Information transfer during embryonic induction in amphibians

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SUMMARY

Neural induction and differentiation has been studied using Concanavalin A, cyclic AMP, tunicamycin and calcium ionophore A 23187. Competent ectoderm of *Xenopus laevis* treated with Concanavalin A differentiates into neural (archencephalic) structures. Binding studies with gold-labelled ConA indicate that the superficial ectodermal layer contains fewer ConA-sensitive sites (α-D-mannoside and α-D-glucoside residues of glycoproteins) than the inner ectodermal layer. The small number of ConA-sensitive sites can be correlated with the fact that the isolated superficial ectoderm layer, in contrast to the inner layer, does not differentiate into neural structures. The gold-ConA particles bound to inner ectoderm are quickly (within 30 minutes) internalized, presumably by receptor-mediated endocytosis. However, endocytosis is not a prerequisite for neural induction. On the contrary ConA apparently must be bound to the plasma membrane for a certain period to initiate neural induction. The rapid internalization of ConA could explain why neural inductions are evoked only if ectoderm is incubated in ConA-containing medium for longer than 30 minutes.

On the other hand cyclic AMP or calcium ionophore A 23187 does not elicit neural inductions. On the contrary calcium ionophore A 23187 apparently inhibits neural and mesodermal differentiation. This effect could be correlated with an increase of intracellular calcium level of the ectodermal target cells, which could influence the permeability of gap junctions resulting in a loss of cell communication, followed by a change of differentiation and pattern formation.

INTRODUCTION

By the well-known implantation experiment of Spemann & Hilde Mangold in 1924 it was shown that the chordamesoderm during amphibian gastrulation is responsible for the neural induction of the overlying ectoderm, which results in the differentiation of the central nervous system (Spemann & Mangold, 1924). Since this time the interest of embryologist has been focused on the chemical nature of the inducer(s) and the transfer of inducing signals to the ectodermal target cells. A main aim was the isolation of inducers (morphogenetic factors) and the characterization of their chemical structure. It turned out that isolation from amphibian embryos was extremely difficult, because the factors are present in the yolk-rich embryonic cells only in small quantities. That was the reason why morphogenetic factors with mesodermal/endoermal (vegetalizing) activity have been isolated.
from guinea pig bone marrow, chicken embryos and swim bladder (Yamada 1958, Tiedemann & Tiedemann, 1956, Kawakami, Noda, Kurihara & Okuma, 1977). In 1970 a crude vegetalizing factor was also obtained from amphibians (Faulhaber, 1970). Tiedemann and coworkers have purified a vegetalizing factor (M.W. ~ 13 000 daltons) from chicken embryos to homogeneity, which shows endodermal/mesodermal inducing activity on competent ectoderm (Tiedemann, 1982; Grunz, 1983). Recently also crude neuralizing factors were obtained from amphibian embryos (Janeczek et al. 1984a, 1984b; John, Born, Tiedemann & Tiedemann, 1984). Fractions with neuralizing activity are apparently present in eggs, early embryonic stages and in gastrula ectoderm in biological inactive form. The data of Tiedemann and coworkers support the view that neuralizing factor(s) are not only present in the inducing chordamesoderm but in a masked form in the ectodermal target cells as well. However, the transport of inducing factors from chordamesoderm to the reacting ectoderm is still a matter of speculation. Neuralizing factor could be exported from the inducing chordamesoderm by exocytosis, could then migrate via the intercellular gap between chordamesoderm and neuroectoderm and could finally interact with specific receptors of the plasma membrane of the ectodermal target cells. Transfilter experiments under in vitro conditions corroborated the concept that free diffusible factors, exported from the chordamesoderm will react with the target cells (Saxén, 1961, Toivonen, 1979). This idea is supported by experiments of John et al. (1983), who found proteins with neuralizing activity in the intercellular space between archenteron roof and neuroectoderm in early neurula. The hypothesis that neuralizing factors interact with receptors of the plasma membrane has been supported by the fact that neural inducers are still active, when their internalization is prevented by binding to polysaccharide matrices. On the other hand vegetalizing factor apparently must become internalized to be biologically active (Born, Grunz, Tiedemann & Tiedemann, 1980).

Further evidence for receptors of the plasma membrane as targets for neuralizing factors came from experiments with Concanavalin A. ConA, a plant lectin, which binds to α-d-mannoside and α-d-glucoside residues of glycoproteins of the plasma membrane of intact cells, is able to induce neural structures in competent ectoderm (Takata, Yamamoto & Ozawa, 1981, Grunz, 1984, 1985). Like Takata's group we found that ConA (100 to 200 µg ml⁻¹) must be bound to the ectoderm for a certain period (3 h in Cynops, about 1 h in Xenopus) to evoke neural inductions. Using Xenopus ectoderm as the test system we obtained further information about the interactions of the lectin with the target cells. The ectoderm of Xenopus consists of two cell sheets (Fig. 1), which can be separated microsurgically by fine glass needles (Asashima & Grunz, 1983). We demonstrated that only the inner ectodermal sheet, but not the superficial layer is able to react to the ConA stimulus. In normogenesis
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Fig. 1. Schematic representation of the test-methods (A) Isolation of the animal cap (ec, ectoderm) from *Xenopus laevis* gastrula for TEM-studies with gold-labelled Concanavalin A. s, superficial ectoderm layer; i, inner ectoderm layer; a,b,c,d, outer and inner surfaces of the two ectodermal sheets checked in the electron microscope. (B) Sandwich method (Holtfreter, 1933): Combination of ectoderm (target cells) with upper blastopore lip (bl) as neural inducer or vegetalizing factor as endodermal/mesodermal inducer. (C) Filter platelet method: The inner side of ectoderm is covered with small pieces of filter paper (fi) to prevent curling up, since most test substances (i.e. inducers, cAMP, actinomycin D, etc.) cannot enter the explant from the external side a.

only the inner layer comes into contact with the underlying chordamesoderm. While the inner ectoderm layer forms the main part of the brain and neural tube, the outer layer without cell contact to the inducing tissue develops only into the ependymal part of the brain. By isolation of the outer from the inner ectoderm sheet and exposing them separately to ConA-containing medium we excluded the possibility that the inner ectoderm acts like a barrier which inhibits the transport of inducers to the outer layer. From our ConA experiments could be concluded that the superficial ectoderm layer in fact possesses a lower neural competence than the inner ectoderm layer. However, xenoplastic experiments with *Xenopus* ectoderm and chordamesoderm of *Triturus vulgaris* as inducer, which allow a clear distinction between self differentiation of inducer tissue and induced structures in the ectodermal target cells, showed that the superficial ectoderm is also able to differentiate into archencephalic neural structures (Grunz, 1985). That the outer ectoderm does not react to the ConA stimulus could be correlated with the fact that it binds much less ConA than the inner ectoderm. This was shown by experiments with gold-labelled ConA performed together with Dr Tacke in my laboratory. The results will be published in detail elsewhere. We have treated all surfaces of inner and outer ectoderm (Fig. 1A) with gold-labelled ConA (Horrisberger & Rosset, 1977) and
have checked its pattern in the electron microscope. The distribution of the gold grains on the plasmamembrane of the different ectodermal sheets can be seen in Figure 2A,B. The amount of bound gold-ConA at the different surfaces of the ectodermal layers is as follows: \( a < b < c < d \). In the same series of experiments we found out that gold-labelled ConA is very quickly internalized (Fig. 2C). This result corresponds to the data of Takata et al. (1981) and from our laboratory (Grunz, 1984, 1985) which shows that ConA must be present in the culture medium for a certain period to evoke neural inductions. Already 30 minutes after the commencement of the incubation at room temperature gold particles can be found in the intercellular spaces (Fig. 2C). In material fixed after 2 h incubation gold particles are localized in endosomes and lysosomal structures (Fig. 2D). We can assume that ConA bound to ConA-specific receptors are internalized by receptor-mediated endocytosis. From experiments of Takata et al. (1981) with ConA immobilized by binding to Sepharose beads it can be concluded that internalization is not, however, a prerequisite for the neuralizing activity of ConA. In contrast the rapid internalization could explain the toxicity of ConA, which is evident when ectoderm is exposed to high concentrations of ConA (>200 \( \mu \text{g ml}^{-1} \)) for more than 2 h. Of interest is the fact that the treatment of competent ectoderm with 10 \( \mu \text{g ml}^{-1} \) cytochalasin B (CB) prior to the ConA treatment enhances the neuralizing effect. The explants treated with CB and ConA contained substantially more neural (archencephalic brain) structures than control explants treated with ConA alone (Grunz, 1985). The explanation of the results is not simple. We know that after CB treatment the microfilament structure is reversibly disrupted. During the incubation for 5 minutes the cells round up, but still remain in focal contact with their neighbour cells resulting in an increase of free cell surface (including parts of the former intercellular contact areas) accessible to ConA. On the other hand, assuming that ConA receptors are associated with cytoskeleton structures, it cannot be excluded that after disruption of microfilaments the ConA receptors arrange themselves on the plasma membrane in a particular way which is favourable for neuralization. The fluorescent pattern of ectoderm incubated with FITC-ConA after CB treatment shows patches in the intercellular areas, while the incubation of control explants with FITC-ConA alone results in a ring-like fluorescence. The

![Fig. 2. (A) Superficial ectoderm layer (side a, Fig. 1B). Only few gold grains could be found after an incubation of ectoderm in gold-ConA for 30 minutes; \( au \), gold grains. (B) Inner ectoderm layer (side d, Fig. 1B). After the same treatment as in Fig. 2A a high amount of gold-ConA has been bound to the plasma membrane; \( d \), former blastocoelic side. (C) Within 30 minutes incubation gold-ConA(Au) can be found in the intercellular space between two ectodermal cells of the inner layer. Also internalization has already started via coated vesicles; \( ve \), vesicle; \( d \), former blastocoelic side. (D) Gold particles could be observed within lysosomal structures (\( lys \)) 2 h after the beginning of the incubation of inner ectoderm; \( d \), former blastocoelic side; \( g \), glycogen; \( i \), intercellular gap between two ectodermal cells. Sides b and c (Fig. 1B), not shown, bind less gold-Con A than side d, but more than side a. Scale bars 0.5 \( \mu \text{m} \).](image-url)
recent results support the view that after CB-treatment the quantity and the patching of ConA bound to the ectodermal cells is higher than in ectodermal cells incubated in ConA alone.

COMPETENCE OF ECTODERMAL TARGET CELLS

It is well known that the competence of ectoderm to react on inducing stimuli decreases during gastrulation. In the early neurula, ectoderm no longer reacts to endodermal/mesodermal or neural inducing factors and develops into ciliated epidermis (Chuang, 1955; Nieuwkoop, 1958; Leikola, 1963; Grunz, 1968, 1970). Although the probability is high that the loss of competence is correlated with regulatory mechanisms at the genome level, it cannot be excluded that specific receptors on the plasma membrane for inducer molecules are modified or lost in correlation to the decrease of competence during gastrulation. We have therefore incubated early and late gastrula ectoderm respectively with gold-labelled ConA. However, we could not observe a significant difference. Both early and late gastrula ectoderm bound similar amounts of ConA. We still cannot exclude the possibility that the observed binding sites in late gastrula ectoderm represent glycoproteins of the extracellular matrix (in this stage responsible for increased cell adhesion), which differ from those in early gastrula ectoderm functioning as receptors for neural induction. Nevertheless the probability seems higher that the loss of competence is correlated with intracellular regulatory processes.

EFFECT OF TUNICAMYCIN ON UPPER BLASTOPORE LIP AND COMPETENT ECTODERM

Assuming that neuralizing factor and/or the receptors on the target cells are glycoproteins, an inhibitor of glycosylation (tunicamycin) should severely interfere with the neural induction processes. Upper blastopore lip isolated from early gastrulae was incubated for 12 h at 11 °C in Holtfreter solution containing 1 μg ml⁻¹ tunicamycin. It was then combined with early gastrula ectoderm by the sandwich-method (Fig. 1 B). In a second series isolated ectoderm of midblastula (stage 8, Nieuwkoop & Faber, 1956) was treated with 1 μg ml⁻¹ tunicamycin for 2 h at 21 °C (by when control embryos have reached late blastula, stage 9). After combination with ectoderm the sandwiches were kept for further 3 h in 1 μg ml⁻¹ tunicamycin. After incubation in Holtfreter solution for up to 5 days at 18 °C the histological examination showed that both the untreated recombinates (controls) and the experimental series formed well-organized brain structures. That means that under our experimental conditions neural differentiation could not be suppressed by tunicamycin. This result is in agreement with data of Sánchez & Barbieri (1983), who observed in tunicamycin-treated embryos, which showed partial exogastrulation, the formation of small neural tubes. On the other hand the aggregation of dissociated, presumptive neural cells in the presence of phytohaemagglutin and
ConA is decreased (Barbieri, Sanchez & del Pino, 1980). From their results the authors conclude that the carbohydrate-containing binding sites for these lectins are not involved in neural induction. On the other hand the data of Takata et al. (1981) and our laboratory indicate that ConA and UEA under certain experimental conditions are able to evoke neural inductions. However, it can be argued that for cell aggregation and morphogenetic movement during gastrulation a higher amount of ConA-binding glycoproteins on the plasma membrane (accumulation of ECM) or other ConA-sensitive glycoproteins may be necessary than for neural stimulation. Since tunicamycin in our experiments does not prevent neural induction, it could be postulated that the "natural" inducer, chordamesoderm, interacts with different receptors than ConA. On the other hand it cannot be excluded that an inhibition of the de novo glycosylation by tunicamycin is without influence, because there exist a high intracellular pool of future receptor glycoproteins. A delayed effect of tunicamycin at least in whole embryos has been reported by Romanovsky & Nosek (1980).

EFFECT OF CYCLIC AMP ON COMPETENT ECTODERM AND THE PROBLEM OF AUTONEURALIZATION

In previous experiments we have shown that in Triturus alpestris ectoderm cyclic AMP or its mono- or dibutyryl cyclic AMP to form neural structures (Løvtrup & Perris, 1983; Wahn, Lightbody & Tchen, 1975). It was shown by Holtfreter (1944) that Ambystoma ectoderm can form neural structures without interaction with a specific inducer under unphysiological conditions. However, in addition, culture conditions which are without effect on other species (Triturus alpestris, Xenopus laevis) can cause autoneuralization in Ambystoma (Slack, 1984). We should mention also that changes in the sodium and potassium ratio after treatment with ouabain did not evoke neural inductions in competent ectoderm of Triturus alpestris (Siegel et al. 1985).

To prevent further discussions about the neuralizing activity of cyclic AMP, we recently have repeated the experiments under exactly the same conditions as described by Løvtrup and co-workers, i.e. the same concentrations of cAMP(10^-5 and 10^-6M) and the same size of explants (tiny pieces of 20-30 ectodermal cells). In further series we also tested again the reaction of much bigger explants. As in our earlier experiments curling up of the isolated ectoderm was prevented by covering with a small piece of filter paper during the treatment with cAMP (Fig. 1C). All experimental series incubated with cAMP differentiated into ciliated epidermis as did the untreated controls (Fig. 3). A very important point is that we performed all experiments with ectoderm of Xenopus laevis instead of Axolotl ectoderm, because we know from other experiments that Xenopus ectoderm does not show any tendency towards autoneuralization (Asashima & Grunz, 1983; Grunz, 1984, 1985).
Fig. 3. (A) Early gastrula ectoderm (animal cap) of *Xenopus laevis* treated with cyclic AMP ($10^{-6}$ M) for 22 h in the filterplatelet method (Fig. 1C) and cultured for 5 days. The ectoderm has differentiated only into ciliated epidermis without any neural structures. Inset: for size comparison; description see Fig. 3C. (B) A small piece of ectoderm (20–30 cells) was isolated from middle blastula, incubated for 24 h in cAMP ($10^{-6}$ M) and cultured for 10 days. The micrograph shows a living explant with cilia (ci). (C) Histological section of a small explant treated in the same way as described under Fig. 3B. The ectoderm has differentiated into epidermis only without any other differentiations. Scale bars 0.1 mm.

Using very small ectodermal pieces of ectoderm, like Løvtrup’s group, (20–30 cells) we never observed permanent contact to the substratum (culture Petri dishes or Thermanox cell culture slides). Our explants containing epidermal cells with beating cilia remained freely floating in the medium after 5–10 days culture (Fig. 3B). It is possible that Axolotl ectoderm, in contrast to *Xenopus* ectoderm, will easily stick to the substratum, especially at unphysiologically high pH (> 7-6). Cyclic AMP could support such adhesion (Ortiz & Yamada, 1975). Other authors have shown that teratocarcinoma cells (embryoid bodies) will differentiate into neural cells only after adhesion to the substratum (Martin & Evans, 1975). There exists the possibility that the neuralization of the *Ambystoma* ectoderm could be correlated with interactions of the plasma membrane and the substratum. On the other hand it cannot be excluded that some of the delicate Axolotl cells within the explant will be damaged by the isolation and by the contact to the uncoated Petri dishes. Neuralizing factor present in masked form in the ectodermal cells could be liberated by cell leakage and could induce neighbouring cells. Probably a neuralizing effect can be evoked especially in tiny explants, since the ratio of damaged cells...
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at the periphery of the explant to vital cells in the centre is relatively higher than in “normal” explants (one animal cap or sandwich). However, in our opinion the culture conditions using tiny pieces of ectoderm on Petri dishes is not comparable with the situation in normogenesis and is not suitable as test system for neural induction. It can be concluded that cyclic AMP or its mono- or dibutyryl derivatives cannot be considered as primary signals for neural induction.

Influence of calcium ionophore A 23187 on neural induction

The ability of calcium ionophore A 23187 to facilitate the calcium exchange across membranes and its intracellular release resulting in an increase of intracellular calcium concentration has been described (Pressman, 1976). Furthermore, this ionophore induces parthenogenesis in sea urchin eggs (Chambers, Pressman & Rose, 1974). Löftrup & Perris (1983) reported that calcium ionophore A 23187 evoked neural differentiation in ectoderm of Ambystoma mexicanum. However, in Triturus alpestris ectoderm the treatment with the ionophore is without effect (Tiedemann, 1984). Also in our recent experiments with ectoderm of Xenopus laevis we did not observe neural induction. In contrast, our results indicate that the ionophore inhibits both neural and mesodermal differentiation. Early gastrula ectoderm treated simultaneously with vegetalizing factor and calcium ionophore A 23187 for 4 h differentiated into yolk-rich tissue only (Fig. 4B). On the other hand, ectoderm combined with inducer omitting the incubation with ionophore formed heart structures and muscle (Fig. 4A). The differentiation of mesodermal structures is probably the result of secondary cell interactions (Grunz, 1979, Minuth & Grunz, 1980). In a further series we treated recombinates of competent ectoderm and upper blastopore lip with $5 \times 10^{-6}$ M calcium ionophore A 23187 for 5 h at 20°C. The explants formed only neural tubes in contrast to untreated controls which contained well-developed archencephalic brain structures (Fig. 4C, D). The change of the pattern of mesodermal and neural differentiation by calcium ionophore A 23187 can be interpreted as follows. On the basis of earlier data, it can be postulated that mesodermal and complex neural structures are formed by secondary cell interactions (Asahi, Born, Tiedemann & Tiedemann, 1979, Grunz, 1979, Minuth & Grunz, 1980). These secondary interactions could in part depend on the communication between ectodermal target cells via gap junctions. It could be concluded that the permeability of gap junctions is reduced by raising the intracellular calcium concentration (Rose & Rick, 1978). That the undisturbed cell-to-cell communication via gap junctions is a prerequisite for normal morphogenetic movements during gastrulation and for the formation of normal brain structures can be concluded from the results of Warner, Guthrie & Gilula (1984), who injected antibodies to gap junctional protein in early embryos of Xenopus. Although we cannot exclude in our experiments that the calcium ionophore A 23187 elicits also other metabolic effects, which are not correlated with gap-junctional permeability i.e. calcium effects on actin filaments correlated with cell motility and cell adhesion,
Fig. 4. (A) Early gastrula ectoderm was treated with vegetalizing factor for 5 h by the sandwich method (Fig. 1B). The explant has differentiated into heart structures and somites. (B) Early gastrula ectoderm was treated in the sandwich-method simultaneously with vegetalizing factor and the calcium ionophore A 23187 for 5 h and was then cultured for 5 days. The explant contains yolk-rich material, but no mesodermal differentiations. (C) Recombinates of early gastrula ectoderm and upper blastopore lip (Fig. 1B). After 5 days' culture well-differentiated archencephalic and deuterencephalic brain structures have been formed. (D) Recombinates of early gastrula and upper blastopore lip treated for 4½ h with 5 × 10⁻⁶ M-calcium ionophore A 23187 and then cultured for 5 days. With exception of a neural tube no other neural structures could be observed. h, heart structures; neu, brain; no, notochord; nt, neural tube; so, somites. Scale bars 0·1 mm.

The results support the view that gap junctions could participate in processes needed for pattern formation. However, it is unlikely that the primary signals of neuralizing and mesodermal factors are transmitted via gap junctions, because the relatively large size (vegetalizing factor: 13 000 daltons) does not permit their passage through gap junctions. On the other hand secondary events between cells within the ectodermal target tissue could be initiated by small molecules via gap junctions. These coordination signals probably must be succeeded by the release of secondary inducing factors (M.W. > 1000 daltons) by exocytosis. Such factors could interact with receptors on the plasma membrane of neighbouring cells or could be
internalized by endocytosis. This chain of events could play an important role in the formation of mesoderm by interaction of ectodermal and endodermal cells or deuterencephalic and spinocaudal structures (Nieuwkoop, 1969, Asahi et al. 1979, Grunz, 1979, Minuth & Grunz, 1980).

CONCLUSIONS

The experiments carried out on *Xenopus* embryos support the view that neural inducing factors interact with receptors of the plasma membrane of the ectodermal target cells. Neural stimulation of competent ectoderm by Concanavalin A suggests that glycoproteins of the plasma membrane are receptors for neuralizing signals. On the other hand we can exclude the possibility that cyclic AMP or ions play any role as factors initiating neural induction.

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DISCUSSION

Speaker: H. Grunz

Question from G. Malacinski (Indiana):
You mentioned that the size of the ectodermal responding tissue piece seemed to govern the response to the vegetalizing factor. Is it really something supracellular about the ectoderm piece, or is it simply the ratio of the responding tissue to the inducer?

Answer:
I think that is a question of much research. It could be that, in the immediate surroundings of the inducer, you get stimulation of the cells and that the cells further away respond to secondary interactions. In our experience, in the immediate zone around the inducer you find endoderm, then mesoderm, then neural. It is really like the pattern which you have also in normal development: first you get endoderm, then you get mesoderm and, finally, you get neural.

Malacinski:
So the inducer is not acting directly on all the responding tissue?

Answer:
Under certain conditions, such as a short contact time or using a giant sandwich, a certain number of cells are triggered and then there are indications that further inducing factors are liberated.

Question from J. Cooke (NIMR, London):
I wonder about the spatial order of the structures within the induced mesoderm. I am rather puzzled by your observation that in a small explant a relatively brief exposure gives you blood islands and the more prolonged one gives the structures we think of as more dorsal. When one considers the fate map of the whole embryo, it is the prospective lateral plate and blood tissue which lies nearer to what we would have thought was the natural source of the vegetalizing factor.

Answer:
It could also be that if you remove the inducer after a short time, you have only certain cells triggered. If you prolong, perhaps every cell gets a threshold amount. We could show in other experiments with radioactively labelled inducer that it distributes more or less uniformly in the small explants.

Question from J. Smith (NIMR, London):
Have you had any success in making the vegetalizing factor from Xenopus embryos?
Answer:
Not myself, but you remember the experiments of Faulhaber in 1970. [Hoppe Seyler's Z. Physiol. Chim. 351, 588–594]. It is very difficult to isolate this factor, but the indications are that it is present. It has a different molecular weight, perhaps about 60K instead of 13K for the chick factor.

J. Smith:
Is there anything known about where it is in the embryo?

Answer:
It should be in the vegetal part, but you could only answer this for sure with a specific antibody. There is some evidence that animal cells also contain it but in small amounts and in a masked form.

Question from H. Woodland (Warwick):
It has been argued in the classical literature that the mesoderm of the tail is actually formed from the neural plate. It would be interesting to look with vegetalizing factor to see if those cells re-acquire an ability to respond. Do you think the old observations are correct?

Answer:
You mean the experiments of ter Horst [Wilhelm Roux' Arch. EntwMech. Org. 143, 275–303 (1948)]. She reported that the very caudal part of the neural plate forms notochord and somites. I think that these results are correct.