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Factors responsible for the establishment of the body plan in the amphibian embryo

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ABSTRACT A central topic of embryology is the establishment of the body plan during embryogenesis. Starting with maternal factors distributed in the early cleavage stages in distinct patterns and gradients cell-to-cell interactions including early embryonic induction result in the formation of mesoderm and the organizer area. While many facts are known about the role of growth factors like activin (closely related to the vegetalizing factor), processed Vg1, BMPs and FGF for mesoderm formation, the establishment of the central nervous system is not yet well understood. However, there is growing evidence that neural induction is a multistep process at the level of the dorsal mesoderm (organizer) and the reacting neuroectoderm. Therefore the existence of only one neuralizing factor is unlikely. We report about data that follistatin protein is not a direct neural inducer. Furthermore our comparative studies of *Xenopus* and *Triturus* exogastrulae indicate that planar signals are unlikely in the *Triturus* embryo (urodeles) during the early steps of neural induction. Vertical signals emanating from the chordamesoderm are essential for the terminal neuralization and regionalization of the central nervous system during gastrulation for both *Xenopus* and *Triturus*. The putative role of neuralizing factors and BMP/ activin-like molecules for the stabilization or shift of neuroectoderm into different pathways of differentiation (epidermis or neural default state) is discussed.

KEY WORDS: *mesodermal and neural induction, gradients, pattern formation, vertical and planar signals, species-differences*

Introduction

The establishment of the basic body plan of the amphibian embryo as well other vertebrates or evertebrates takes place by maternal factors and complicated cell-cell interactions (Asashima *et al.*, 1991a; Dohrmann *et al.*, 1993; Regabgliati *et al.*, 1993). The latter ones include processes described as embryonic induction. A key experiment was the implantation (Einsteck-Versuch, Fig. 1A) of the dorsal blastopore lip into the blastocoel of a host gastrula, which induced a secondary axis (Spemann and Mangold, 1924). Because of its central role in the organisation of the embryonic body axis, the dorsal blastopore lip has been called organizer (Spemann's organizer). Although the interest of embryologist in the 30ies and 40ies concentrated mainly on the problem of neural induction which is responsible for the formation of the central nervous system, the strongest progress in the last decades has been made in the study of the formation of the mesoderm. This process is one of the earliest events in embryogenesis.

In the unfertilized amphibian egg we can distinguish an animal/vegetal polarity, which can be recognized by the maternal pigment with the highest concentration at the animal pole and the nearly pigment free vegetal pole. The sperm entry causes cortical rotation and the establishment of the dorsal ventral polarity is indicated by the so called grey crescent, the future dorsal side of the embryo. However, it should be pointed out that a grey crescent, a zone of intermediate amounts of maternal pigment in the dorsal equatorial zone of the egg, can be observed in a few amphibian species only. The formation of the mesoderm (area of the marginal zone) is thought to occur by the interaction of the vegetal and animal hemisphere (Nieuwkoop, 1969; Tiedemann, 1993; Tiedemann *et al.*, 1995). It has been shown that the vegetal endoderm of blastula and gastrula stages induces in isolated ectoderm mesodermal tissues (Nakamura *et al.*, 1971; Asashima, 1975). Diffusible factors are responsible for this induction (Grunz and Tacke, 1986). It was concluded from these experiments that in the embryo mesoderm is induced by the endoderm. Recent experiments have shown that factors determining mesoderm are prelocated or processed in the dorsal vegetal zone (Kessler and Melton, 1995).

Abbreviations used in this paper: FGF, fibroblast growth factor; BMP, bone morphogenetic protein.

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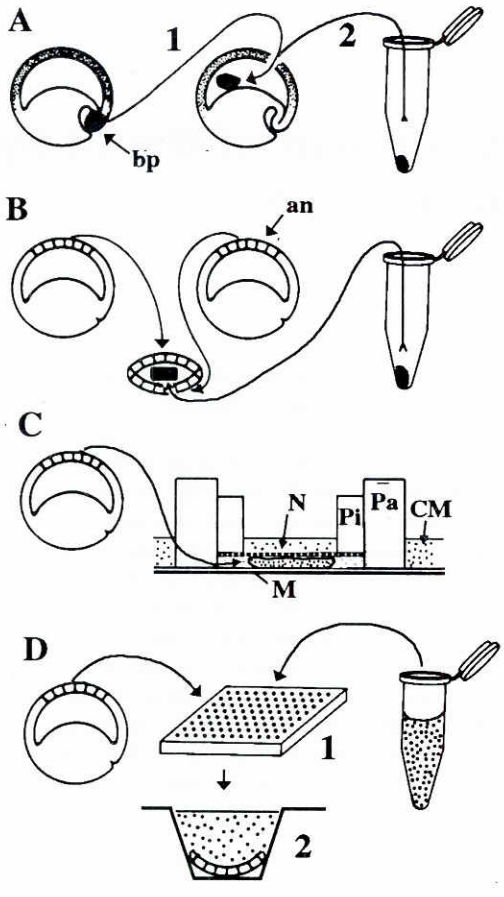

Fig. 1. Test methods. **(A)** *Implantation-method (Einsteck-Experiment after Spemann and Mangold, 1924): 1) blastopore lip; 2) pellet (insoluble factors or so-called heterogeneous inducers).* **(B)** *Sandwich-Technique (Holtfreter, 1933a) (insoluble factors or heterogeneous inducers). an, animal cap (ectoderm).* **(C)** *Nuclepore®-chamber (Grunz and Tacke, 1986; modified after Saxén, 1961) for tests of soluble factors, which can only act on the former blastocoelic side of the explant. The Nuclepore®-filter prevents the curling up of the ectoderm.* **(D)** *Test plate (1) with flat -bottom wells (Terasaki-plate) Test of soluble factors in small amounts of medium (10 µl) like FGF or activin (animal cap assay); (2) one well at higher magnification. N, Nuclepore-membrane; Pa, outer Plexiglas-ring; Pi, inner Plexiglas-ring; CM, culture medium; M, O_2 and CO_2-permeable membrane (Petriperm®, Fa. Heraeus). Further test methods are described by Hildegard Tiedemann (1986).*

Factors controlling the mesoderm formation

Good candidates as factors in the formation of mesoderm are activin and Vg 1, which belong to the TGFß superfamily of proteins. It could be shown that Vg1 is mainly located at the vegetal pole in early cleavage stages (Weeks and Melton, 1987; Tannahill and Melton, 1989). Recently it has been shown that Vg1 may be processed from an inactive precursor molecule (cleavage by a prelocalized protease) after cortical rotation mainly at the future dorsal vegetal side (Thomsen and Melton, 1993). From *in vitro* recombination experiments it has been proposed that Vg 1 and further factors may be present in a distinct center in the dorsal vegetal area of the egg (sometimes called Nieuwkoop center). However, there exist strong indications that mesoderm inducing factors are more likely distributed in gradients rather than in centers. Very recent reports of Doug Meltons' group indicate that mature Vg1 (processed Vg1) is found in a restricted area (dorsal vegetal zone) at the late blastula stage (Kessler and Melton, 1995). It could be shown that the four animal cells of eight cell stages are able to form dorsal mesodermal structures, which should not be the case if all mesoderm inducing factors are exclusively located in the subequatorial zone (Grunz, 1977; Elinson and Kao, 1989; Grunz, 1994). That growth factors and/or their receptors are distributed in the developing embryo as distinct gradients could be shown by antibodies against a bFGF receptor. The bFGF receptors could be mainly found in the animal and the equatorial zone of blastula and gastrula stages (Ding *et al.*, 1992). An important factor which plays a part in mesoderm formation is activin(Asashima *et al.*, 1990). A protein homologous to activin was at first isolated from 11-day-old chicken embryos by the group of Tiedemann (Tiedemann, 1959; Geithe *et al.*, 1981; Grunz *et al.*, 1989; Tiedemann *et al.*, 1992). Since it induced in the animal cap assay (Fig. 1B,C,D) differentiations derived from the vegetal part of the embryo (mesodermal and endodermal derivatives) it has been called vegetalizing factor. If activin-mRNA is injected into the ventral vegetal blastomeres of an eight-cell-stage or into the blastocoel a secondary embryonic axis with deficiencies in the head area is induced (Thomsen *et al.*, 1990; Ariizumi *et al.*, 1991). A complete secondary axis with head structures is formed after the injection of BVg1 in early cleavage stages. A secondary axis is also induced when an animal cap of middle blastula is treated with activin prior to its implantation (Einsteck-Versuch) into the blastocoel of an early gastrula (Ruiz i Altaba and Melton, 1989). This indicates that Vg1 and/or activin-like proteins play a central role in the establishment of the dorsal mesoderm. By our earlier results could be shown that activin(vegetalizing factor) is able to induce most of the dorsal structures in a dose dependent manner. While untreated animal caps (ectoderm) differentiate into ciliated epidermis, so called atypical epidermis (Grunz, 1973; Grunz *et al.*, 1975), increasing concentrations of activin result in the formation of mesenchyme, blood precursor cells, coelomic epithelium, muscle, notochord (Grunz, 1983). It could also be shown that the vegetalizing factor (homologous to activin) is able to change the surface structure and the cell affinity of treated ectoderm (Kocher-Becker *et al.*, 1965; Grunz, 1972, Grunz *et al.*, 1975). These results could be confirmed more recently, using recombinant activin (Moriya and Asashima, 1992; Moriya *et al.*, 1993; Green *et al.*, 1992). Under certain experimental conditions (dissociated ectodermal cells treated with activin) only one cell type (notochord) can be induced (Grunz and Tacke, 1989). A dorsal signal which may participate in the formation of Spemann's organizer is noggin (Smith *et al.*, 1993), while fibroblast-growth factors, factors of the Wnt-family (Christian *et al.*, 1991) and bone morphogenetic protein like BMP-4 and BMP-2 are considered as ventral mesoderm signals or mesoderm modifiers (Knöchel *et al.*, 1987, 1989; Grunz *et al.*, 1987; Köster *et al.*, 1991; Suzuki *et al.*, 1994; Graff *et al.*, 1994). There are many indications that the formation of Spemann's organizer is a multistep process starting shortly prior to gastrulation and lasting until the middle gastrula. The dorsalization of the organizer and its acquisition of activity to induce neural structures in the overlaying ectoderm takes place during the involution of the Spemann's

TABLE 1

DIFFERENTIATION OF DISSOCIATED ECTODERMAL CELLS AFTER TREATMENT WITH DIFFERENT SUBSTANCES

Series	treatment/ source of tissue	problem	conc.	stage	method	number of cases	atypical epidermis	cement gland	neural arch	neural deut.	notochord
1	control	shift of neural type	-	9	40 animal caps disaggregated, 2 h as single cells, then reaggregated, divided in 6 pieces and cultured for 3 days	6	-	6	6	-	-
2	retinoic acid (RA)	from anterior to posterior	10^{-7} M	9	40 animal caps disaggregated, 1 h as single cells, then addition of RA for 1 h and reaggregation	6	-	-	-	6(?)	
3	retinoic acid		10^{-6} M	9	40 animal caps disaggregated and treated as series 2	6	-	-	-	6(?)	
4	retinoic acid		10^{-7} M	9	40 animal caps disaggregated, 2 h as single cells, reaggregated and reaggregates treated for 4 h with RA	6	-	6	6	-	-
5	retinoic acid		10^{-6} M	9	40 animal caps diaggregated and treated as series 4	6	-	-	-	6(?)	-
6	retinoic acid		10^{-5} M	9	40 animal caps disaggregated and treated as series 4	6	-	-	-	6(?)	-
7	dorsal ectoderm	differences of dorsal or	-	9	50 animal caps disaggregated, 2 h as single cells, then reaggregated, divided in 6 pieces and cultured for 3 days	6	-	6	6	-	-
8	ventral ectoderm	ventral ectoderm	-	9	50 animal caps disaggregated, 2 h as single cells and then reaggregated and divided in 6 pieces and cultured for 3 days	6	-	6	6	-	-
9	control	neuralization by follistatin	-	9	40 animal caps disaggregated, immediately reaggregated divided in 6 pieces and cultured for 3 days	6	6	6	-	-	-
10	follistatin (FS)		500 ng/ml	9	40 animal caps disaggregated, immediately reaggregated, during and after reaggregation treatment with follistatin for 5 h	6	6	6	-	-	-
11	blastula-ectoderm	competence	-	8	40 animal caps disaggregated, immediately reaggregated	6	6	6	-	-	-
12	blastula-ectoderm			8	40 animal caps disaggregated, 3 h as single cells	6	-	6	6	-	
13	gastrula-ectoderm		-	9	40 animal caps disaggregated, immediately reaggregated	6	6	6	-	-	-
14	gastrula-ectoderm		-	9	40 animal caps disaggregated, 3 h as single cells	6	-	6	6	-	-
15	control	inhibition of	-	8+	40 animal caps disaggregated, immediately reaggregated	6	6	6	-	-	-
16	follistatin (FS) 250 ng/ml+EDF	neuralization	2 ng/ml EDF	8+	40 animal caps disaggregated, treated as single cells for 3 h simultaneously with EDF and FS prior to reaggregation	6	-	6	6	-	-
17	activin (EDF)		2 ng/ml	8+	40 animal caps disaggregated, treated as single cells with EDF for 3 h prior to reaggregation	6	-	-	-	-	6 large
18	activin (EDF)		0.2 ng/ml	8+	40 animal caps disaggregated, treated as series 17	6	-	6	6	-	6 medium
19	activin (EDF)		0.02 ng/ml	8+	40 animal caps disaggregated, treated as series 17	6	-	6	6	-	6 tiny
20	bFGF		10 ng/ml	8+	40 animal caps disaggregated, single cells with bFGF for 3 h	6	-	-	-	6	-
21	Suramin		50 µM	9	40 animal caps disaggregated, single cells with Suramin (3 h)	6	-	6	6	-	-
22	Suramin		150 µM	9	40 animal caps disaggregated, single cells with Suramin (3 h)	6	-	6	6	-	-

organizer (chordamesoderm) (Grunz, 1992, 1993a). These observations are in agreement with the fact that dorsal blastopore lip prior to involution induces trunk and tail structures, while the chordamesoderm after involution induces mainly head structures (Holtfreter 1936, 1938; Kaneda and Suzuki, 1983).

The dorsal mesoderm and the formation of the central nervous system

While there exist already many facts which may fairly well explain the processes of mesoderm formation, our knowledge about the formation of the central nervous system is still not well understood (reviewed by Saxén, 1989; Gilbert and Saxén, 1993; Green, 1994). Transfilter experiments indicate that diffusible factors are transmitted from the chordamesoderm to the reacting overlaying neuroectoderm (Saxén, 1961). This information transfer takes place by short distance migration of the factors supported by the close apposition of chordamesoderm and the neuroectodermal target cells (Grunz and Staubach, 1979; Tacke and Grunz, 1988). It could be shown that diffusible factors are located in the gap between the inducing chordamesoderm and the neuroectoderm (John *et al.*, 1983). Neuralizing factors have been partially purified from *Xenopus* gastrula and neurula stages and from the brain of chicken embryos (Mikhailov *et al.*, 1995). In the cytosol a small protein with neuralizing activity (15-25 kDa) prevails (Janeczek *et al.*, 1992). It could be shown that neural specific genes are expressed very early in development as a result of neural induction (Richter *et al.*, 1988; Oschwald *et al.*, 1991). Up till now it could not be finally decided, which and how many factor(s) are responsible for the neural induction of the neuroectoderm. Many data support the view that neural induction is a multistep process, in which several neural inducing factors may participate. Noggin, a protein, which is suggested to be a secreted protein induces in dorsal ectoderm neural structures without mesoderm formation (Smith and Harland, 1982; Lamb *et al.*, 1993; Smith *et al.*, 1993). However, it must be pointed out that neuralization of competent ectoderm takes place after treatment with relatively high ("unphysiological") concentrations of this protein (in the range of micrograms). It should be mentioned that even bFGF (a ventral mesodermal inducer in *Xenopus*) in high concentrations causes neural induction in *Triturus* (Tiedemann *et al.*, 1994).

Genes, which are expressed in the organizer area and during involution in the chordamesoderm like goosecoid (Cho *et al.*, 1991; Blumberg *et al.*, 1991), the forkhead homologs XFD-1, XFH1 (Knöchel *et al.*, 1992, Dirkson and Jamrich, 1992), pintallavis (Ruiz i Altaba, 1992) and XLIM1 (Taira *et al.*, 1992) are thought to play important roles in embryogenesis. Several genes containing a homeobox coding for transcription factors can be considered as important members of the hierarchy of various genes expressed mainly in the organizer area. In this context should be mentioned that the expression of the forkhead homolog XFD-1 is suppressed by the treatment with Suramin, while the expression of α-globin and BMP-4 - mRNA is increased (Oschwald *et al.*, 1993; Fainsod *et al.*, 1994). These results together with our earlier histological observations indicate

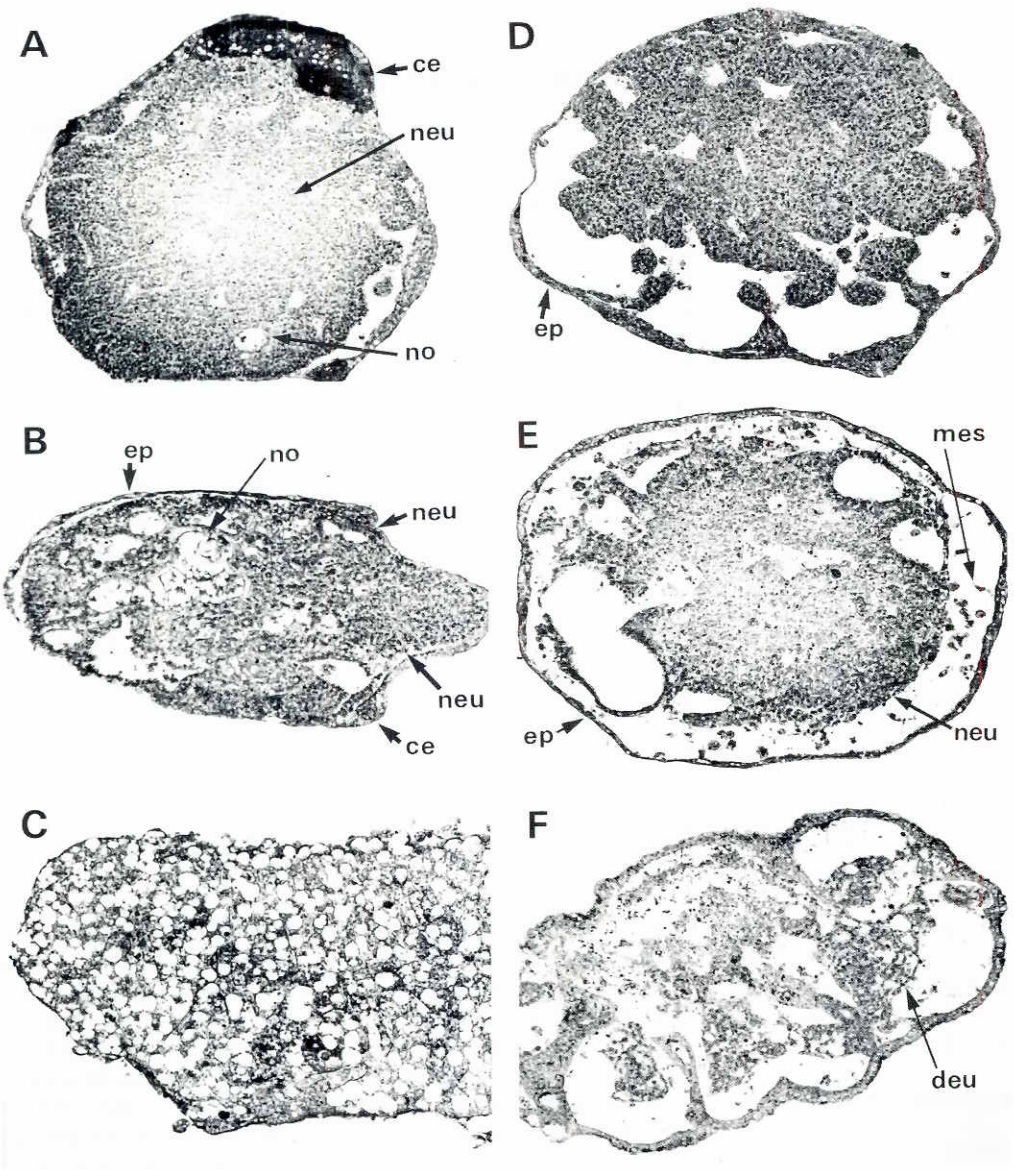

Fig. 2. Differentiation of ectoderm after dissociation and reaggregation *(details of the treatment see Table 1).* **(A)** *Disaggregated cells treated with 0.02 ng/ml activin (EDF) (Table 1, series 19). The reaggregated cells differentiated mainly into neural (archencephalic brain, neu) structures. A tiny notochord (no) was found in addition. ce, cement gland.* **(B)** *Disaggregated cells treated with 0.2 ng/ml activin (EDF) (series 18). Both archencephalic brain structures (neu) and a substantial amount of notochord (no) were found in this series 18. ce, small cement gland; ep, epidermis.* **(C)** *Disaggregated cells treated with 2 ng/ml activin (EDF) (series 17). The reaggregated cells differentiated totally into notochord. No other differentiation could be found in addition.* **(D)** *Disaggregated cells treated with 150 µM Suramin (series 22). The reaggregated cells differentiated into neural derivatives as untreated controls.* **(E)** *Disaggregated cells treated with 10 ng/ml basic fibroblast growth factor (bFGF) (series 20). The reaggregated cells formed mesenchyme (me) in addition to the brain structures (neu). The presence of mesenchyme and the histological features suggest that these differentiations are deuterencheplic brain structures. FGF is able to shift the pattern from anterior to posterior brain structures. ep, epidermis.* **(F)** *Disaggregated cells treated with 10 -7 M retinoic acid (series 2). The reaggregated cells formed neural derivatives, which differ distinctly from the archencephalic structures shown in* **(A and B)**. *We suggest that the structures are similar to deuterencephalic (deu) brain structures.*

that the dorsal blastopore lip differentiates after treatment with Suramin into ventral mesodermal derivatives like heart structures rather than into dorsal mesodermal structures like notochord and somites (Grunz 1992, 1993a). These results support the view that the dorsalization of the dorsal blastopore lip takes place during gastrulation (during the involution of the dorsal mesoderm).

It could be shown that the ectoderm of *Triturus alpestris*, *Triturus vulgaris* and *Ambystoma mexicanum* can be triggered into mesodermal derivatives by Lithiumchlorid (Masui, 1961; Grunz 1968, 1993b) and into neural structures by disaggregation of *Xenopus* ectoderm into single cells (Grunz and Tacke, 1989, 1990). Apparently such treatments interact with different steps in the signaling chain downstream of the receptor level on the plasma membrane, where in normogenesis proteineous ligands will initiate the inducing processes. So the activation of protein kinase C by the treatment with phorbolesters leads to the neuralization of ectodermal cells (Davids *et al.*, 1987; Davids, 1988; Otte *et al.*, 1988). Furthermore it could be shown that the activation of both protein kinase C and adenyl-cyclase followed by an increase of the level of cyclic AMP results in the neuralization of competent ectodermal cells (Otte *et al.*, 1989). However, the increase of cAMP alone does not result in neural induction (Grunz and Tiedemann, 1977; Otte *et al.*, 1989). Whether the neuralization of ectoderm after disaggregation and delayed reaggregation (Grunz and Tacke, 1989) is correlated with an activation of protein kinase C and cAMP is not yet known. Also a significant increase of the DAG could not be observed (Grunz and Geilen, unpublished results). There is good evidence that the extracellular matrix in concert with growth factors (inducing factors) plays an important role for the stabilization of determination of non induced ectoderm into epidermis (Grunz and Tacke, 1990, see discussion about a possible default state below). It is generally accepted that growth factors are associated with glycosaminoglycans (low affinity receptors) and may be presented to the tyrosine kinase recep-

tors (high affinity receptors) for initiating the further steps in the signal transmitting chain.

It has been speculated for many years (Holtfreter, 1934) that a masked (repressed) neuralizing factor is present in the ectoderm, which is activated by induction of the ectoderm to the nervous system (Grunz *et al.*, 1986). Recently it has been postulated that ectoderm will differentiate into epidermis, when a low level of activin will interact with its receptors on the ectodermal target cells (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.*, 1994). This conclusion has been drawn from experiments in which a truncated form of the activin receptor was expressed in the ectoderm resulting in the neuralization (expression of neural specific markers) of the ectoderm (Hemmati-Brivanlou and Melton, 1992, 1994). On the basis of these results it has been argued that the default state of the ectoderm is neural. We have tested in large number of series the influence of several substances and parameters on disaggregated cells (Table 1). The detailed dissociation method is described elsewhere (Minuth and Grunz, 1980; Grunz and Tacke 1989, 1990). It should only be mentioned that dispersed ectodermal cells immediately reaggreagated differentiate into atypical epidermis, while dissociated cells kept single for 2-3 h prior to the reaggregation will form neural (archencephalic brain) structures. We speculated that there may be a difference in the degree of neuralization when we isolated animal caps from middle blastulae or early gastrulae, respectively, and kept them as single cells for 2-3 h prior to reaggregation (problem of competence). However, there was no difference in neuralization (Table 1, series 11-14). In a further series we could show that there is no difference in the degree of neuralization, when the dorsal or ventral part of the animal cap has been cultivated for 2 h as single cells (series 7,8). These results are in contrast to other reports, which observed a different reaction of intact dorsal and ventral ectoderm to mesodermalizing factors (Sokol and Melton, 1991). Since it has been reported that the quality of neural structures is shifted from anterior to posterior by retinoic acid (Durston *et al.*, 1989), we speculated that also the neural structures in our disaggregation system could be shifted from archencephalic to more posterior neural structures. Although a clear histological analysis is quite difficult, the data suggest that the treatment with different concentrations of retinoic acid results in a shift from archencephalic to denterencephalic structures (Table 1, series 1-6; Fig. 2F). Since it has been reported that follistatin may be a neural inducer we treated dispersed ectoderm immediately after reaggregation with follistatin (series 10). However, also under these conditions (increased surface in contrast to intact ectoderm; compare Fig. 3) we did not observe a neuralization by the activin-antagonist. It has been suggested that the default state of the ectoderm is neural and that the neuralization is prevented by low concentrations of activin (Hemmati-Brivanlou and Melton, 1992, 1994). In our earlier paper we could show that the supernatant of disaggregated cells can prevent the neuralization of disaggregated ectodermal cells (Grunz and Tacke, 1990). It can be speculated that the extracellular matrix contains factors which have a suppressing effect on the default neural state. Therefore we wanted to find out if activin can prevent the neuralization of disaggregated cells and shifts the determination into epidermal pathway of differentiation To test this hypothesis, we added various concentrations of activin to dispersed ectodermal cells. At

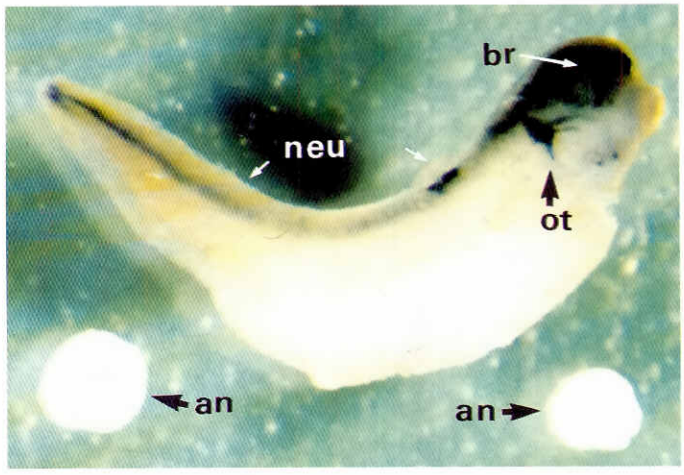

Fig. 3. Animal caps (competent ectoderm) of *Xenopus*- or *Triturus* - middle or late gastrulae were treated with 1, 10, 100 and 1000 ng/ml follistatin (recombinant human follistatin) for 6-8 h. *Several explants were cultured for 3 days for histological analysis. The cases shown above (Xenopus-early gastrula ectoderm treated with 1000 ng/ml follistatin) were fixed in HEMFA after 24 h for whole-mount in situ preparation with a neural specific ß-tubulin (Richter et al., 1988). In contrast to the larva (control) the explants (an) show no neural specific signals. Also by histological analysis (not shown) we could confirm that Xenopus- and Triturus- ectoderm treated with different concentrations of follistatin (1-10.000 ng/ml) differentiated in atypical (ciliated) epidermis only. neu, neural tube; br, brain with eye vesicle; ot, otic vesicle.*

lower concentrations activin did not prevent neuralization. Only a tiny notochord together with the large amount of archencephalic brain structures was formed (series 19, Fig. 2A). Increasing concentrations resulted in the differentiation of notochord in addition to neural structures (series 18, Fig. 2B). The highest concentrations in our experiments 2-4 ng/ml caused the total mesodermalization of the dispersed and reaggregated cells (series 17, Fig. 2C). The ectoderm formed exclusively notochord as has been reported earlier with XTC (crude activin) (Grunz and Tacke, 1989; Green and Smith, 1990). However, at no concentration of activin epidermis was formed. These results are in agreement with the report of Wilson and Hemmati-Brivanlou (1995). Dispersed ectodermal cells treated simultaneously with activin and follistatin differentiated into neural structures as expected (series 16). In a further series we could show that another growth factor, basic fibroblast growth factor (b FGF) cannot prevent neuralization. At relatively high concentration (10 ng/ml) mesenchyme is formed besides the neural structures (series 20, Fig. 2E). The presence of mesenchyme and the histological features suggest that these differentiations are deuterencephalic brain structures. These results are in agreement with data of other authors, who could show an anteroposterior shift after the treatment with FGF (Cox *et al.*, 1995; Doniach, 1995). However, a shift from neural type structures to epidermis was not observed. It should be mentioned that FGF alone can stimulate neuralization under certain experimental conditions (Kengaku and Okamoto, 1993; Tiedemann *et al.*, 1994; Lamb and Harland, 1995). Recent results of several authors indicate that the disruption of BMP signals in the animal cap is the essential step for the formation of neural tissue (Wilson and Hemmati-Brivanlou, 1995;

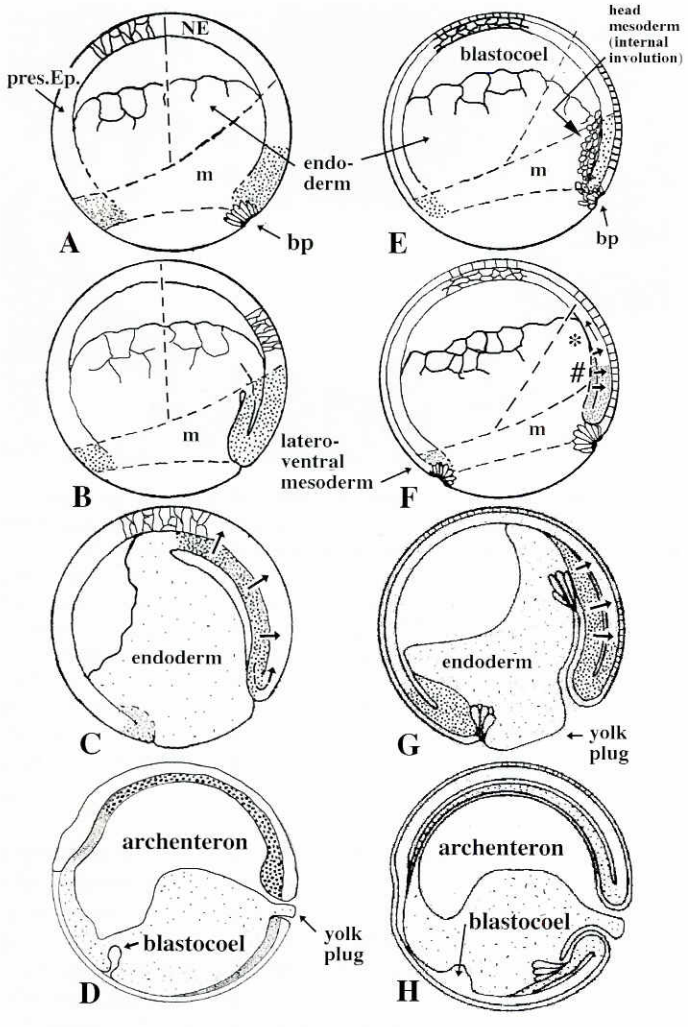

Fig. 4. Comparison of the gastrulation processes of Urodela *(Triturus alpestris)* (A-D) and Anura *(Xenopus laevis)* (E-H). *bp, blastoporus; m, marginal zone; neu, neuro-ectoderm; pres. Ep., presumptive epidermis.* **(A,E)** *Very early gastrula (stage 10, Nieuwkoop and Faber, 1956) In Xenopus the ectoderm and the marginal zone consists of two cell layers. Mesoderm is already located inside of the embryo.* **(B,F)** *Early phase of neural induction. Rolling-in of the marginal zone at stage 10-10.5. In Xenopus* **(F)** *the internal marginal zone only is moving to the animal pole (compare with plate 18, Hausen and Riebesell, 1991). #, zone of vertical signals; * , zone of postulated planar signals.* **(C,G)** *Advanced stage of neural induction by vertical signals. The primary steps of neural induction are nearly finished.* **(D,H)** *Small yolk plug stage. The primary steps of neural induction are now finished. The 3 germ layers have reached their final position.*

Xu *et al.*, 1995). When we treated dispersed ectodermal cells with 50 or 150 µM suramin, which prevents dorsalization of mesoderm and neural differentiation in dorsal blastopore lip (Grunz 1992, 1993; Oschwald *et al.*, 1993), the reaggregated ectoderm did not form epidermis, but even better differentiated neural structures than the untreated controls (series 21 ,22; Fig. 2D). It can be speculated that suramin blocks the receptors of BMP – or/and activin-like molecules which together with the dilution of BMPs in cultures of dispersed ectodermal results in the realization of the neural default state of the ectoderm. On the other hand it could be shown that suramin stimulates the expression of BMP-4 (Fainsod *et al.*, 1994). Up till now it is an unsolved problem how neural induction takes place in normogenesis. It can be argued that neural inducers emanating from the chordamesoderm interrupt the signal chain of BMP – or activin like molecules. Neural inducers synthesized by Spemann's organizer (chordamesoderm) could act like competitive inhibitors by binding to BMP - type I – or activin type II receptors, which may initiated the primary steps of neural induction. It could be possible that 'natural' inducers (Mikhailov *et al.*, 1995; Tiedemann *et al.*, 1995) bind to the receptor of BMP without initiating the dimerization of the receptor needed for the suggested BMP-signal transfer. Alternatively neuralizing factors could directly interact with BMP-like molecules, secreted from the ectodermal cells and stored in the extracellular matrix, preventing their migration to their receptors. It has been reported that noggin (Knecht *et al.*, 1995) and follistatin act as neural inducers. If mRNA of follistatin, an inhibitor of activin, is injected in one blastomere of the two-cell-stage, neural specific markers are expressed in the ectoderm of the early gastrulae (Hemmati-Brivanlou *et al.*, 1994). We have treated blastula and gastrula ectoderm of *Triturus alpestris* and *Xenopus laevis* with recombinant human follistatin (1-10,000 ng/ml for 4 h). The protein with a over 80% homology to *Xenopus* follistatin was a gift of Prof. Makoto Asashima, Tokyo (Fukui *et al.*, 1993). However, the treated ectoderm of both species (*Triturus* ectoderm is considered to be even more sensitive to neural stimuli than *Xenopus* ectoderm) did not express a neural specific marker (Fig. 3). Also in histological sections we did not observe neural structures, but atypical (ciliated) epidermis only. These data (Grunz and Schüren, unpublished) are in agreement with the results of Asashima's group received on *Xenopus laevis* (Asashima *et al.*, 1991b). The discrepancy between the results of Melton's group and Asashima's or our data can be explained by the use of different techniques and developmental stages. If follistatin mRNA is injected in early cleavage stages many secondary interactions could take place up till the early gastrula, which will cause the expression of neural specific markers in the gastrula ectoderm. It is unlikely that in our experiments the external follistatin could not interact with an activin-like factor located at the outside of the ectodermal cells. Therefore it can be ruled out with high probability that follistatin is a direct neuralizing factor. Also with the so-called scatter factor which was postulated to play a part in neural induction, we received negative results. It has been reported that hepatocyte growth factor/scatter factor may play a role in axis formation in the chicken embryo (Stern, 1993). This factor, a glycoprotein, has been primarily described besides its growth-factor-activities as a protein, which stimulates epithelial cells to leave their cell layer (Weidner *et al.*, 1990, 1991). We have tested recombinant scatter factor in different concentrations on both *Triturus* and *Xenopus* ectoderm. However, we did not observe any neural induction (Grunz, Weidner, Sachs and Birchmeier, unpublished results). Of special interest is the observation of Rao (1994) that a truncated form of brachyury (Xbra) consistently increased the amount of the neural specific marker NCAM in concert with bFGF (in low and high concentrations) or activin (in low concentrations). In these experiments bFGF or the truncated Xbra (so-called 304) did not induce

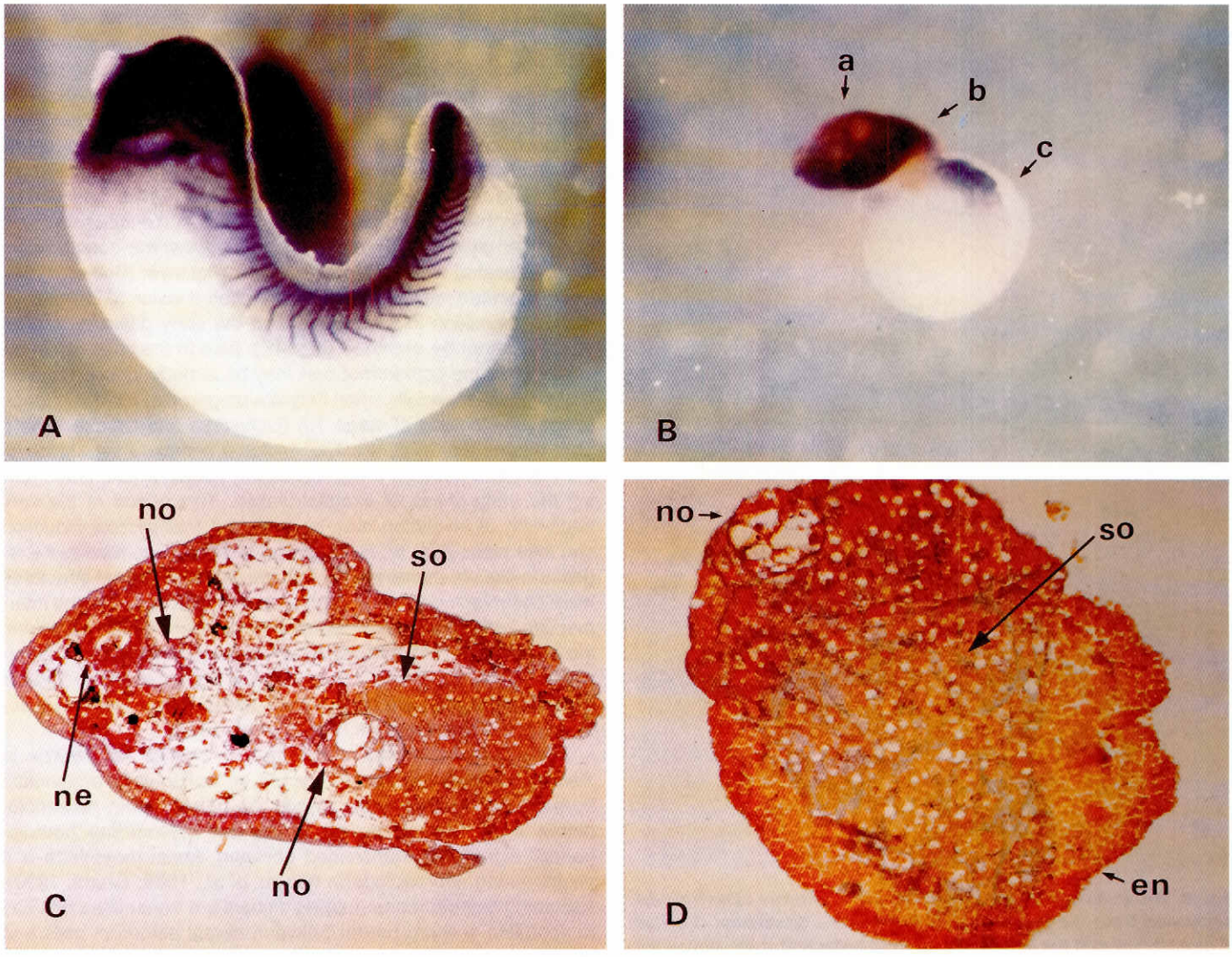

Fig. 5. Exogastrulae of *Xenopus laevis* have formed neural structures in addition to mesoderm derivative tissue. (A) *Whole-mount in situ preparation with the neural-specific marker np 187 (control embryo). (Dissertation C. Schüren, Essen).* **(B)** *Exogastrula, which shows the marker not only in the ectodermal zone (a) but also in the intermediate (b) and the meso-endodermal part (c).* **(C)** *Histological section of an exogastrula of the same series after 3 days' culture at 20°C. The transversal section (see arrow b in 5B) shows that neural structures are accompanied by notochord.* **(D)** *Transversal section of an exogastrula in the area (c) (see 5B). In this part of the endo-mesoderm neural structures were not observed. Transversal sections [not shown] of the distal part of the ectoderm (zone (a) in 5B) contain neural structures only. However, notochord together with neural structures is found in the neighbouring sections in the zone between (b) and (c). The differentiation of neural structures accompanied by notochord in both the ectodermal and meso-endodermal part explains the expression of neural markers and indicates that the neural structures were induced by vertical signals emanating from the chordamesoderm (notochord). no, notochord; ne, neural structures; so, somites; en, yolk-rich material (endoderm-derivative)*

NCAM expression by themselves, suggesting a synergy between 304 and bFGF or activin.

As already mentioned above the induction of the central nervous system is apparently a multistep process with many participating factors. The isolation, chemical characterization and test of one factor alone may not be sufficient to explain the complex process of neural induction. The number of factors responsible for neural induction is not yet known. Also the exact mechanism of the interaction between inducing tissue (organizer/chordamesoderm) and the reacting cells (dorsal ectoderm) is still obscure. The classical experiment of Spemann and Hilde Mangold (1924) and the exogastrula-experiment of Holtfreter (1933b) suggest that mainly vertical signals between chordamesoderm and overlaying neuroectoderm are the prerequisite for neural induction. In several recent articles has been postulated that planar signals starting from the dorsal blastopore lip and travelling through the ectoderm are as important as diffusible vertical signals migrating from the chordamesoderm to the overlaying neuroectoderm. These conclusions are based on three different approaches using *Xenopus* embryos, i.e. experiments with exogastrulae, so called Keller-sandwiches and open-faced explants of the dorsal blastopore lip (Doniach 1992; Doniach *et al.*, 1992; Keller *et al.*, 1992;

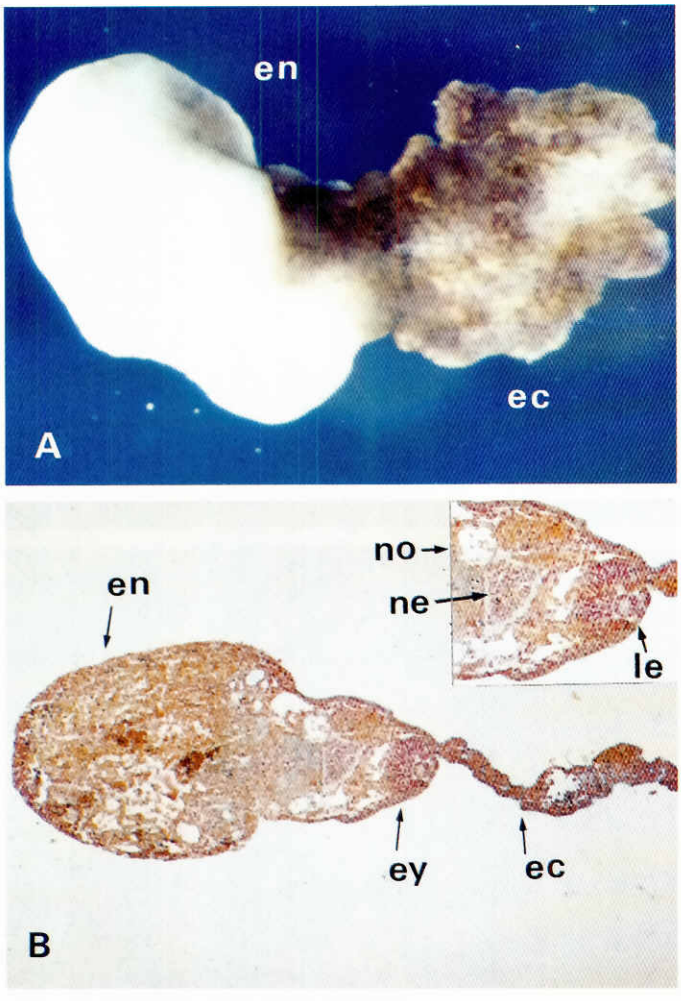

Fig. 6. The ectoderm of an early gastrula of *Triturus alpestris* was separated from the meso-endoderm by micro dissection. *A bridge remained between the ectoderm and meso-endoderm in the area of the blastopore (Grunz et al., 1995).* **(A)** *Pseudoexogastrula after 11 days' culture at 20°C. The ectoderm separated from the endo-mesoderm has differentiated into ciliated (atypical) epidermis.* **(B)** *Neural structures (brain structures and an eye with lens) have differentiated in the intermediate zone between endo- mesoderm and ectoderm only. The neural structures are found close to notochord (see inset). en, endoderm or derivatives; ec, ectoderm (atypical epidermis); no, notochord; ne, brain structures; ey, eye; le, lens.*

Ruiz i Altaba, 1992). In all sets of experiments it has been postulated that the ectoderm adjacent to the dorsal blastopore lip never had received a vertical signal, but nevertheless neural specific genes are expressed in the ectoderm in a anterior-posterior pattern similar to their expression in normogenesis (Doniach *et al.*, 1992). The authors argued that planar signals starting from the dorsal blastopore lip travel through the plane of ectoderm and induce four position - specific neural markers: engrailed-2, Krox-20, XlHbox 1 and XlHbox 6. In contrast to Holtfreter, Ruiz i Altaba (1990, 1992) received also in exogastrulae expression of neural specific markers in the ectodermal part. It must be pointed out that Holtfreter performed his exogastrulation experiments with Axolotl-embryos (Ambystoma mexicanum), while the recent data were received with *Xenopus*. We think that experiments with *Xenopus* alone are not sufficient to decide the exact role and importance of planar (horizontal) and vertical neuralizing signals (see also Nieuwkoop, 1993; Grunz *et al.*, 1995; Nieuwkoop and Koster, 1995). In contrast to urodeles (*Triturus* and Axolotl) in *Xenopus* ectoderm the area of the ectoderm and the dorsal blastopore lip consists of two distinct cell layers (see Fig. 4) (Asashima and Grunz, 1983; Grunz, 1985; Tacke and Grunz, 1986). Furthermore in contrast to urodeles in *Xenopus* and also in another pipid frog *(Hymenochirus boettgeri)* the dorsal mesoderm is located under a superficial epithelial layer (Bolker, 1994). Since the head mesoderm is already located inside of the embryo in close aposition to the ectoderm at the early gastrula, vertical signals cannot be excluded (Fig. 4E). Also in the case of Keller-sandwiches the ectodermal part may be already induced by vertical signals, especially when they are prepared at a too late stage (stage 10+ instead of stage 10) (Uchiyama and Otsuka, 1995). That means that in contrast to Axolotl a complete separation of the ectoderm from the endo-mesoderm does not take place during the early phase of exogastrulation. This period of the very beginning of involution may be sufficient that vertical induction can take place (Fig. 5). By a special technique we received exogastrula-like structures also in *Triturus alpestris*. Neural structures were observed close to chordamesoderm (notochord) in the intermediate zone between ectoderm and meso-endoderm. However, the distal part of the ectoderm differentiated into atypical epidermis only (Fig. 6). These results support the view that in *Triturus* neuralizing signals cannot be transmitted in the plane of ectoderm over a long distance (Grunz *et al.*, 1995).

The question is why in contrast to Axolotl or *Triturus* in *Xenopus* neuralizing signals should be horizontally transmitted from the dorsal blastopore lip into the neighbouring neuroectodermal layer. It would be more likely that mesodermal homoiogenetic signals are transmitted between dorsal mesoderm and neighbouring neuroectoderm (Grunz *et al.*, 1986; Grunz, 1990). Assuming that planar neuralizing signals are transmitted one has to postulate a sharp border between dorsal ectoderm cells and neighbouring neuroectoderm cells which have already lost mesodermal but not neural competence, which is the case at about stage 10.5 in *Xenopus* (Nieuwkoop and Faber, 1956). Since in Axolotl or *Triturus* ectoderm mesodermal competence is present up till mid-gastrula the dorsal mesoderm may in exogastrulae induce neighbouring ectoderm into the mesodermal rather than into the neural pathway of differentiation. Both the absence of the superficial epithelial sheet covering the mesoderm and the longer presence of mesodermal competence may prevent neuralization of ectoderm in Axolotl and *Triturus*. Experiments with *Triturus alpestris* indicate that planar signals during the early phase of neural induction are extremely unlikely in urodeles (Grunz *et al.*, 1995). It must be pointed out that also in *Xenopus* planar signals are unlikely for the terminal differentiation of neural structures. Vertical signals emanating from the involuting chordamesoderm are essential for the final determination of neuroectoderm into brain structures during gastrulation.

Conclusions

Seventy years after the discovery of the organizer phenomena the study of mesoderm induction and axis formation in the

amphibian embryo has attracted again the interest of many laboratories. In the last decade experiments using both molecular and traditional embryological techniques tremendously increased our knowledge about the early steps of embryogenesis. There exists quite a lot of information about maternal factors including transcription factors, which are already present in the unfertilized egg and early cleavage stages and which are thought to be important for the epigenetic development of the embryonic structures (Asashima *et al.,* 1991; Dohrmann *et al.,* 1993; Regabgliati and Dawid, 1993) Several proteineous factors have been identified belonging to the FGF-, TGFß- or Wnt-superfamilies. There are strong indications that these factors and/or their receptors are located in distinct gradients during the early cleavage, blastula and gastrula stages. The prelocation and the activation of different factors in restricted areas of the embryo are the prerequisite for the temporal and spatial expression of certain genes. Of particular interest was the observation that the expression of several genes takes place predominantly in the organizer area or the marginal zone. Purely understood is still the chain of events leading to neural induction and formation of the central nervous system. Recent data indicated that the suppression of epidermal tendencies will cause neuralization of competent ectoderm (neural default state of the ectoderm). There is growing evidence that neural induction is a multistep process. "Natural" neuralizing inducers emanating from the chordamesoderm during the involution process could interact with factors (BMP-like factors), which are synthesized in the intact ectoderm and which prevent the shift from the epidermal to the neural default state.

Therefore we suggest that neuralizing factor(s) transmitted as vertical signals from the chordamesoderm to the overlaying ectodermal target cells act as inhibitors (repressors) rather than instructive inducers on an uncommitted ectoderm (permissive reaction) during the primary steps of neural induction.

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