

Fig. 17. *Schema of the transfilter technique of Saxén (1961).*

connecting cell projections between the ectoderm and the inductor during midgastrula stage, but fail to demonstrate «cytoplasmic bridges...which would allow a free transfer of inducing substances without passing through membranes». A true electric coupling of these two cell populations during gastrulation has been demonstrated by Ito and Ikematsu (1980). This coupling, measurable during early gastrulation, gradually disappears concomitantly with the completion of induction and the differentiation of the interacting tissue components.

The results of Suzuki *et al.*, (1984a, b) are in good agreement with these observations: cells of the posterior archenteron roof experimentally combined to those from the competent ectoderm become electrically coupled in 3 to 6 h. This lag closely corresponds to the time needed for completion of neural induction (above). The author also demonstrated that coupling did not occur when non-competent ectoderm was used and that induction with Con-A prevented coupling. Thus, electric coupling of the interacting cells can in several ways be associated to neural induction, but it remains to be shown whether we are dealing with a prerequisite or a consequence of induction.

Extracellular materials in the interspace between the presumptive neuroectoderm and the inducing mesenchyme have naturally received attention as candidates for the carrier substances. Tarin (1972, 1973, 1978) describes both granular and fibrillar material in the interphase. The spatial and temporal distribution of this material corresponds quite well to the inductive events. The granular material contains RNA, and there is circumstancial evidence that it represents extracellular accumulation of ribosomes. The fibrils, on the other hand, consist mainly of glycosaminoglycans. Enzymatic digestion experiments, however, cast some doubt on the significance of these materials for induction. After enzyme injections granules and fibrils disappear and are not reconstituted until 3 to 6 h later, respectively. Since this treatment did not affect the normal development of the embryos, Tarin (1973, 1978) concluded that these interfacial

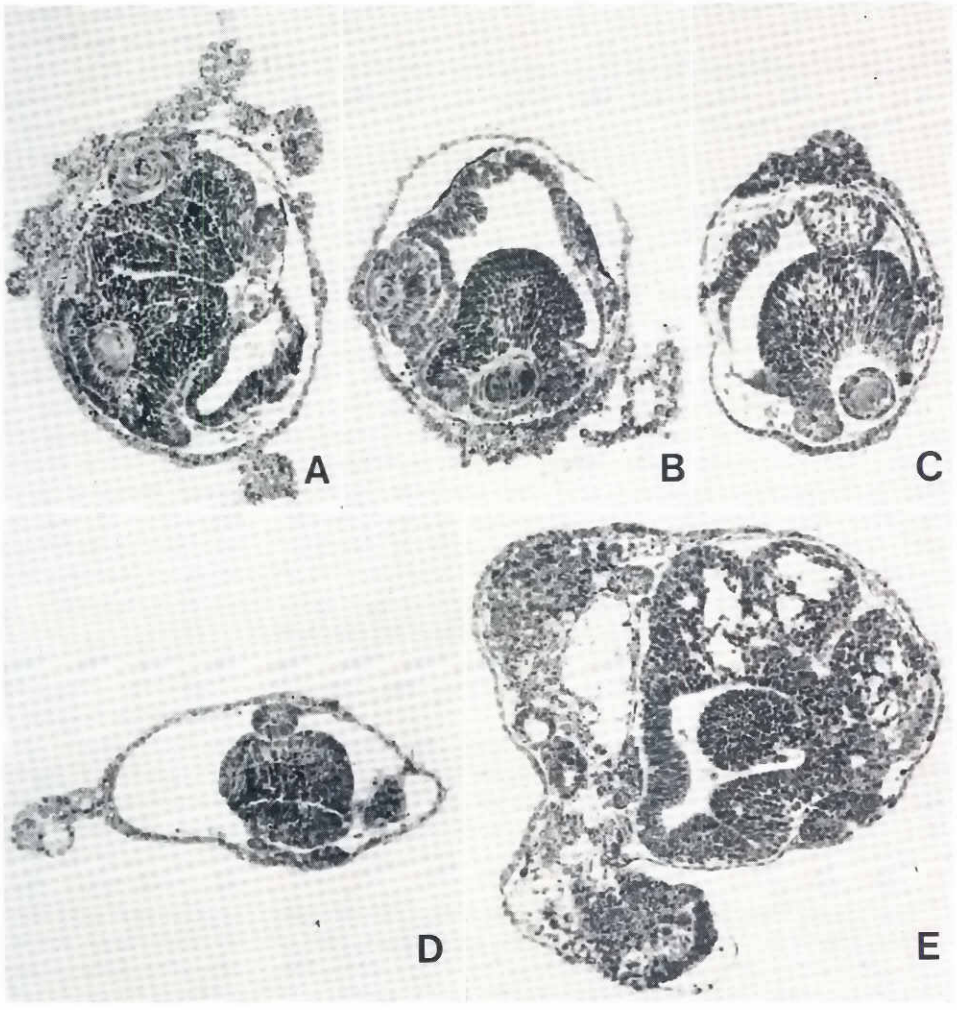

Fig. 18. Examples of ectodermal transfilter-induced explants. *A and B: forebrain structures and optic vesicles induced by the dorsal lip tissue through a Nuclepore filter with an average pore diameter of 0.05 microns. C and D: a similar response obtained by the same inductor through a dialyzing membrane. D: caudal neural structures and mesenchymal derivatives (myotomes and notochord) obtained after a prolonged contact through a filter with 0.6 micron pores (Toivonen, 1979).*

materials are not essential for induction, and their role remains open. The demonstration of interfacial RNA is, however, of potential interest. Suggestive evidence for its existence has been obtained from direct biochemical analyses of the RNA classes in the cells and in their interphase (Sanchez *et al.*, 1983).

To test the developmental significance of the interfacial material, different types of experiments have been devised. Since the fibrillar network coating the inner surface of the ectoderm is known to contain fibronectin, a glycoprotein with adhesive properties, Boucaut and others (Boucaut and Darribere, 1983; Boucaut *et al.*, 1984) injected antibodies to fibronectin into the blastocoele of early gastrulae. Gastrulation and subsequent development were blocked, and the antibody treatment apparently interfered with the morphogenetic movements of the invaginating cells. Since the normal relationships between the presumptive ectoderm and the inductor tissue were distorted, no conclusions could be drawn as to the effect on the induction process itself.

Further studies on the possible role of the ECM that coats the inner surface of the ectodermal cells were based on findings that in many amphibians the ectoderm is double-layered with an inner layer which consists of less competent cells. In experimental conditions, however, both layers are responsive (Asashima and Grunz, 1983; Duprat and Gualandris, 1984). Because there were no apparent differences in the neural response when the ECM-coated inner surface of the ectoderm and the uncoated outer surface were exposed to the inductor, it was concluded that «this ECM is not implicated in the process of neural determination» (Duprat and Gualandris, 1984). The fact that the outer cell layers show definitely higher responsiveness *in vitro* (when directly exposed to the inductor) than *in vivo* might reflect a slow or insufficient transfer from the inductor to the outer layers which are separated by the inner layer. This does not, however, distinguish between the prevailing diffusion hypothesis and the views of contact-mediated transfer as pointed out by Asashima and Grunz (1983).

An approach to test the possible inductive effect of the interfacial, soluble compounds was made by John *et al.*, (1983) who collected the interfacial material from the early neurula stages of *Triturus* embryos. The protein isolated from the phenol extraction of this material displayed neural inductive action, whereas proteoglycans of the aqueous phase were inactive.

From the various observations and results related to the transfer of the hypothetic signal substances from the mesodermal inductor to the overlying neuroectoderm, only tentative partial conclusions can be suggested. The majority of observations speak for a free diffusion of the neuralizing factor released from the inductor and acting on the overlying ectodermal cell surfaces. The transforming effect exerted by the mesoderm might require actual cell contacts provided by the cell processes traveling over the interphase (or through the filters in experimental conditions).

Chemistry of the Signal Substances

The concept of the chemical nature of primary induction was originally based on experiments by Bautzmann, Holtfreter, Spemann and Mangold (1932), who claimed that the «active factor» could be deposited in agar after contact with an inductor tissue. Efforts to reproduce this experiment have failed, and it has been felt that the original experiment was biased by contamination of the agar with inductor cells. However, we still believe that the basic conclusion is valid and that the inductive messages are indeed carried by chemical signal substances. The basic contention in the thirties was certainly influenced by the accumulating information concerning hormones, molecules carrying messages from their site of synthesis to the target cells. Today, an analogous situation is found in the rapidly-expanding field of growth factors, molecules synthesized in the organism and affecting the proliferation of cells in a more or less specific manner. Many of the growth factors are already reasonably well characterized, and their mode of action can be roughly outlined. As for a third control mechanism for embryonic development, the system or morphogenetic tissue interactions and signals transmitting them, there is still a lack of knowledge. Before examining the available data on neural inductors, we will list three candidates for inductor molecules in other interactive situations.

The mesenchymal factor (MS) acts upon the epithelium of the developing exocrine pancreas and is necessary for its differentiation into secretory acini. In experimental conditions this factor replaces the mesenchymal stroma of the developing epithelium, the natural inductor. The effect of the MS is not affected by covalent binding to Sepharose beads, suggesting an action of the cell surface and a second messenger mechanism. The dependence of this action on cAMP has, in fact, been shown (Pictet *et al.*, 1974, 1975). The MS seems primarily to exert a proliferation-stimulating effect upon the target cells. It might be considered a growth factor with a specific target and thus necessary for cytodifferentiation and organogenesis. Many of the same features are carried by transferrin in at least one model-system for determination and differentiation, namely the fetal mouse kidney. Here induction is closely linked to the appearance of transferrin sensitivity, which again is a prerequisite for the expression of the newly-gained differentiative potentials (Ekblom *et al.*, 1983; Thesleff and Ekblom, 1984; Saxén, 1987).

A third extensive analyzed morphogenetic factor is the chondrogenic inductor enhancing cartilage formation in the somitic mesenchyme. Extracellular proteoglycans seem to be the key molecules in this event of differentiation (Kosher *et al.*, 1973; Kosher and Lash, 1974). With this rather meager background information on «signal substances» during embryogenesis, we will now examine present knowledge of such molecules acting during primary induction.

Primary induction

As already related, the hunt for specific inductor molecules in the thirties was largely based on the prevailing hypothesis of one key molecule, the evocator (or the organicine) presumably distributed as a concentration gradient in the embryo. Extensive experiments with heterogeneous inductors soon changed this view, and it became evident that the search would be made with two or more signal substances in mind. When the first fractionation experiments were started in the forties, those expectations were soon confirmed, but even before that Chuang (1940) had shown that heat treatment could change the inductive action of a heterogeneous tissue. The first fractionation experiments with guinea pig kidney as starting material showed how short-term treatment at 70 °C to 90 °C altered the inductive action from a spinocaudal type to an archencephalic one (Toivonen and Kuusi, 1948; Toivonen, 1950). It was further shown that extraction of the tissue with petrol-ether enhanced induction and dif-

ferentiation of secondary mesodermal structures. Digestion with either trypsin or pancreatin abolished the spinocaudal inductive action, while RNase seemed to have no such effect (Kuusi, 1951a, b, 1953; Brachet *et al.*, 1952).

At the beginning of the fifties it had become evident from such experiments as well as from biological combination experiments that there was a factor causing exclusively archencephalic inductions and another one leading to the induction of spinocaudal formations. Many investigators also believed in a «deuterencephalic» inductor, a factor causing the induction of the mid-CNS-derivatives. This situation was finally schematized by the hypothesis of two factors, the neuralizing and the mesodermalizing inductor. This hypothesis will be followed here as far as possible.

The mesoderm-inducing factor

Most of the classic work done to characterize the mesoderm-inducing factor(s) has been performed with heterogeneous inductors tested upon competent gastrula ectoderm. The ectoderm responds to these inductors and their soluble fractions by mesodermalization (with or without neural derivatives). Since many tested fractions also induce endodermal structures, it has been recommended that the inductor be called the «vegetalizing factor». According to Tiedemann and his group, this factor is a protein of m.w. 28,000 and can be dissociated into subunits of 13,000 to 14,000 daltons. The factor is alkali-labile but resistant to acid treatment, sensitive to proteolytic treatments and heat but resistant to RNase. Approximately $2x10^{-4}$ ug of this preparation is needed for one gastrula to induce detectable trunk/tail structures in 50% of the explants - i. e. 10^5 to 10^6 molecules per cell (summarized by Tiedemann, 1981, 1982, 1984).

The relation of this vegetalizing factor to the normal mesoderm inductor acting upon the animal pole cells during blastula stages is not known. Its effect should, however, be strictly distinguished from the second-step transforming action of the mesoderm acting upon neuralized ectoderm. This probably contact-mediated induction occurs towards the end of gastrulation and during neurulation and is quantitative in nature.

Recent experiments with soluble mesoderm-inducing factors acting upon the animal pole cells have brought the analysis closer to the normal *in vivo* situation. Smith (1987) showed that the *Xenopus*-derived cell line XTC produces a soluble-factor-inducing mesoderm of the isolated animal pole cells. The factor called XTC-MIF is a hydrophobic protein with an isoelectric point of 7.8 and apparent relative molecular mass of 23,500. The factor can be dissociated into inactive subunits with a relative mass of 15,000. The mesoderm-inducing capacity of this factor is dose-dependent, since low concentrations induce mesenchyme and mesothelium and the highest concentrations, axial mesodermal structures (Smith *et al.*, 1988).

Several heparin-binding growth factors have likewise been shown to be mesodermal inductors when tested against isolated animal pole cells: the acidic and the basic fibroblast growth factors (FGF) and the embryonal-carcinoma-derived growth factor (Slack *et al.*, 1987). The active compound of these factors differs, however, from another group of MIFs recently isolated by Godsave and collaborators (1988) from three sources: chick embryos, the XTC cells and the WEHI-3 cell line. The active proteins obtained from these sources acted upon the blastula ectoderm in the same way as did the heparin-binding growth factors, but they are chemically distinct.

After Kimelman and Kirschner (1987) reported the presence of an mRNA of the fibroblast growth factor in early *Xenopus* embryos, Slack and collaborators (1988) performed a detailed study of the effect of FGF upon the blastula ectoderm. Both acidic and basic FGF exerted a dose-dependent mesoderm-inducing action on the target cells, and the kinetics of this process corresponded to that of normal induction by the vegetal cells.

In conclusion, there are several well characterized compounds acting as mesodermal inductors when tested against the blastula ectoderm. Their effect mimics that of the normal inductor, and it remains to be seen whether one or several of them are really involved in normal development.

The neuralizing factor

The neuralizing factor(s) is found throughout the animal kingdom, and most of the fractionation results so far published have started from heterogeneous tissues: Tiedemann and his group mainly employ 11-13-day chick embryos, while many others have used mouse and guinea pig tissues, both embryonic and adult. Neuralizing and mesodermalizing activities have also been demonstrated in young amphibian embryos, in extracts from cleavage- and gastrula-stage *Xenopus* embryos (Faulhaber, 1972; Faulhaber and Lyra, 1974; Wall and Faulhaber, 1976). We have already referred to the recent examination of the extracellular material from the neuroectoderm-mesoderm interphase.

In the forties the prevailing idea concerning the chemical nature of the neuralizing factor was that it was related to RNA (Brachet, 1942, 1944), but many subsequent experiments with RNase-treated inductors failed to support this idea. The most convincing experiment was made in 1959 by Hayashi (1959): starting with a purified ribonuclearprotein sample from liver tissue, he showed that an almost complete (99%) removal of RNA from the sample did not affect its inductive capacity. Instead, trypsin was able to inactivate this sample. Inactivation was also reported by Tiedemann *et al.* (1961) who tested a soluble fraction from amphibian embryos.

The neuralizing action of various samples is not inactivated by treatment with thioglycolic acid or mercaptoethanol, and it is, as already mentioned, more heat stable than the mesodermalizing principle (Tiedemann, 1976, 1978, 1981).

More recently Tiedemann (1982, 1984) and his group have shown that the neuralizing factor is not an integral part of the plasma membrane, as isolated membrane material expresses only a weak inducing activity. In various embryonic tissues, the main activity was found in the RNP particles. Again, this activity was abolished after trypsin treatment, whereas RNase had no effect. Correspondingly, RNA isolated from these samples showed no activity, but this was found in the protein fraction. SDS electrophoretic separation experiments suggested that the active protein has a molecular weight of 10,000 to 16,000 daltons (Janaczek, 1984a, b).

In vivo, neural inductors can be almost completely inactivated by ultraviolet irradiation. If the vegetal hemisphere of a fertilized *Xenopus* egg is irradiated prior to the first cleavage, subsequent neural induction is affected and the embryos become defective (Chung and Malacinski, 1975; Malacinski *et al.*, 1977).

To conclude this fragmentary information: the neuralizing factor, widely spread in the animal kingdom, both in embryonic and adult tissues, is most probably a ribonucleoprotein, the activity of which is in the protein part. This factor has not yet been

isolated in purified form and its effect (when acting alone) is the induction of forebrain structures (archencephalic induction).

Towards the end of the fifties, scientists were still searching for another neuralizing inductor, the deuterencephalic agent (Tiedemann, 1959), and many relatively pure samples exhibited this action. Following results obtained from various combination experiments with heterogenous inductors (Toivonen and Saxén, 1955, 1961), Tiedemann and his group could split such «deuterencephalic» inductors into a neuralizing (archencephalic) and a mesodermalizing (vegetalizing) component (Tiedemann *et al.*, 1963).

In another set of investigations, Tiedemann's group has demonstrated an interesting difference in the mode of action of the neuralizing and vegetalizing (mesodermalizing) factors. In these experiments two crude inductor preparations were used: a vegetalizing fraction prepared from chick embryos (Born *et al.*, 1972) including both deuterencephalic and spinocaudal structures and a corresponding neuralizing fraction prepared from the same source. The two protein preparations were then bound to BAC-Cellulose or to CNBr- Sepharose matrix, and the competent ectoderm was tested for these preparations (Tiedemann and Born, 1978; Born *et al.*, 1980). The results were striking: covalent binding of the vegetalizing factor to the beads inactivated the action almost completely - an occasional weak action was apparently caused by the small amount of the inductor released from the Sephadex during exposure. The binding did not affect the inducing action of the neuralizing principle tested for two different neuralizing preparations (Born *et al.*, 1986). After an enzymatic degradation, a fully active vegetalizing fraction could be released from the beads. The authors reached the important conclusion that the neuralizing factor acts on the cell surface of the ectoderm, while the vegetalizing factor must become internalized to act upon the target cells. We will shortly return to this finding.

The decisive role of the mesoderm for the development of the neural tube has been known since the long series of experiments by Takaya (Takaya, 1956a, b, 1959, 1977, 1978), who cultured pieces of branchial parts of the neural plate with various amounts of mesoderm at various ratios to the neural tissue. By stimulating proliferation of the adjacent neural tissue, the mesoderm determines the thickness of the wall of the neural vesicle formed and thus creates the region-specific, morphological features of the neural tube (Fig. 19). This contact-mediated stimulation of proliferation brings us back to the growth factors definitely involved in differentiation. We have recently demonstrated an analogous situation during kidney tubule development, where induction is associated to a contact-mediated stimulation of proliferation of the target cells (Saxén *et al.*, 1983).

To conclude, the actual neural determination of the prospective neuroectoderm during gastrulation is brought about by a chemical signal substance released from the mesoderm. This diffusible factor acts upon the surface of the ectodermal cells. It is a protein, probably rather small (as suggested by dialysis experiments), and resistant to RNase treatment.

An interaction between the neural plate and the underlying mesoderm determines the regionality of the CNS by an unknown mechanism. Suggestive evidence is available that a contact-mediated stimulation of cell proliferation is involved here. The following discussion on the possible mechanism of action of the inductor substances is largely based on these conclusions, tentative as they may be.

Induction and Response

As emphasized earlier under «Model-systems», most of the studies related to primary induction still follow the classic way of experimentation, which means that the actual response of the target cell population is monitored as morphogenesis of definite

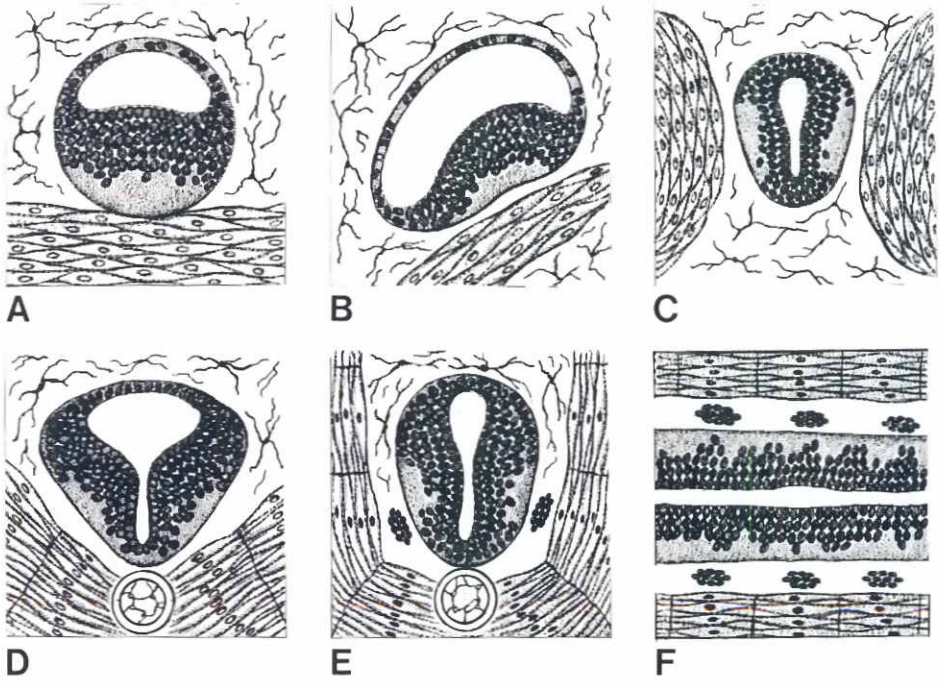

Fig. 19. *Various types and shapes of neural vesicles developed after combination with mesoderm in various ways (Takaya, 1956a, 1959).*

structures. In most cases this analysis is performed 6 to 12 days after the exposure of the ectodermal cells to the inductor. Thus very little can be learned from the early steps of induction and from the response to it at various levels (tissue level, cell level and molecular level). Here we will summarize some rather recent observations on such early events following induction, and an effort will be made to explain some of these events in molecular terms.

The role of cell surface in neural induction has gradually become quite obvious. The inductor released by the mesoderm can apparently traverse a certain distance (Spemann, 1916; Løvtrup *et al.*, 1978), and it acts directly on the plasma membrane without having to become internalized (Tiedemann and Born, 1978; Born *et al.*, 1980, 1986). Various approaches have been used to examine the significance of the cell surface during recent years: direct morphological observations on postinductory changes of the cell surface, follow-up of changes in the surface charge density of the induced cells, use of lectins to alter the surface characteristics, and, ultimately, tests for the second-messenger hypothesis by direct experimental manipulations.

Changes in the cell surface

At gastrulation, the ectodermal cells already carry an epidermal bias (Grunz, 1973) and form well-developed epidermal cells when cultured *in vitro* (Fig. 13). Their development closely resembles that *in vivo* and leads to the formation of cells with a convex, villus-coated surface which occasionally develops cilia. Experimentally vegetalized cells differ from these uninduced controls already at 24 h by showing horn-like protrusions of the membrane (Fig. 20) and further differentiation in prolonged cultures. Ciliated cells do not develop in these conditions (Grunz *et al.*, 1975).

Detailed descriptions are available on changes in cell morphology during gastrulation (Moran *et al.*, 1975; Monroy *et al.*, 1976; Nakatsuji, 1976). Disaggregation/reaggregation experiments with induced vs. uninduced ectodermal cells suggest an increased cell adhesiveness following induction (Suzuki *et al.*, 1986), and the same conclusion might be drawn from observations of an early expression of the neural cell adhesion molecule, N-CAM in neuralized cells.

Artificially neuralized cells have been examined in detail by Yamamoto and collaborators (1981). Treatment of isolated ectodemal cells with the lectin Concanavallin-A (Con-A) leads to early morphological changes associated with neuralization of the target. In treated cells, intercellular connecting filopodia disappeared, and after 60 h a neuroid pattern of the cells could be recognized. Throughout cultivation the surface topography differed definitely from the controls (cells treated with non-neuralizing succinyl-Con A or untreated) (Fig. 21). The initially disrupted intercellular relations of the induced cells were restored around 20 h, but the marked differences in the surface structures remained. The authors conclude that their lectin, Con A,

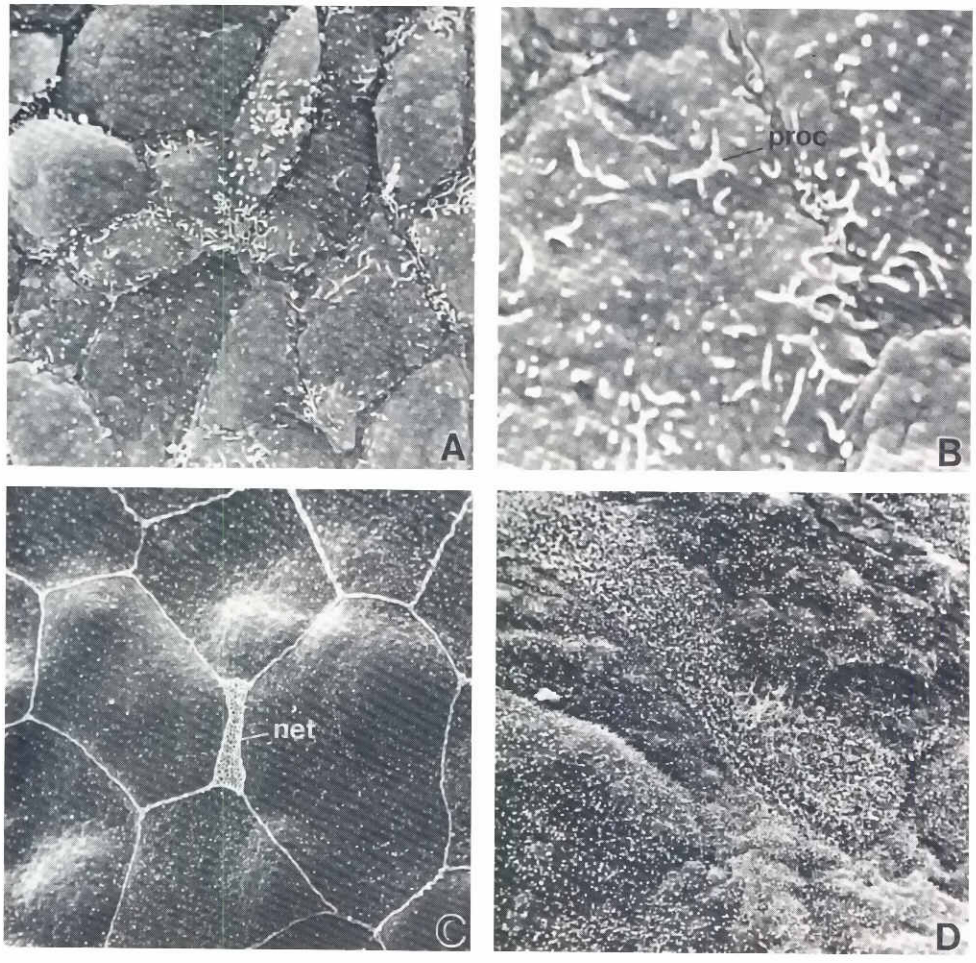

Fig. 20. *Scanning electron micrographs of isolated ectodermal cells treated with the vegetalizing factor for 24 h (A and B) and subcultured for 10 days (C and D). Various surface structures can be identified after the initial induction, and during subsequent cultivation the cells form an irregular epithelium of flattened cells many of which are covered with microvilli. Magnifications A: 1200 x, B: 3000 x, C and D: 1400 x (Courtesy of Dr. Horst Grunz).*

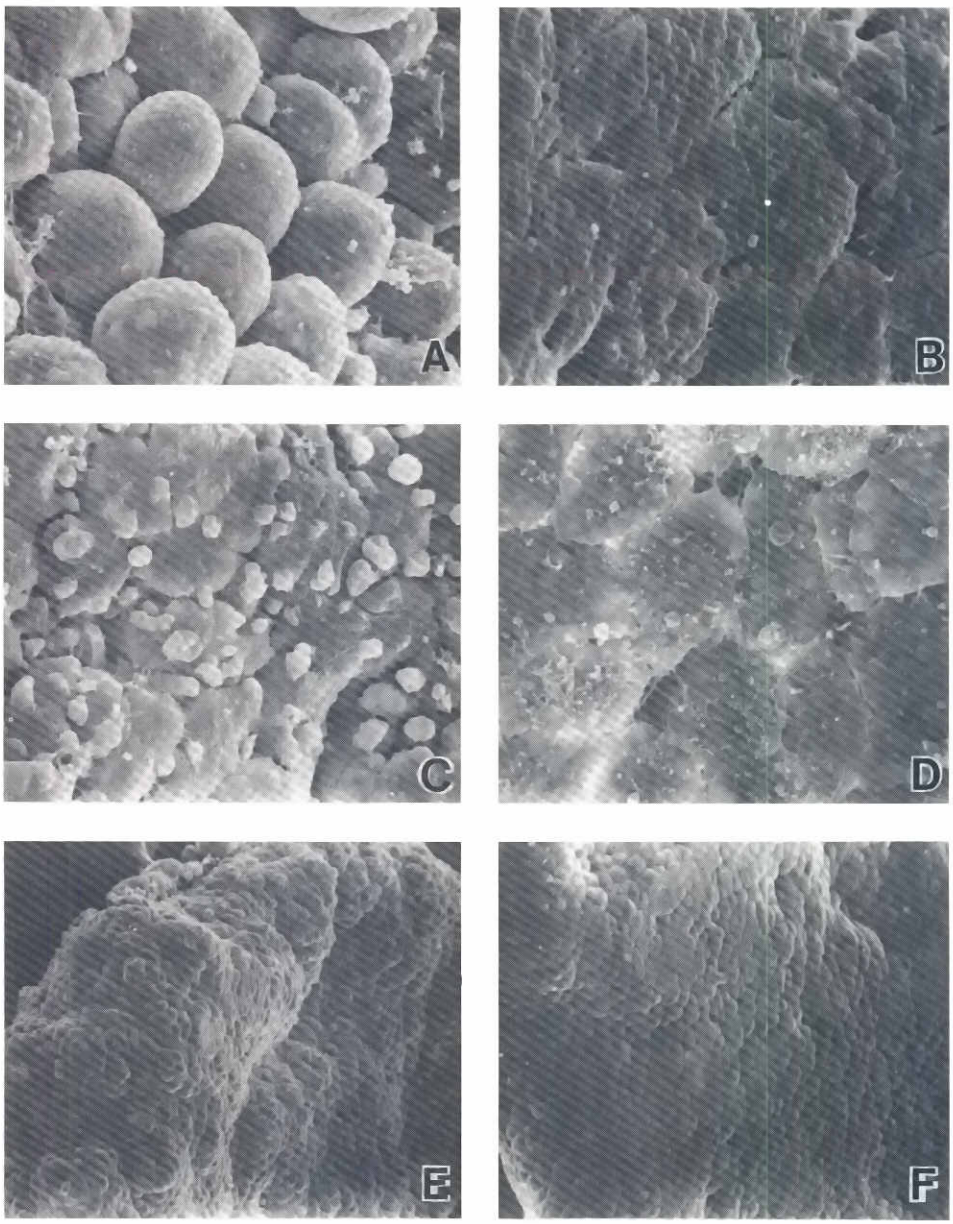

Fig. 21. *Scanning electron micrographs of the interior (A, C) and exterior (E) surfaces of ectoderm «induced» with Con A for 3 h (A), and subcultured for 20 h (C and D). Uninduced, corresponding controls on the left (B, D, F) (From Yamamoto* et al., *1981) (Courtesy of Dr. K.Y. Yamamoto).*

might act on the same surface receptors involved in normal induction, a view not very different from those expressed by more recent investigators using lectin probes (below).

In order to examine early changes in the cell surface charge, Grunz and Staubach (1979a) used cationized ferritin binding to the negatively charged groups at the cell surface. When uninduced isolated ectodermal cells were compared with those induced with the vegetalizing factor, qualitative and quantitative differences were detected: while in uninduced cells the ferritin was seen as a thin layer covering the entire cell surface, in induced cells the amount was reduced, and it was distributed in an uneven, patchy fashion. The author correlate this change in the negatively charged groups at the cell surface to parallel changes in the cell affinity after induction (Kocher-Becker *et al.*, 1965). These also seem to correlate temporally to an increase in the surface charge density of vegetalized cells as shown by the electroporetic mobility studies by Togashi and Asashima (1980). Further experiments comparing the cation concentration and the membrane resting potential of induced with uninduced ectodermal cells led to the conclusion that the induced membrane changes result in ionic selectivity (Siegal *et al.*, 1985).

Experiments with lectins

The apparent action of the neuralizing factor on the cell surface (Tiedemann and Born, 1978; Born *et al.*, 1980, 1986) and the many morphological and physiological changes there (above) strongly suggest a central role for cell surface in the induction process. Hence, it was only natural that several investigators recently picked lectins as tools to manipulate the cell surface conformation during and after induction. Duprat *et al.*, (1984, 1985c) chose two lectins known to have their receptors

on the gatrula ectodermal cells: soybean lectin (SBA) binding to N-acetylgalactosamine and pea lectin (PSA) binding to N-acetylglucosamine. These lectins have no inducing effects on the competent ectoderm, but when the latter was pretreated for 30 min with the lectins, neural competence was almost completely lost when tested during a four-hour contact with the dorsal lip inductor. If, however, the contact time was extended to 24 h, induction occurred, and the author concluded that the prolonged culture after lectin treatment had allowed repair of the structural integrity of the target cell surface. This structural integrity was considered necessary for neural induction, but it remains to be explored how the various surface receptors are linked to it. A spheric hindrance of the inductor receptors or nearby lectins is possible, but we may also be dealing in part with the same reactive and binding sites.

Takata (1983, 1985), having shown that competent ectoderm can be neuralized by treatment with Con A, has proposed that the neural receptors on the ectodermal cell surface might, in fact, consist of glycoproteins. Various types of experiments were performed to examine this hypothesis. After measuring the uptake of radioactive Con A, he tested whether an extended contact between surface-bound Con A and the target cells was required for the induction. For this, Con A-treated cells were chased with mannoside which removed more than 50% of the labeled compound. The fact that this blocked the induction led the author to conclude that the remaining, apparently internalized Con A molecules were not essential for the induction whereas the surface-bound molecules were. The role of the presumably mannose-type sugar-containing receptors was further elucidated in the following experiment. The ectodermal explants were pretreated with neuraminidase and, subsequently, with glycopeptidase to remove the receptors. When these explants were then exposed to the inducing Con A, only 13% responded by neuralization while the percentage in adequate controls was 71% (Takata, 1983).

To us, these results of careful experiments are of considerable interest, as they show (for the first time) at least one pathway whereby an exogenous agent acting on the cell surface can act on the competent ectoderm and lead to its neuralization. As for the relation of this finding to normal neural induction, we still prefer to be cautious. In fact, recent observations by Grunz (Grunz, 1985a, b) suggest that the situation is more complex. He treated his blastoporal lip/competent ectoderm sandwich-type explants with tunicamycin, an inhibitor of glycosylation, but could not prevent neural induction. He suggests that the natural inductor could interact with receptors other than Con A. Finally, the possibility still exists that Con A used in rather high concentrations might be toxic to the target cells and cause autoneuralization through a release of the inductively active compounds found in the ectoderm (Gualandris *et al.*, 1985). This explanation was not, however, accepted by Tacke and Grunz (1986), who did not detect any signs of a toxic effect in their Con A-treated cells.

Second messenger hypothesis

The results suggesting the role for surface receptors in neural induction raise the question of the possible role of a second messenger. A straight-forward attempt to test this would be the exposure of the ectoderm to various exogenous cyclic nucleotides. Thus, Grunz and Tiedemann (1977) used several such compounds (mono- and dibuturyl-derivatives of cyclic AMP), but did not obtain differentiation of the ectoderm - in contrast to earlier reports with *Axolotl* material (Wahn *et al.*, 1975; Løvtrup and Perris, 1983). The authors conclude that their results do not support the idea of a second messenger being involved in the implementation of neural induction, and they refer to the extreme sensitivity of the *Axolotl* ectoderm to toxic treatments leading to autoneuralization (Grunz, 1984).

Activation of «neural» genes

The search for early markers for neural differentiation following induction is ultimately focused on «neural» molecules, the expression of which would be spatially and temporally associated to early neurogenesis. The detection of such molecules would allow an analysis of the gene activation reflected in the expression of the «new» molecule in its enhanced transcription. (Another strategy would be the one successfully adopted by Gurdon's group: screening the cDNA library of certain developmental stages and correlating the transcripts to neurogenesis.

Neural cell adhesion molecule N-CAM is a member of a larger family of compounds with adhesive properties showing developmental changes in their expression and localization (Edelmann, 1985). N-CAM was recently detected in the nervous system of *Xenopus* embryos. Both in primary neural tissue and in experimentally-induced ectoderm, N-CAM expression was greatly enhanced some 18 h after the start of induction - in fact, no detectable amounts of the compound were seen in immunohistology before this stage (Jacobson and Rutishauser, 1986). These results were soon confirmed by Kintner and Melton (1987) who isolated *Xenopus laevis* N-CAM cDNA to use this probe for showing a definite increase in N-CAM RNA levels when competent ectoderm was brought into contact with the normal inductor. *In situ* hybridization showed that the early expression of N-CAM RNA was localized in the neural plate in whole embryos. The results thus indicate that neural induction is followed by gene activation - a finding opening promising possibilities for analysis of the basic mechanism in neural induction and, ultimately, for the characterization of the inducing molecules.

Neural induction - A sequential process

The abundant data acumulated during 60 years of research in the field of neural induction do not yet warrant a unifying concept of the process. However, we can summarize this review in the form of a working hypothesis that might be used when planning further investigation - and which most probably will require modification in the future (Fig. 22).

Neural induction is a multistep process. Inducing factors are already present in the egg, and they are widely spread in early embryos, apparently in an inactive form. Their activation in the organizer region at the blastula stage is associated with the determination of this particular tissue. This, in turn, seems to be controlled both by an interaction between the animal and the vegetal cells and by determinants with specific localization already in the egg.

After the chordamesoderm acquires its inductive properties, the presumptive neuroectoderm acquires its responsiveness, or competence, to respond to inductive stimuli. Whether this is implemented by the formation of membrane-associated receptors or by some other mechanisms remains open. There is evidence, however, that the signal substances released from the inductor will be transferred to the surface of the ectodermal tar-

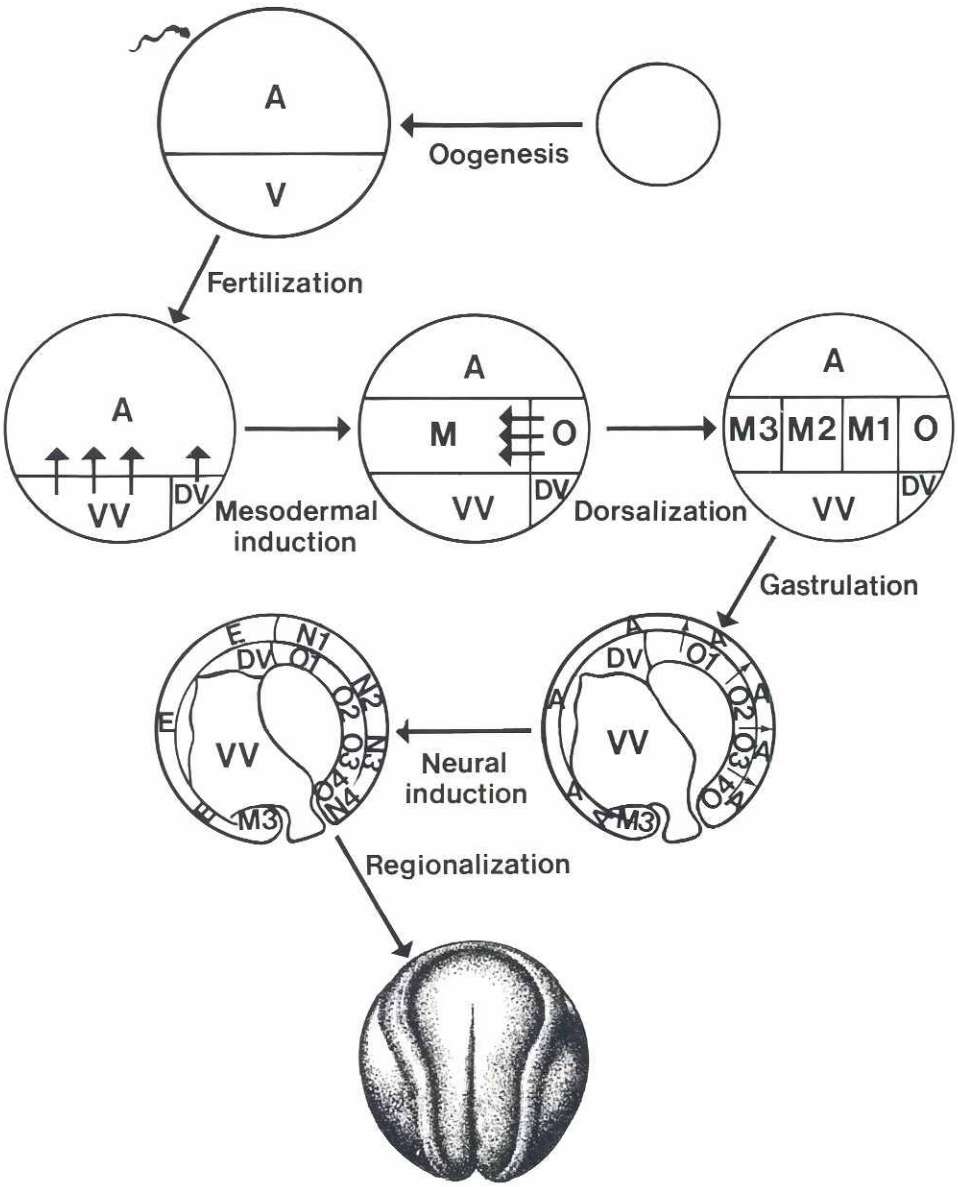

Fig. 22. *Schema of the sequential induction processes leading to the formation of the CNS. The schema and terminology borrowed from Smith* et al., *(1985) but completed with the last stage of regionalization.*

After fertilization the vegetal half becomes subdivided into dorso-ventral (DV) and ventral-ventral (VV) portions. The dorso-ventral region then induces the organizer (0) from the animal half while the ventral region induces the ventral mesoderm (M). The latter is then induced by the organizer into subdivisions. During gastrulation the organizer acquires craniocaudal «positional values» (01 to 04) which become transmitted to the competent ectoderm, there to produce the homologous craniocaudal positional values (N1 to N4). These are implemented by a secondary interaction between the neuralized ectoderm and the axial mesoderm leading to the regionalization of the CNS.

get cells where they act on the plasma membrane without being internalized. This interaction leads to an overall neuralization of the presumptive neural ectoderm without definite regional segregation.

The last step in the chain of interactive events governing early neurogenesis is the segregation of the neuralized ectoderm into the various territories of the CNS. This secondary action of the inductor tissue is quantitative in nature, probably mediated by actual cell-to-cell contacts, and stimulates proliferation of the neuroblasts.

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