Int. J. Dev. Biol. 39: 317-325 (1995)

Original Article

Partially purified factor from embryonic chick brain can provoke neuralization of *Rana temporaria* and *Triturus alpestris* but not *Xenopus laevis* early gastrula ectoderm

ALEXANDER T. MIKHAILOV[1#], NIKOLAI A. GORGOLYUK[1], LOTHAR TACKE[2], MIKHAYL M. MYKHOYAN[1] and HORST GRUNZ[2*]

[1]*Laboratory of Organogenesis, Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia and* [2]*Department of Zoophysiology, University GH Essen, Essen, Germany*

ABSTRACT A high neuralizing activity has been determined in forebrain of 7.5-day old chick embryos using *Rana temporaria* early gastrula ectoderm as reacting tissue (Mikhailov and Gorgolyuk, *Soviet Scientific Reviews, Section of Physiology and General Biology, Vol. 1:* 267-306, 1987). The corresponding protease-sensitive agent was extracted, partially purified by chromatography on DEAE-Toyopearl and Heparin-Ultragel columns, and its neuralizing activity was tested *in vitro* on ectoderm isolated from early gastrulae of *R. temporaria, Triturus alpestris,* and *Xenopus laevis* at different concentrations and for different periods of time (animal cap assay). Induction of neural structures was found in *R. temporaria* and *T. alpestris* explants (up to 100 and 60%, respectively), but not in cultures of *X. laevis* ectoderm. Under our experimental conditions, so-called "autoneuralization" of the ectoderm explants can safely be excluded. The results are discussed in relation to the neural competence of amphibian ectoderm and the mechanisms of neuralizing actions of different factors which might be involved in neural induction and patterning.

KEY WORDS: *amphibian ectoderm, neuralizing factors, neural competence, specie-specific differences, neural induction*

Introduction

In amphibian gastrula, the primordium of the central nervous system is induced in the ectoderm by the signals from the dorsal mesoderm (Spemann and Mangold, 1924). Although studies on the mechanisms of neural induction have a long history, the direct attempts to isolate the extracellular mediators of this process using biochemical purification have not been successful yet (for reviews see Saxén, 1989; Tiedemann, 1990; Tiedemann *et al.*, 1993; Dawid *et al.*, 1990; Yamada, 1990; Grunz, 1992; Slack and Tannahill, 1992; Mikhailov and Gorgolyuk, 1992a).

It has been suggested that the early gastrula ectoderm (a target tissue for neuralizing factors) balances between epidermal and neural differentiation pathways. A variety of agents can shift this balance toward neural direction (see Slack, 1993). Moreover, neural differentiation of the amphibian gastrula ectoderm *in vitro* can proceed without any additional influences. In particular, axolotl ectoderm can be easily neuralized even under physiological culture conditions (so-called "autoneuralization"; see Tiedemann, 1984). In contrast to that, early gastrula ectoderm of *Xenopus laevis, Triturus alpestris, Pleurodeles waltl,* and *Rana temporaria* is resistant to such "autoneuralization" (Grunz and Tiedemann, 1977; Siegel *et al.*, 1985; Mikhailov, 1988). However, neuralization of *X. laevis* (Grunz and Tacke, 1989) and *P. waltl* (Saint-Jeannet *et al.*, 1990) gastrula ectoderm can result from its dissociation into single cells followed by delayed reaggregation.

Furthermore, *Xenopus* and *Triturus* ectoderm can be stimulated by phorbol esters to differentiate into neural structures (Davids *et al.*, 1987; Otte *et al.*, 1988, 1989). Under these conditions the neuralization of the ectoderm is mediated by several transduction pathways into target cells. However, it is still an open question, how the binding of extracellular ligands with their receptors on the plasma membrane of ectodermal cells could initiate the corresponding intracellular "neuralizing cascade". It has been shown that diffusible macromolecular factors are transmitted between the inducing chordamesoderm and the reacting ectodermal

Abbreviations used in this paper: ECM, extracellular matrix; PMSF, phenylmethylsulfonyl fluoride; MEM, minimal essential medium; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; PAAG, polyacrylamide gel; NGF, nerve growth factor; FGF, fibroblast growth factor; BSA, bovine serum albumin; Con A, Concanavalin A; TPA/PMA, tetradecanoyl phorbol acetate; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; ABC, archencephalic brain complex.

***Address for reprints:** Department of Zoophysiology, University of GH Essen, Universitätsstr. 5, 45117 Essen, Germany. FAX: 49.201.1833173.

#Present address: Department of Cell and Molecular Biology, University of Coruña, Campus de Zapateira s/n, E-15071 La Coruña, Spain.

0214-6282/95/$03.00

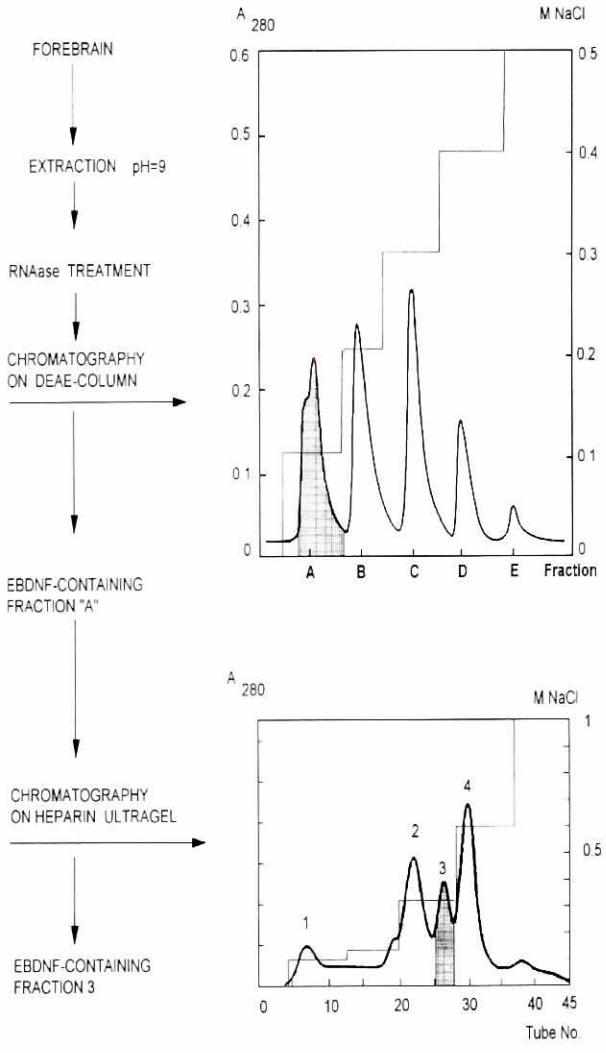


Fig. 1. Partial purification of the neuralizing activity from forebrain of 7.5-day old chick embryos. (Left) *Steps of extraction and purification.* **(Right)** *Profile of brain extract elution from a DEAE-Toyoperl 650 M column; neuralizing activity of fraction "A" (450 μg/ml) - 80%. Earlier the neuralizing activity of chick embryo brain was designated by us (Mikhailov and Gorgolyuk, 1989) as EBDNF (embryonic brain derived neuralizing factor). Profile of fraction "A" elution from a Heparin-Ultragel column; neuralizing activity of fraction 3 (80 μg/ml) - 40%.*

cells (Saxén, 1961, 1989; John *et al.*, 1983; Tacke and Grunz, 1988), but so far such factors could not be precisely characterized.

On the other hand, it has been demonstrated that chick embryonic brain extracts have a high neuralizing effect on *R. temporaria* early gastrula ectoderm (Mikhailov and Gorgolyuk, 1987). Several indirect data have shown that these extracts contain not dialysable, protease-sensitive agent(s), which provoked the neuralization of *R. temporaria* ectodermal explants in dose-dependent manner (Mikhailov and Gorgolyuk, 1989). Subsequent experiments demonstrated that it can be partially purified by ion-exchange and affinity chromatography (see Fig. 1) and the resulting preparation is capable of binding heparin. These facts suggest that the neuralizing "components" of this preparation can be bound to ECM produced by gastrula ectoderm cells.

This paper deals with data on the neuralizing activity of chick embryo brain fractions as tested on the early gastrula ectoderm of not only *R. temporaria* but also *X. laevis* and *T. alpestris*.

Results

Separation procedure and characterization of the fractions with neuralizing activity

The high neuralizing activity has been identified in the brain and neural retina of 7-8-day-old chick embryos using *R. temporaria* ectoderm as a test-tissue (Mikhailov and Gorgolyuk, 1987, 1989, 1992a; Mikhailov, 1990). These extracts induced archencephalic complexes and "unspecified" well-differentiated bran tissue (without mesodermal structures) in 80-90% of cultures. The isolation procedure of partially purified neuralizing factor from chick embryo forebrain included (Fig. 1): extraction with deionized water, pH 9.0; treatment with RNAse; ion-exchange chromatography on a DEAE-column, pH 8.0; affinity chromatography on a Heparin-Ultragel column. It could be extracted with distilled water at pH 9,0 but not with acidic (pH 2-4) solutions. The activity was found in the supernatants after ultracentrifugation of the extracts. Sediments obtained after the centrifugation of brain extracts at 20000g and then at 105000g (microsomal fraction) did not show any neuralizing activity in the animal cap assay. Neuralizing activity of the brain supernatants is termolable, resistant to lyophilization and RNAse treatment but sensitive to proteinase K.

Incubation with Protein A-Sepharose did not affect the neuralizing activity of the brain fraction (see Fig. 1, fraction "A"), while the treatment with Concanavalin (Con A)- or Heparin-Sepharose reduced its neuralizing effect by 30% and 50%, respectively. Addition of heparin (1 mg/ml) to the culture medium with brain extract or fractions (see Fig. 1, fraction "A") significantly decreased neuralizing activity.

Ion-exchange chromatography experiments have shown that the entire neuralizing activity of the "starting" brain extract is bound to the carrier although the amount of adsorbed protein was about 50% of the total protein applied on the DEAE-column. Fraction «A» (elution with 100 mM NaCl) proved to be most active. SDS-PAGE analysis demonstrated that this fraction still contains many polypeptides present in the initial extract (Fig. 2, lane 3). According to the analytical isoelectrofocusing data (5% PAAG-slab containing 7 M urea and ampholytes, pH 3-10), fraction "A" consists of several "bands" located in a 5.0-6.0 pH range (Mikhailov and Gorgolyuk, 1992a). The yield of fraction "A" was about 6% of the total soluble protein of brain extract or 0.3-0.4% of wet tissue weight. The neuralizing activity of fraction "A" calculated per total protein was in 4-5 times higher that of the initial brain extract. At the concentration levels of 1000 μg/ml, this fraction could provoke the formation of archencephalic brain complexes (ABCs) in 80-90% of cultures (see Tables 1, 2 and Fig. 3), while the starting brain extract induced ABCs in only 30-50% of explants (Mikhailov and Gorgolyuk, 1987).

In the next stage of separation, fraction "A" was applied onto a column with heparin. About 50% of the fraction only protein was bound with column. The highest neuralizing activity was found in a fraction eluted by 250 mM NaCl (see Fig. 1, fraction 3). Its neuralizing activity (50%) did not statistically differ from that of total heparin binding protein (elution in bulk by 1 M NaCl), although the concentrations in culture medium were 100 and 1000 μg/ml, respectively (Mikhailov *et al.*, 1993). The yield of fraction 3 was about 10% (by protein) of fraction "A", 0.3-0.4% of extracted brain protein or 0.01-0.02% of the tissue wet weight. In the obtained

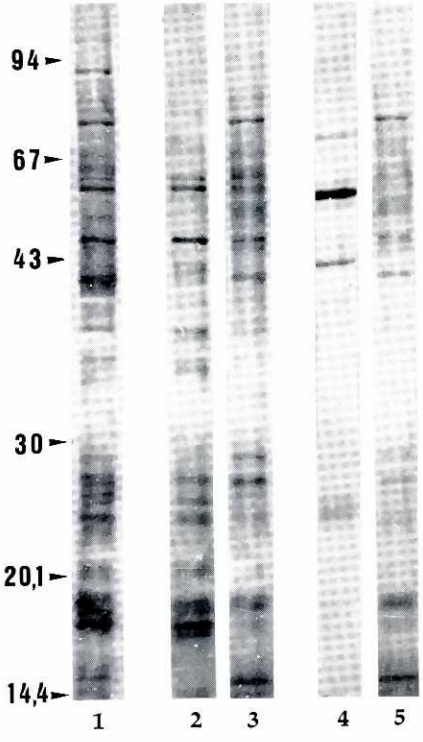


Fig. 2. Silver stained gel of the extract and chromatographic fractions of chick embryo brain, separated using 10% SDS-PAAG. (Lane 1) *Initial brain extract, neuralizing activity (2000 μg/ml) – 80%.* **(Lane 2)** *DEAE-unbound "fraction" of brain extract, neuralizing activity (1500 μg/ml) – 0%.* **(Lane 3)** *Fraction "A" eluted from DEAE column by 100 mM NaCl, neuralizing activity (450 μg/ml) – 80%.* **(Lane 4)** *Fraction 3 eluted from heparin column by 250 mM NaCl, neuralizing activity (100 μg/ml) – 40%.* **(Lane 5)** *Heparin-unbound proteins of fraction "A", neuralizing activity (1000 μg/ml) – 50%. 14,4 - 94, Molecular weight standards, kDa.*

preparation SDS-PAGE revealed two major fractions of about 40 and 60 kDa (see Fig. 2, lane 4). The neuralizing fraction containing 30 kDa- and 60 kDa-polypeptides had previously been electrophoretically isolated by us from the same brain extract (Mikhailov, 1988).

At the concentration levels of about 100 μg/ml, the neuralizing effect of fraction 3 was 40-50%, while fraction "A" provoked the neuralization in 20% of explants only (see Table 2). However, in the explant culture experiments (animal cap assay, see below) we used fraction "A" obtained after ion-exchange chromatography because it has been shown that the total neuralizing activity of the brain extract was bound to the DEAE-column and could be completely eluted from the carrier as a separate peak (see Fig. 1).

At the same time, no more than 50% (by protein) of fraction "A" was bound with heparin-containing column and the neuralizing effect of fraction 3 was about 40% of the total neuralizing activity of fraction "A". It should be remembered that Heparin-Sepharose treatment of fraction "A" (Mikhailov *et al.*, 1993) decreases its neuralizing activity by about 50% as well.

Biological tests with R. temporaria ectoderm

The neuralizing effect of fraction "A" depends on its time-exposure with the explants and concentration in culture medium.

TABLE 1

TISSUES FORMED IN THE EXPLANTS OF *R. TEMPORARIA* GASTRULA ECTODERM TREATED WITH FRACTION "A" (900 μg/ml) FOR DIFFERENT PERIODS OF TIME (1-18 h)

Exposure time (h)	Number of cultures	Induced structures, n (%) Brain tissue*	ABC	Cartilage	Muscle	Epidermis**
1	18	4(22)	2(11)	0	0	8 (44)
3	17	6(35)	4(23)	1 (6)	0	8 (47)
18	14	14(100)	11(80)	0	0	9 (64)

*ABCs and "unspecified" brain tissues; **linear epidermis in addition to atypical epidermis.

When *R. temporaria* explants were treated with fraction "A" (900 μg/ml) for 1, 3 or 18 h the neuralization was 20%, 35% and 100%, respectively (Table 1).

At 18-h exposure, the neuralizing effect of fraction "A" appeared to be dose-dependent. Fraction "A", at the concentration of 50.0 μg/ml did not lead to any neuralization. A minimal effect was first observed at about 100 μg/ml. When the concentration of fraction "A" was increased, the percentage of neural inductions increased as well and reached the maximum (100%) at 900 μg/ml (Table 2).

Using high concentrations of fraction "A" (900-1000 μg/ml), we could also obtain the neuralization (100%) of the explants (n 5) which were not covered with the rings preventing their "closing" (the time of complete "closing" of the inner ectodermal layer was 6-7 h).

In addition to neural tissue (Fig. 3), cartilage and muscle occasionally formed in the *R. temporaria* explants treated with fraction "A". The initial extract from the forebrain of 7-8-day-old chick embryos induced these structures in 10-20% of cultures (Mikhailov, 1988). We suggest that the ion-exchange chromatography of the brain extract did not result in complete separation of neuralizing and mesodermalizing activities.

TABLE 2

TISSUES FORMED IN THE EXPLANTS OF *R. TEMPORARIA* EARLY GASTRULA ECTODERM TREATED WITH DIFFERENT CONCENTRATION (50-1000 μg/ml) OF FRACTION "A" FOR 18 h

Fraction "A" (μg/ml)	Number of cultures	Induced structures, n (%) Brain tissue*	ABC	Cartilage	Muscle	Epidermis**
0	30	0	0	0	0	6(20)
50	15	0	0	0	0	4(27)
112	14	3(21)	1(7)	0	0	4(29)
225	14	9(64)	5(36)	0	0	9(64)
450	16	13(81)	9(56)	0	0	10(63)
750	18	15(83)	10(55)	3(16)	4(22)	11(64)
900	14	14(100)	11(79)	0	0	9(64)
1000	35	34(97)	31(89)	2(6)	0	21(63)

*ABCs and "unspecified" brain tissues; **linear epidermis in addition to atypical epidermis.

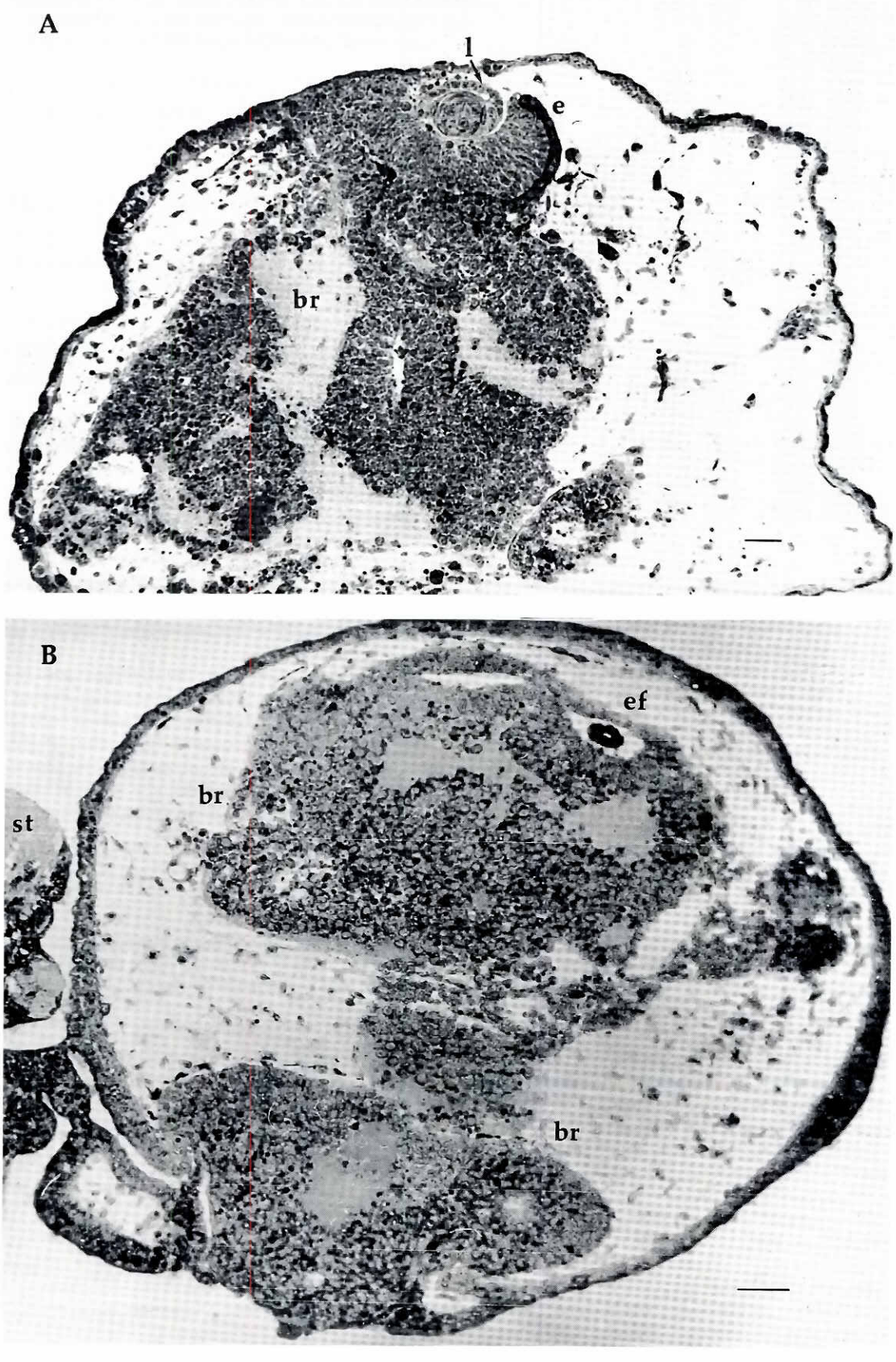


Fig. 3. Brain tissue induced in *R. temporaria* early gastrula ectoderm by fraction "A". (A) *Archencephalic brain (br) complex with well formed eye (e) containing the lens (l) (fraction "A", 450 µg/ml);* **(B)** *The explant was completely transformed into large epithelial vesicle containing brain (br) tissue and eye fragment (ef); st, sucker tissue (fraction "A", 750 µg/ml). Bars, 50 µm.*

TABLE 3

NEURAL TISSUE FORMED IN EXPLANTS OF *T. ALPESTRIS* EARLY GASTRULA ECTODERM TREATED WITH FRACTION "A" (1000 μg/ml) FOR 12 h

Fraction "A" (μg/ml)	Number of cultures	Induced structures, n (%) Brain tissue*	ABC	Linear epidermis	Atypical epidermis
0	15	0	0	0	15(100)
1000	18	11(61)	11(61)	11(61)	18(100)

*ABCs and "unspecified" brain tissues.

A weak mesodermalizing effect of brain extract and fraction "A" is probably due to the presence of trace amounts of fibroblast-like growth factors (FGFs) in brain tissue of 7-8-day old chick embryos (Risau *et al.*, 1988) or to a touch of blood vessels and blood elements in isolated forebrains, because neuroblastoma cell lines were shown to have almost no mesodermalizing activity (Lopashov *et al.*, 1992). This view is supported by the fact that *Xenopus* ectoderm differentiated into ventral mesodermal structures after treatment with fraction "A" (see Table 4).

In control explants cultivated in MEM/2 (n 30) or MEM/2 with rabbit gamma-globulin (n 15) no neuralization was observed (Table 2).

Biological tests with T. alpestris ectoderm

Animal caps of *T. alpestris* treated with fraction A (1000 μg/ml) for up to 12 h differentiated in 60% of the cases into ABCs only (Fig. 4) without any mesodermal structures (Table 3). On the other hand, in the control series, animal caps (n 15) incubated in Barth solution with 0.1% BSA differentiated into ciliated (atypical) epidermis.

Biological tests with X. laevis ectoderm

Animal caps of middle and late blastulae (stage 8 or 9, respectively) or early gastrulae (stage 10) treated with fraction "A" (500-4000 μg/ml) did not show any neural differentiations. However, the ventral-type mesoderm inductions (blood precursor cells, coelomic epithelium, heart structures and small amounts of somite muscle) at relatively high percentage were detected in these experiments (Fig. 4). The highest induction of heart structures (43-44%) could be observed in explants treated with fraction "A" at the concentrations of 1 and 2 mg/ml during 6 or 3 h, respectively (Table 4). These differentiations may be caused by the mesodermalizing activity of a component (perhaps FGF-like) in the not highly purified preparation. However, the same concentration of fraction "A" (1000 μg/ml) had no mesodermalizing activity on *T. alpestris* explants (see Table 3).

Untreated control explants (n 15) differentiated into ciliated (atypical) epidermis only.

Discussion

In this paper we describe the isolation procedure of a neuralizing factor from brain of chick embryos. The high molecular anionic complex in the 105000g supernatant of chick brain extract was partially purified. In this complex the neuralizing factor(s) is probably present primarily in inactive form. However, under our experimental conditions it could be partially activated by the extraction procedure at high pH (pH 9) and following RNAase treatment. The binding of 50% (by protein) of fraction "A" to Heparin-Ultragel column representing 50% of its neuralizing activity, indicates that either several complexes are present in fraction "A" or that a single complex is modified (and/or partially inactivated) by the isolation procedure. The first possibility is more likely.

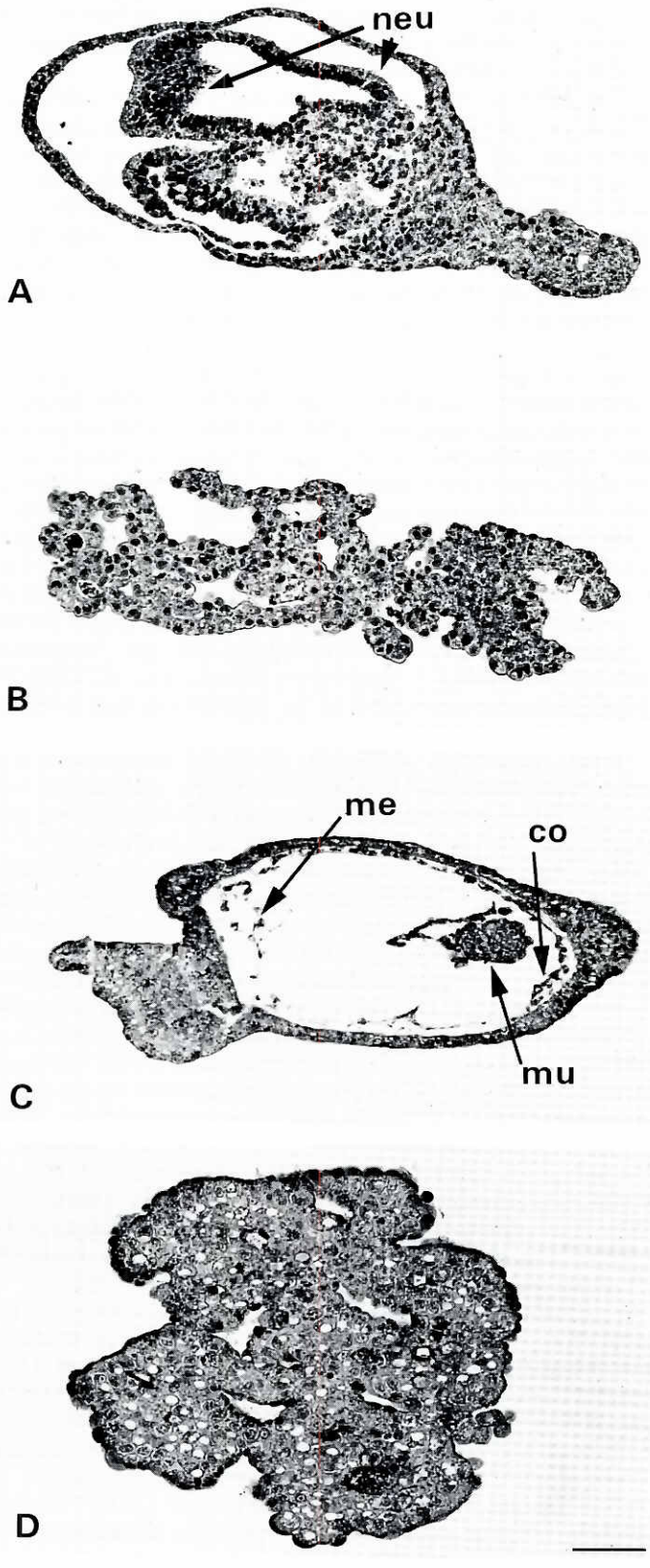
The biological test (animal cap assay) shows that middle/late blastula and early gastrula ectoderm of different amphibian species is characterized by "selective sensitivity" to the treatment with partially purified neuralizing factor. We found neural induction in *R. temporaria* and *T. alpestris* explants but no neuralization was detected in ectodermal cultures of *X. laevis*. This was observed in explants treated for different periods of time with different concentrations of brain fractions, which ranged from very high (1-4 mg/ml) to subphysiological (50-100 μg/ml) levels.

A possible explanation of these results is that the ectoderm of *X. laevis* is more resistant to "unspecific" neuralization than *R. temporaria* and *T. alpestris* ectoderm (see Slack, 1993). However, in controls we could not detect the neuralization of *R. temporaria* and *T. alpestris* ectoderm. Moreover, it has been shown that *R. temporaria* early gastrula ectoderm does not become neuralized *in vitro* after treatment with the following agents: cyclic nucleotides, forskolin, calcium ionophores, ouabain, Hepes-buffer, venom nerve growth factor (NGF) and NGF-like factors from chick embryos, chorionic gonadotropins, heparin, serum proteins, etc. (Mikhailov and Gorgolyuk, 1987, 1992a,b; Mikhailov, 1988, 1990). Similar results have been obtained in analogous tests with *T. alpestris* early gastrula ectoderm (Siegel *et al.*, 1985; Grunz, 1987) with the exception of the neuralization of the explants exposed to Hepes buffer (Tiedemann, 1986).

Using monoclonal antibodies, we could demonstrate that mammalian-like cytokeratin N 8 (MW 57 kDa) is expressed at very low level only in animal pole ectoderm but not in blastopore lip and vegetal pole cells of *R. temporaria* early gastrula (Simirskyi *et al.*, 1991). The expression of this epidermal marker is significantly increased during *in vitro* cultivation of *R. temporaria* animal caps under neutral conditions (Mikhailov and Gorgolyuk, 1992a); similar results have been obtained (using anti-cytokeratin N 8 antibody) with ectoderm of *P. waltl* (A-M. Duprat, personal communication).

Application of the tumor promoter, tetradecanoyl phorbol acetate (TPA or PMA), which is an activator of protein kinase C (PKC), had a strong neural inducing effect on *Triturus* (Davids *et al.*, 1987) and *Xenopus* (Otte *et al.*, 1988) early gastrula ectoderm, when used at high concentrations exceeding the pharmacological doses of the agent. In analogous experiments performed with *R. temporaria* explants (TPA concentration in culture medium 2-150 nM), no neuralization could be observed and the PKC activity of control and TPA-treated cultures was practically indistinguishable. Oleoyl acetyl glycerol, an activator of PKC, did not produce any neuralization of *R. temporaria* explants (Mikhailov and Gorgolyuk, 1992a).

It can also be suggested that the early gastrula ectoderm of the amphibian species studied is characterized by different developmental dynamics of neural competence. There is some indirect evidence that the neural competence of ectoderm of *R. temporaria* and *X. laevis* is quite different (Holtfreter, 1938; Servetnick and Grainger, 1991). We have observed the induction of neural tissue in *R. temporaria* explants treated with fraction "A" for 1 or 3 h (see Table 1). *Xenopus* animal caps started to curl up about 40 min after explantation and the inner ectoderm layer (the receptive surface) became completely hidden within 4-5 h. It is



unlikely that during this period of time *Xenopus* ectodermal cultures were inaccessible to fraction "A" and could completely lose neural competence (Lamb *et al.*, 1993). On the other hand, animal caps isolated from early gastrulae of *X. laevis* (Grunz, 1985), *T. pyrrhogaster* (Takata *et al.*, 1981) and *R. temporaria* (Mikhailov and Gorgolyuk, 1988) were neuralized *in vitro* when the explants were treated with Con A (100 μg/ml) for 1 or 3 h. Hence, the absence of neural induction of *X. laevis* ectoderm after the exposure to fraction "A", in contrast to *R. temporaria* and *T. alpestris* ectoderm, cannot be explained by a quick loss of neural competence in *Xenopus*.

This view is supported by the fact that we have detected the ventral mesodermal structures in *Xenopus* explants treated with fraction "A" which shows that the ectoderm was still competent during at least 3 hours after explantation (Table 4). It should be pointed out that in *Xenopus* and also in other amphibian species the mesodermal competence is lost even faster than the neural competence of the ectoderm. At the same time, we could not detect any mesodermal inductions in *Triturus* explants treated with the same concentrations of fraction "A". These results cannot be interpreted as a quick loss of mesodermal competence in *T. alpestris* ectoderm. It has been shown that the mesodermal competence of isolated early gastrula ectoderm of *T. alpestris* declines after about 15 h of cultivation *in vitro* and has completely vanished after 18-20 h (Nieuwkoop *et al.*, 1985).

Furthermore, it could be shown that *Xenopus* ectoderm differentiates into neural structure in 100 % of the cases, when animal caps are dissociated into single cells and reaggregated after 2-4 h in Calcium/Magnesium containing medium (Grunz and Tacke, 1989). These data suggest that inhibitory molecules are present in the intact ectoderm tissue. This view is supported by the observation that extracellular matrix components are able to prevent neural differentiation of dissociated *Xenopus* ectodermal cells (Grunz and Tacke, 1990). A neuralizing signal might represent a removal or lifting of such inhibition. We have suggested (Mikhailov and Gorgolyuk, 1992a) that the removal of neural inhibition could be achieved via several pathways in which different factors have an essential role to play.

Recent reports corroborate this standpoint. The expression of a dominant negative mutant of the activin receptor not only prevents the response of the ectodermal cells on mesodermalizing stimuli (activin), but shifts the ectoderm into neural pathway of differentiation (Hemmati-Brivanlou and Melton, 1992, 1994). Similar results (neuralization of *Xenopus* animal caps) were received with a truncated form of the mouse gene *Brachyury, Xbra* (Rao, 1994). Of interest is the fact that the "wildtype" molecules of both, activin and *Brachyury* are able to initiate mesodermal induction in amphibian ectoderm. From our disaggregation experiments (neuralization of *Xenopus* ectodermal cells) could be speculated that activin-like

Fig. 4. Tissues induced in *T. alpestris* and *X. laevis* early gastrula ectoderm by fraction "A". **(A)** *Archencephalic brain complexes (ABCs) induced in T. alpestris early gastrula ectoderm (fraction "A", 1000 μg/ml). neu, archencephalic brain structures.* **(B)** *Untreated early gastrula ectoderm of T. alpestris. The explant has differentiated into ciliated (atypical) epidermis only.* **(C)** *Xenopus ectoderm treated with fraction "A" (1000 μg/ml) was differentiated into ventral mesodermal structures. However, no neural tissue was induced. me, mesenchyme; co, coelomic epithelium; mu, muscle (presumably heart muscle).* **(D)** *Untreated early gastrula ectoderm of X. laevis. The explant has differentiated into ciliated (atypical) epidermis only. Bars, 100 μm.*

TABLE 4

VENTRAL-TYPE MESODERM TISSUES FORMED IN EXPLANTS OF *X. LAEVIS* LATE BLASTULA/EARLY GASTRULA ECTODERM TREATED WITH FRACTION "A" (500-4000 μg/ml) FOR DIFFERENT PERIODS OF TIME

			Induced structures, n (%)						
Fraction "A" (μg/ml)	Exposure time (h)	Number of explants	Atypical epidermis	Epidermis	Mesenchyme	Muscle (somites)	Heart structures	Blood-like cells	Coelomic Epithelium
0 (control)		16	16 (100)	0	0	0	0	0	0
500	3	15	15 (100)	7 (44)	7 (44)	0	0	2 (13)	3 (20)
1000	3	18	18 (100)	9 (50)	9 (50)	0	0	6 (33)	7 (39)
2000	3	14	14 (100)	11 (79)	11 (79)	4 (29)	6 (43)	0	11(79)
4000	3	18	17 (94)	9 (50)	9 (50)	1 (6)	3 (17)	0	7(39)
1000	6	16	16 (100)	12 (75)	12 (75)	3 (19)	7 (44)	0	12(75)
2000	6	10	9 (90)	7 (70)	7 (70)	3 (30)	2 (20)	0	7(70)

molecules are lost or unable to diffuse between the dispersed single cells to maintain the necessary threshold concentration.

A further interesting approach was the injection of follistatin mRNA into *Xenopus* embryos and animal caps cut from these embryos, which causes the expression of neural specific markers in ectoderm (Hemmati-Brivanlou *et al.*, 1994). The authors postulate that the expression of follistatin inactivates activin molecules present in/on the ectoderm cells, which results in the neural differentiation. In this concept, the formation of epidermis is considered as a weak activin influence upon the ectoderm. However, no neuralization could be observed when *X. laevis* or *T. alpestris* explants were treated *in vitro* with human recombinant follistatin at the concentrations of 1-1000 ng/ml (Asashima *et al.*, 1991; Grunz and Schüren, unpublished results).

Meanwhile, the mentioned results are not contradictory to the view that exogenous neuralizing factors may primarily initiate the neuralizing cascade in normogenesis by binding to specific receptors on the plasma membrane of ectodermal cells. In our experiments with chick brain fraction "A", the ectoderm of both, *R. temporaria* and *T. alpestris* showed a high percentage of neural inductions. Negative results obtained with *Xenopus* may reflect the specie-specific differences ("prevalences"): (1) in the mechanisms of acceptance of exogenous neuralizing agent(s) by ectoderm, and/or (2) in the intracellular transductions of neural signals. In particular, neural induction provoked in *Xenopus* explants is positively mediated by cross-talk between the PKC and cAMP pathways (Otte *et al.*, 1989). However, the neuralization of *R. temporaria* explants could be inhibited by forskolin (an activator of adenylate cyclase) and dioctanoyl-cAMP (Mikhailov and Gorgolyuk, 1992b). Moreover, during normal development the role of alternate pathways of neural induction may be different in *R. pipiens*, *T. alpestris* and *X. laevis* (Saint-Jeannet and Dawid, 1994).

The results of xenoplastic transplantations of the dorsal blastopore lip (the organizer region) have indicated that the similar signals could be involved in neural induction in different amphibians. However, fraction "A" had neuralizing activity in *R. temporaria* and *T. alpestris* but not *X. laevis*. This suggest that the neuralizing "components" of fraction "A" are not related with physiological neural inducers. Conversely, the neuralizing activity of the concrete agent depends on (more that of organizer region) the dynamic response of the target cells which could be different in different amphibian embryos (Slack and Tannahill, 1992; Saint-Jeannet and Dawid, 1994).

Neural induction is a multistep process and depends on the interactions of many different factors. It is appropriate to remind that the different "factors" (fractions) with neuralizing activity have been biochemically isolated from various sources (for reviews see: Tiedemann, 1990; Tiedemann *et al.*, 1993; Mikhailov, 1990; Janeczek *et al.*, 1992). Even FGFs, a ventral mesodermal inducers, cause neural differentiation under certain experimental conditions (Tiedemann *et al.*, 1994).

A good candidate for a molecule, which can play an important role in the determination of the central nervous system in amphibian embryos, is the secreted protein noggin (Lamb *et al.*, 1993). Like FGF, this protein induces neural structures in amphibian ectoderm at high, probably unphysiological, concentrations only. If this factor is related to the fractions isolated by us from chick brain is not yet known. This can be decided after further purification of our factor.

At present, all data available suggest that neural induction is a very complex process (even in comparison to mesoderm induction). Many factors and mechanisms in concert may be involved in neural induction including diffusible exogenous factors, released by distinct processes from the inducing chordamesoderm, ECM components, receptors on the plasma membrane, cell adhesion molecules and various agents inside of the ectodermal target cells (Dixon and Kintner, 1989; Grunz and Tacke, 1989, 1990; Durston and Otte, 1991; Mikhailov and Gorgolyuk, 1992a; Otte and Moon, 1992; Harland, 1994). Neural induction may be initiated by several "determinants" (factors) in a spatial and temporal specific manner. This could explain why a single universal inducer (the dream of the "old" embryologists) has not yet been found. Noggin, activin, FGFs, follistatin (Gurdon *et al.*, 1993; Harland, 1994) and soluble polypeptide factors characterized by certain specie-specificity of action may be involved in the multistep processes resulting in the formation of mesoderm (including the organizer area) and the central nervous system in different amphibians.

Materials and Methods

Preparation of extracts

Chick embryos incubated for 7.5 days (stage 32; Hamburger and Hamilton, 1951) were placed into sterile Petri dishes with chilled (4°C) Hanks' solution containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Forebrains were dissected and meninges removed. Isolated tissue was placed into a 20-fold (w/v) volume of deionized water containing, in addition to PMSF, other protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM p-tosyl-1-lysine-chloromethyl ketone, and 1 mM 6-

aminocaproic acid; Sigma) and adjusted to pH 9.0 by adding ammonia. The tissue was homogenized in a glass-glass homogenizer on an ice bath and the homogenate kept overnight at 4°C. Then the homogenate was centrifuged (20000g, 60 min, 2°C) and the supernatant was lyophilized. The lyophilized preparation was dissolved in 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA (Merck) and centrifuged at 105000g for 90 min at 2°C. RNAse A (Serva) was added to the supernatant to a final concentration of 50 μg/ml, and the preparation was kept at room temperature for one hour. Thereafter the preparation was transferred into a dialysis sac, and treatment with RNAse continued overnight at 4°C along with simultaneous dialysis against distilled water. The resulting extract was used for chromatography.

Ion-exchange chromatography

Chromatography was performed on a C10/25 column (Pharmacia Fine Chem.) packed with DEAE-Toyopearl 650M (Toyo Soda) according to recommendations of the manufacturer and equilibrated with 10 mM Tris-HCl, pH 8.0. After loading of the sample, the column was washed with three volumes of the same buffer, and bound proteins were eluted with a stepwise NaCl gradient (100-500 mM, 5 steps); NaCl concentration in eluates was measured using a Radelkis OK-104 conductometer (Hungary).

As the result, five fractions were obtained. In tests with *R. temporaria* gastrula ectoderm, the highest neuralizing activity (70-90%) was characteristic of the fraction "A" eluted with 100 mM NaCl (Fig. 1); the effect of the other four fractions either did not exceed 25% or was absent (Mikhailov and Gorgolyuk, 1992a; Mikhailov *et al.*, 1993).

The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) or rabbit gamma-globulin (Sigma) as a standard.

Affinity chromatography

Chromatography was performed on a Heparin-Ultragel column (Pharmacia Fine Chem.) in 10 mM Tris-HCl buffer containing 10 mM NaCl. After the sample (fraction "A"; see Fig. 1) was loaded, the column was washed to the base line and the bound proteins were eluted by 1000 mM NaCl (as a bulk) or stepwise NaCl gradient (50-1000 mM). As a result of stepwise elution, fore fractions were obtained. The highest neuralizing activity (about 50%; *R. temporaria* animal cap assay) was found in the fraction 3 (see Fig. 1) eluted by 250 mM NaCl.

SDS-electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the procedure of Laemmli (1970) using 10% and 15% polyacrylamide gels (PAAG), which were stained by silver nitrate (Rabilloud, 1990). The Pharmacia low molecular weight electrophoresis kit was used for determining the apparent molecular weight of fractions.

Biological tests

Fraction "A" and fraction 3 were dialyzed against deionized water at 4°C, sterilized by ultrafiltration (Millipore GSWP, 0.22 μm) and lyophilized. The lyophilizate was dissolved in culture medium containing antibiotics (penicillin, 100 U/ml; gentamycin, 70 μg/ml; streptomycin, 50 μg/ml).

Rana temporaria

Mature *R. temporaria* eggs obtained using hypophysial injections were artificially inseminated and embryos reared until the early gastrula stage (stage of "early crescent" blastopore). After mechanical removal of the jelly coat, early gastrulae were sterilized by placing for 15 sec into 70% ethyl alcohol and then washed in 3-5 changes of culture medium containing antibiotics. Vitelline membrane was mechanically removed and central portions of animal pole ectoderm (animal caps) were isolated microsurgically. Explants (about 1 mm in diameter) were placed upside down in glass cultivation chambers (Mikhailov and Gorgolyuk, 1987) containing 200 μl of the test fractions in MEM (Flow Lab.) diluted by half with deionized water (MEM/2) and, to prevent closing of the explants, covered with Plexiglas rings with the attached Millipore HAWP filter, pore size 0.45 um. The weight of the ring (about 8 mg) prevented ectoderm from rolling up during 10-12 hours but did not damage its cells. The chambers were closed with coverslips and explants cultivated 1, 3 or 18 h at 21°C. The concentration of fraction "A" in culture medium ranged from 50 μg/ml to 1 mg/ml (see Table 2). Then rings were removed, explants washed with fresh MEM/2 and cultivated for 6-8 days in medium that replaced every 48 h. In the control series the explants were cultivated in the presence of rabbit gamma-globulin (1 mg/ml) or in MEM/2 only. Each animal cap assay was also accompanied by control cultivation of 20 dejellied embryos from the same batch of the "donors" of early gastrula ectoderm.

Xenopus laevis

Mature *X. laevis* eggs were obtained by injecting female frogs with 600-1000 U chorionic gonadotropin (Schering AG, Berlin) into the dorsal lymph sack. After *in vitro* fertilization the embryos were dejellied for 4-6 min using 3% cysteine-chloride in Holtfreter solution. Embryos were carefully washed in sterile Holtfreter solution and raised until middle-late blastula/ early gastrula stages (stage 8, 9 and 10; Nieuwkoop and Faber, 1967). The vitelline membrane was mechanically removed using sharpened watchmaker's forceps. Animal caps were isolated with fine glass needles. Operations were performed in agarose-coated Petri dishes. Then the explants were placed in Terasaki-plates containing (10 μl per well) of fraction «A» dissolved in Barth solution (containing 0.1 % BSA) at final concentration of 500-4000 μg/ml (pH 7.35). The explants were incubated for 3 or 6 h prior to the transfer to Holtfreter solution and further cultivated for up to 3 days at 20°C.

Triturus alpestris

The jelly coat of late blastula or early gastrula of *T. alpestris* was removed by an irisectomy knife and embryos were washed in sterile Holtfreter solution. The vitelline membrane was removed by sharpened watchmaker's forceps. Animal caps were isolated by fine glass needles and incubated in Terasaki plates containing (10 μl per well) of fraction «A» (final concentration 1000 μg/ml) dissolved in Barth solution containing 0.1 % BSA. After 12-hour exposure, the explants were transferred to Holtfreter solution and cultured for 8 days at 20°C.

Light microscopy

R. temporaria explants were fixed in Bouin's solution, processed histologically, and serial sections (5 μm) were stained with azocarmine G and Mallory's mixture. Explants of *Xenopus* and *Triturus* were also fixed in Bouin's solution, gross-stained in borax-carmine and dehydrated in ethanol series. Paraffin sections (6 μm) were stained with aniline blue/orange G.

Statistical analysis

The results were processed using Fisher test for binomial distribution with a small n.

Acknowledgments

We thank S. Effenberger for skilful technical assistance and Prof. Dr. A.-M. Duprat for testing the expression of cytokeratin N 8 in Pl. waltl ectoderm cells. This investigation was supported by the Deutsche Forschungsgemeinschaft (436RUS113/14/1), Germany, Russian Foundation for Basic Research, Russia and in part by the Forschungspool of the University GH Essen, Germany.

References

ASASHIMA, M., NAKANO, H., UCHIYAMA, H., SUGINO, H., NAKAMURA, T., ETO, Y., EJIMA, D., DAVIDS, M., PLESSOW, S., CICHOCKA, I. and KINOSHITA, K. (1991). Follistatin inhibits the mesoderm-inducing activity of activin-A and the vegetalizing factor from chicken embryo. *Roux Arch. Dev. Biol. 200:* 4-7.

DAVIDS, M., LOPPNOW, B., TIEDEMANN, H. and TIEDEMANN, H. (1987). Neural differentiation of amphibian gastrula ectoderm exposed to phorbol ester. *Roux Arch. Dev. Biol. 196:* 137-140.

DAWID, I.B., SARGENT, T.D. and ROSA, F. (1990). Role of growth factors in embryonic induction in amphibians. In *Current Topics in Developmental Biology* (Ed. M. Nilsen-Hamilton). Academic Press Inc., New York, pp. 380-396.

DIXON, J.E. and KINTNER, C.R. (1989). Cellular contacts required for neural induction in *Xenopus* embryos: evidence for two signals. *Development 106:* 749-757.

DURSTON, A.J. and OTTE, A.P. (1991). A hierarchy of signals mediates neural induction in *Xenopus* laevis. In *Cell-Cell Interactions in Early Development* (Ed. J. Gerhart). Wiley-Liss Inc., New York, pp. 109-127.

GRUNZ, H. (1985). Effect of Concanavalin A and vegetalizing factor on the outer and inner layers of early gastrulae of *Xenopus laevis* after treatment with cytochalasin B. *Cell Differ. 16:* 83-98.

GRUNZ, H. (1987). The importance of inducing factors for determination, differentiation and pattern formation in early amphibian development. *Zool. Sci. 4:* 579-591.

GRUNZ, H. (1992). Suramin changes the fate of Spemann's organizer and prevents neural induction in *Xenopus laevis. Mech. Dev. 38:* 133-142.

GRUNZ, H. and TACKE, L. (1989). Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Differ. Dev. 28:* 211- 218.

GRUNZ, H. and TACKE, L. (1990). Extracellular matrix components prevent neural differentiation of disaggregated *Xenopus* ectodermal cells. *Cell Differ. Dev. 32:* 117-124.

GRUNZ, H. and TIEDEMANN, Hi. (1977). Influence of cyclic nucleotides on amphibian ectoderm. *Roux Arch. Dev. Biol. 181:* 261-265.

GURDON, J.B., KATO, K. and LEMAIRE, P. (1993). The community effect, dorsalization and mesoderm induction. *Curr. Opin. Genet. Dev. 3:* 662-667.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol. 88:* 49-92.

HARLAND, R.M. (1994). Neural induction in *Xenopus. Curr. Opin. Genet. Dev. 4:* 543-549.

HEMMATI-BRIVANLOU, A. and MELTON, D.A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature 359:* 609-614.

HEMMATI-BRIVANLOU, A. and MELTON, D.A. (1994). Inhibition of activin receptor signalling promotes neuralization in *Xenopus. Cell 77:* 273-281.

HEMMATI-BRIVANLOU, A., KELLY, O.G. and MELTON, D.A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell 77:* 283-295.

HOLTFRETER, J. (1938). Veränderungen der Reaktionsweise im alternden isolierten Gastrulaektoderm. *W.R. Arch. Entw.Mech. Org. 138:* 163-196.

JANECZEK, J., BORN, J., HOPPE, P. and TIEDEMANN, H. (1992). Partial characterization of neural-inducing factor from *Xenopus* gastrula. Evidence for a larger protein complex containing the factor. *Roux Arch. Dev. Biol. 201:* 30-35.

JOHN, M., JANECZEK, J., BORN, J., HOPPE, P., TIEDEMANN, H. and TIEDEMANN, H. (1983). Neural induction in amphibians. Transmission of a neuralizing factor. *Roux Arch. Dev. Biol. 192:* 45-47.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

LAMB, T., KNECHT, A., SMITH, W., STACHEL, S., ECONOMIDES, A., STAHL, N., YANCAPOLOUS, G. and HARLAND, R.M. (1993). Neural induction by the secreted polypeptide noggin. *Science 262:* 713-718.

LOPASHOV, G.V., SELTER, H., MONTENARH, M., KNÖCHEL, W., GRUNZ, H., TIEDEMANN H. and TIEDEMANN, H. (1992). Neural inducing factors in neuroblastoma and retinoblastoma cell lines. *Naturwissenschaften 79:* 365-367.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem. 193:* 265-275.

MIKHAILOV, A.T. (1988). *Embryonic Inducers.* (Ed. A.A. Neifakh). Nauka, Moscow.

MIKHAILOV, A.T. (1990). Factors of neural determination in early development of amphibians. In *Mechanisms of Determination* (Ed. A.I. Zotin). Nauka, Moscow, pp. 107-130.

MIKHAILOV, A.T. and GORGOLYUK, N.A. (1987). *Biochemistry of Embryonic Induction: Identification and Characterization of Morphogenetic Factors.* Soviet Scientific Reviews, Section F: Physiology and General Biology, Vol. 1. Harwood Acad. Publ., New York, pp. 267-306.

MIKHAILOV, A.T. and GORGOLYUK, N.A. (1988). Concanavalin A induces neural tissue and cartilage in amphibian early gastrula ectoderm. *Cell Differ. 22:* 145-154.

MIKHAILOV, A.T. and GORGOLYUK, N.A. (1989). Embryonic brain derived neuralizing factor. *Cell Differ. Dev. 29 (Suppl.):* 70 (Abstr.).

MIKHAILOV, A.T. and GORGOLYUK, N.A. (1992a). Plant lectins and factors from embryonic tissues as probes for studying the mechanisms of neural induction in amphibians. *Izv. Akad. Nauk SSSR (Ser. Biol.). 3:* 334-349.

MIKHAILOV, A.T. and GORGOLYUK, N.A. (1992b). Forskolin and cAMP inhibit the neuralizing effect of Concanavalin A on explants of amphibian gastrula ectoderm. *Ontogenez 23:* 624-632.

MIKHAILOV, A.T., GORGOLYUK, N.A. and MYKHOYAN, M.M. (1993). Embryonic brain derived neuralizing factor (EBDNF): Partial purification using ion-exchange and affinity chromatography. *Ontogenez 24:* 24-32.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis (Daudin).* North-Holland, Amsterdam.

NIEUWKOOP, P.D., JOHNEN, A.G. and ALBERS, B. (Eds.) (1985). *The Epigenetic Nature of Early Chordate Development.* Cambridge University Press, Cambridge.

OTTE, A.P. and MOON, R.T. (1992). Protein kinase C isozymes have distinct roles in neural induction and competence in *Xenopus. Cell 68:* 1021-1029.

OTTE, A.P., KOSTER, C.H., SNOEK, G.T. and DURSTON, A.J. (1988). Protein kinase C mediates neural induction in *Xenopus laevis. Nature 334:* 618-620.

OTTE, A.P., VAN RUN, P., HEIDEVELD, M., VAN DRIEL, R. and DURSTON, A.J. (1989). Neural induction is mediated by cross-talk between the protein kinase C and cyclic AMP pathways. *Cell 58:* 641-648.

RABILLOUD, T. (1990). Mechanisms of protein silver staining in polyacrylamide gels: A 10-year synthesis. *Electrophoresis 11:* 785-794.

RAO, Y. (1994). Conversion of a mesodermalizing molecule, the *Xenopus Brachyury* gene, into a neuralizing factor. *Genes Dev. 8:* 939-947.

RISAU, W., GAUTSCH-SOVA, P. and BOHLEN, P. (1988). Endothelial cell growth factors in embryonic and adult chick brain are related to human acidic fibroblast growth factor. *EMBO J. 7:* 959-968.

SAINT-JEANNET, J-P. and DAWID, I.B. (1994). Vertical versus planar neural induction in *Rana pipiens* embryos. *Proc. Natl. Acad. Sci. USA 91:* 3049-3053.

SAINT-JEANNET, J-P., HUANG, S. and DUPRAT, A.M. (1990). Modulation of neural commitment by changes in target cell contacts in *Pleurodeles waltl. Dev. Biol. 141:* 93-103.

SAXÉN, L. (1961). Transfilter neural induction of amphibian ectoderm. *Dev. Biol. 3:* 140-152.

SAXÉN, L. (1989). Neural induction. *Int. J. Dev. Biol. 33:* 21-48.

SERVETNICK, M. and GRAINGER, R.M. (1991). Changes in neural and lens competence in *Xenopus* ectoderm: evidence for an autonomous developmental timer. *Development 112:* 177-188.

SIEGEL, G., GRUNZ, H., GRUNDMANN, H., TIEDEMANN, H. and TIEDEMANN, H. (1985). Embryonic induction and cation concentrations in amphibian embryos. *Cell Differ. 17:* 209-219

SIMIRSKYI, V.N., ALEINIKOVA, K.S., FEDTZOVA, N.G., GORGOLYUK, N.A. and MIKHAILOV, A.T. (1991). Distribution of Concanavalin-A binding proteins and cytokeratins in different tissues of amphibian early gastrula. *Ontogenez 22:* 245-253.

SLACK, J.M.W. (1993). Embryonic induction. *Mech. Dev. 41:* 91- 107.

SLACK, J.M.W. and TANNAHILL, D. (1992). Mechanism of anteroposterior axis specification in vertebrates. Lessons from the amphibians. *Development 114:* 285-302.

SPEMANN, H. and MANGOLD, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *W.R. Arch. Entw.Mech. Org. 100:* 599-638.

TACKE, L. and GRUNZ, H. (1988). Close juxtaposition between inducing chordamesoderm and reacting neuroectoderm is a prerequisite for neural induction in *Xenopus laevis. Cell Differ. 24:* 33-44.

TAKATA, K., YOMAMOTO, K.Y. and OZAWA, R. (1981). Use of lectins as probes for analyzing embryonic induction. *Roux Arch. Dev. Biol. 190:* 92-104.

TIEDEMANN, H. (1984). Neural embryonic induction. In *The Role of Cell Interactions in Early Neurogenesis* (Eds. A-M. Duprat, A.C. Kato and M. Weber). Plenum Publ. Corp., New York, pp. 89-105.

TIEDEMANN, H. (1990). Cellular and molecular aspects of embryonic induction. *Zool. Sci. 7:* 171-186.

TIEDEMANN, H., GRUNZ, H., KNÖCHEL, W. and TIEDEMANN, H. (1993). Inducing factors and the molecular mechanism of differentiation in early amphibian embryos. *Ontogenez 24:* 5-25.

TIEDEMANN, H., GRUNZ, H., LOPPNOWBLINDE, B. and TIEDEMANN, H. (1994). Basic fibroblast growth factor can induce exclusively neural tissue in *Triturus* ectoderm explants. *Roux Arch. Dev. Biol. 203:* 304-309.

TIEDEMANN, Hi. (1986). The molecular mechanism of neural induction: neural differentiation of *Triturus* ectoderm exposed to Hepes buffer. *Roux Arch. Dev. Biol. 195:* 399-402.

YAMADA, T. (1990). Regulations in the induction of the organized neural system in amphibian embryos. *Development 110:* 653-659.

Accepted for publication: December 1994