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Consequences of the Spemann-Mangold organizer concept for embryological research in Russia: personal impressions

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"When we really think we understand development, we should actually be able to take it backwards from the end right to the beginning and then send it out on a different route."
(John Gurdon; see Smith, 2000)

"Science, at least today, is the product of communities of scientists, not individuals... And scientific theories and papers do not grow on trees, but are devised and promulgated by people."
(John Dupré, 2000)

Introduction

The genesis of cell diversity is a major area of interest in modern developmental biology. The early embryo is a mass of similar cells derived from the single fertilized egg, and, whatever the level at which its development is analyzed (cellular, genetic, or molecular), researchers inevitably come against the "puzzle of differentiation," i.e. understanding how can these cells eventually become different. It is generally accepted that two types of determinative signals specify cell fate in the course of development: extrinsic signals coming from the extracellular environment and intrinsic (autonomous) signals. In practice, implementation of this concept means that researchers have to answer at least two questions, namely, (1) how the extrinsic and intrinsic signal systems cooperate in processes determining the cell differentiation pathway and the identity of resulting cell types, and (2) how this cooperation is adequately realized in the concrete embryonic space and time.

Many years ago, Hans Spemann and Hilde Mangold, nee Proscholdt, performed their famous experiments with amphibian embryos, which showed that the interaction of the undifferentiated gastrula-stage ectoderm with the dorsal lip of the blastopore is essential for neural differentiation to occur. In particular, the transplanted dorsal lip proved to induce the formation of an additional neural tube in the host embryo. In some experiments, two entire embryos formed from the host embryo, with both neural tubes derived from the host tissue. These scientists named the dorsal lip *the organizer* because of its ability to cause formation of a complete second embryo (Spemann and Mangold, 1924). Their findings and theoretical conclusions have been repeatedly confirmed and gained worldwide recognition, especially after the award of the Nobel Prize to H. Spemann in 1935.

The idea that the embryo contains signal centers (i.e., organizers) that "instruct" the surrounding cells regarding which differentiation pathway to choose proved to be so attractive that several generations of embryologists throughout the world devoted their lives to the search for the corresponding signal molecules and the analysis of their mechanisms of action at the cell and molecular

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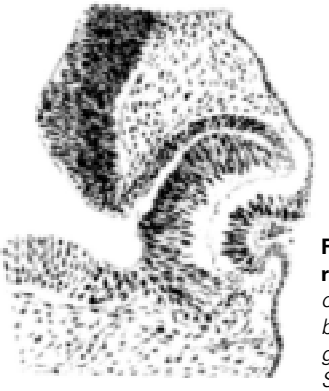


"The eye seems to be an outgrowth of the neural tube, which protudes through the muscle layer as far as the skin layer, and the outer parts of the eye are changes in the skin evoked as a result". Karl Ernst von Baer, 1828.

Fig. 1. On the way to the discovery of lens induction by the eye rudiment (from S.G. Gilbert, modified). *Gilbert notes: "Baer's outer parts of the eye are probably the eyelid and nictitating membrane, not the lens and cornea; so he missed presaging one of the great research programs of experimental embryology" (see Gilbert, S.F.: http://zygote.swarthmore.edu/regul1.html). However, this conclusion is questionable. If von Baer succeeded in identifying an egg cell in a mammalian ovary (see Mikhailov, 1997), he was probably capable of recognizing transformation of the "skin layer" into transparent cornea and lens, the tissues he closely knew as an anatomist. In any case, a more important fact is that von Baer unequivocally concluded that the eye rudiment determines differentiation of the adjacent head ectoderm.*

levels. Thus, Prof. Lauri Saxén, a famous Finnish embryologist, remembers that

> *"... I myself considered developmental biology and the problem of inductive tissue interactions still most exciting. In the late fifties, the original idea of Spemann (Spemann and Mangold, 1924) concerning the determination of the neuroaxis had been repeatedly confirmed, but the exact nature of the molecules which apparently emitted the determinative stimuli were still practically unknown and their mechanisms of action obscure. This was an obvious challenge for a young scientist."*

He also noted that:

> *"To follow daily the operated embryos under the microscope and detecting the gradual formation of the induced supernumerary structures was truly pleasurable. Sometimes you became so much attached to these little objects that it was almost a pity to fix them for further analysis"* (see Lehtonen, 1999).

For a long time, however, the massive and even heroic efforts of many research groups had been virtually fruitless: the inducing molecules were remarkably elusive. Repeated experimental failures gave rise to frustration and disappointment in the theory of embryonic inducers in general: the increasing number of specialists denied their specificity. Studies in this field gradually became outmoded, and only a few scientists proved to have enough courage and stamina to continue the search for molecular messengers of inductive interactions. Among them are Heinz Tiedemann and his collaborators, who identified and characterized the so-called *vegetalizing factor* (see Tiedemann, 1968,1975; 1981; 1982; Tiedemann *et al.*, 1992), the first and, for a long time, the only inducing factor purified from embryonic tissues.

As expected (see Gurdon, 1987; Mikhailov, 1988), stagnation in studies on embryonic inducers was broken largely owing to the integration of molecular genetics and experimental embryology. The resulting "molecular metamorphosis of experimental embryology" (see Fraser and Harland, 2000) allowed scientists to obtain principally new information that does not necessarily fit into the textbook schemes dating back to Spemann's organizer theory. Thus, the organizer proved to secrete both activator and inhibitor signal molecules, the latter playing an important role in the processes of neuralization of the embryonic ectoderm (see Wilson and Hemmati-Brivanlou, 1995, 1997; Zimmermann *et al.,* 1996; Grunz, 2000). These and other experimental findings provide the basis for the attempts to review the organizer theory (for example, see Ruiz i Altaba, 1998; Nieto, 1999), although the basic Spemann's concept that the organizer is the source of patterning signals in the early embryo has not yet been questioned.

Modern molecular-genetic technologies offer new incentives to research on the inductive tissue interactions, and it may well be that the new data will unexpectedly revitalize old ideas and approaches in this field. This is the standpoint from which we attempted to retrospectively analyze investigations of embryonic inductions in Russia.

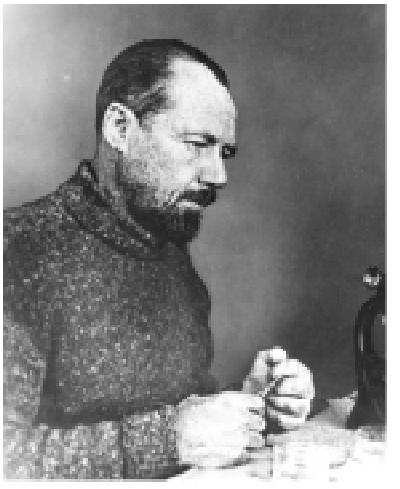
It should be noted that the theme of Spemann's organizers was not very popular in Russia: for a long time, the existence of specific embryonic inducers (morphogens) had been denied or questioned. This may be attributed to the fact that Russian embryologists concentrated on the problems concerning the role of developmental patterns in evolution (the field currently known as Evo-Devo), according to the tradition established by K.E. von Baer and his followers, I.I. Metchnikoff and O.A. Kowalevsky (see Mikhailov, 1997). Later on, A. Gurwitch's theory of embryonic fields came to attention in Russia and abroad (Gurwitch, 1922; see also Belousov, 1997). It is noteworthy that Spemann agreed with many concepts of this theory (see Steinbeisser, 1997). In the early 1920s, with the emergence of the first genetics groups in Moscow and Leningrad, the problem of genetic control of individual development became a most important topic in Russian embryology (see Korochkin *et al.*, 1997). Nevertheless, the development of experimental embryology in Russia actually began with the analysis of morphogenetic tissue interactions in the eye–lens system, i.e., the experimental system in which Spemann demonstrated for the first time the phenomenon of embryonic induction (see Saha, 1991).

Lens induction

It appears that even Christian Heinrich Pander and Karl Ernst von Baer, the founders of the Russian embryological school,

sensed by intuition that the harmonious development of the embryo as a whole is determined by interactions between various tissue rudiments. Thus, von Baer (1828) noted that the eye rudiment, formed as a lateral evagination of the neural tube, subsequently comes in contact with the skin (ectoderm) layer, and this event determines the development of "the outer parts of the eye" (Fig. 1). At that time, this was no more than interesting hypothesis, but von Baer's intuition turned out to be correct: in the beginning of the 20th century, Spemann published the first experimental results providing evidence that the eye rudiment is a source of signals causing lens formation in the embryonic ectoderm (Spemann, 1901).

Spemann cauterized one of the eyecup rudiments in the neurula-stage *Rana fusca (Rana temporaria)* embryos, leaving the second rudiment intact. The subsequent histological analysis of developing tadpoles showed that neither the eyecup nor the lens vesicle developed on the operated side, whereas on the control side these organs were normal. Interestingly, lentoid bodies also formed in cases when the eye rudiment was destroyed incompletely, but only on condition that the remaining eye tissue contacted the overlying ectoderm. Hence, Spemann concluded that, in normal development, the ectoderm is transformed into the lens under the effect of stimuli provided by the prospective eyecup material of the neural plate. This study gave rise to the "lens induction Odyssey," which still continues today (for retrospective problem reviewing, see Lopashov and Stroeva, 1964; Mikhailov, 1978, 1988; McAvoy, 1980; Saha *et al.,* 1989; 1992; Saha, 1991; Granger, 1992, 1996, Grainger *et al.,* 1997; Servetnick *et al.,* 1996).



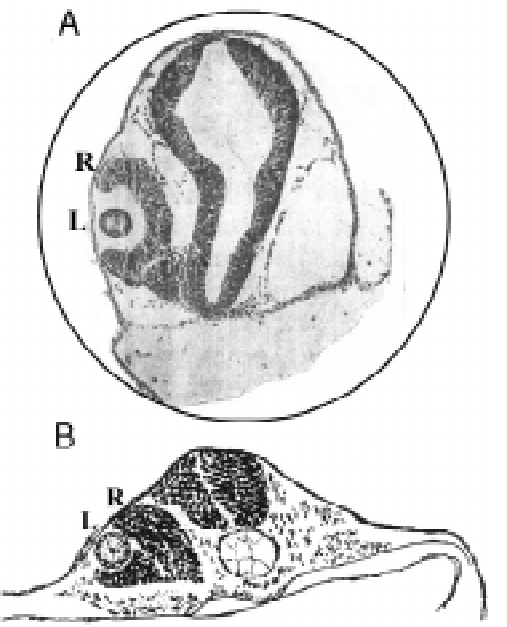


Fig. 2. D.P. Filatov (Filatow), 1935. (Right) Scheme summarizing the results of Filatov's experiments on the removal and transplantation of eye rudiments in pike embryos. **(A)** *When the eye rudiment was removed, neither neural retina (R) nor lens (L) developed on the operated side.* **(B)** *Implantation of the eye rudiment under the trunk ectoderm resulted in lens (L) formation at this site. These results are remarkably similar to those obtained by Spemann in analogous experiments on R. fusca embryos (for details, see the text).*

As in many other cases, experimental verification of this idea by other scientists provided contradictory data. Warren Lewis (1904), who transplanted *R. palustris* optic vesicles under ectopic sites of the ectoderm, observed the formation of lentoids at these sites. On the other hand, Helen King (1905) repeated Spemann's eye-ablation experiment on *R. palustris* and, unexpectedly, revealed the formation of lentoids in the absence of eyecup rudiments (sic!). Thus, she confirmed the results obtained by Emmanuel Mencle (1903) in experiments on trout (*Salmo salar*) embryos, which he interpreted as "free lens development" (i.e., eye-independent lens development). These findings stimulated Spemann to reinvestigate lens induction in *R. palustris,* and the results of these studies provided evidence that both kinds of processes - eye-dependent and eye-independent - have roles in the formation of the eye lens during development. This situation was interpreted as "double assurance" of the lens embryonic formation (Spemann, 1912; see also Fäbler, 1996).

Spemann was still interested in verifying the concept of so-called dependent differentiation (Roux, 1883), but the results of experiments were too ambiguous to draw any valid conclusions. This probably explains the fact that he quit experiments with the lens model, which failed to meet his expectations, and began studies on the mechanisms of neuralization. In this course, he performed the first organizer experiments (Spemann, 1918). The observed phenomena were so remarkable that Spemann's research team concentrated almost exclusively on the induction of the axial complex. Thus, experimental research on lens induction ceased for a while (Harrison, 1920).

The revival of interest in the processes of lens induction in the late 1920s is largely associated with the studies performed by Dmitrii Petrovitch Filatov (Filatow) and his colleagues at the Kol'tsov Institute of Experimental Biology (see Dettlaff and Vassetzky, 1997). Why did Filatov choose this model? In Spemann's organizer experiments, the entire tissue complexes were formed, and, hence, it was difficult or even impossible to differentiate between the effects of primary and secondary inductive stimuli. This interfered with the interpretation of experimental results and made the search for inducing agents still more difficult. As the lens, unlike the secondary axial complex, is a homogeneous cell population, its use as a model, in Filatov' opinion, could allow him to avoid such complications.

Initially, Filatov analyzed the lens-inducing activity of the eye rudiment with respect to its species-specificity. He performed a series of xeno-transplantation experiments, exchanging eye rudiments and prospective lens ectoderm between different amphibian species. The results of these studies provided evidence that the eye-derived lens-inducing activity lacks any strict species-specificity, at least in amphibians. Moreover, Filatov also came to an important conclusion that embryonic lens induction is a multistage process and that the non-eye tissues can exert their determinative influence on the prospective lens ectoderm prior to the effect of the eye vesicle (Filatov, 1925 a, b). By the time when Filatov began his studies, lens induction had already been demonstrated in amphibian embryos, and he decided to evaluate the universality of this

phenomenon in experiments with embryos of various lower vertebrates, primarily fishes. The results of his research on pike embryos confirmed the important role of the optic vesicle in lens induction; the formation of free lenses was not observed in these experiments (see Fig. 2). On this basis, Filatov (1935) noted:

> *"Thus, as for the two basic postulates of the theory of relationships between the eye and the lens, which is currently elaborated in experiments on amphibians, the first is that the eye cup can cause lens formation in the alien* [i.e., ectopic; A.M. & N.G.] *epithelium, and the second is that the lens-forming cells can give rise to a free lens without the effect of the eyecup. Only the first postulate was confirmed in experiments on pike embryos, whereas the second was neither confirmed nor disproved ... and it appears that this postulate is basically not as clear and indisputable as the first one."*

Filatov was among the first to analyze the temporal characteristics of lens induction by the eye vesicle in amphibians. In particular, he experimentally determined the period of the inducing action of the eye required for lens determination (Filatov, 1934a, 1934b). Subsequently (1937–1948), Filatov's students and colleagues - V.V. Popov, N.A. Manuilova, M.F. Nikitenko, M.N. Kislov, and others - continued research in this field (for references and review of corresponding data, see Lopashov and Stroeva, 1964), whereas Filatov himself took an increasing interest in the comparative-morphological field of developmental mechanics. Thus, he regarded inductive interactions in the eye-lens system as the so-called *morphogenetic apparatus* and analyzed probable factors responsible for the development of such a system in the course of evolution (Filatov, 1941, 1943).

Georgii Viktorovich Lopashov, who still uses the eye model in his experimental research (see Lopashov *et al.*, 1997), began his scientific career in the same Kol'tsov Institute of Experimental Biology in the 1930s. His studies, unlike Filatov's, were initially aimed at the analysis of agents inducing eye tissues. By that time, Bautzmann together with Holtfreter, Spemann and Mangold (1932), had already demonstrated on the model of axial complex induction that the so-called *devitalized* (killed) organizers retain their inducing activity, which indicated the chemical nature of the corresponding agents. Using a similar approach, Lopashov implanted devitalized (heated or ethanol-treated) eye rudiments into the blastocoel of gastrula-stage amphibian embryos or placed them between fragments of the amphibian gastrula ectoderm (the sandwich technique). In both experimental variants, he detected portions of the brain with the eyes and free lentoids formed in the ectoderm (Lopashov, 1935, 1936; see Fig. 3). He interpreted these results as evidence that devitalized eye rudiments contain some agents that are capable of inducing both the eye proper (i.e., neural retina and pigment epithelium) and the lens only. These considerations were precursory to Lopashov's future hypothesis that tissue-specific inducers acting upon target cells not only determine their differentiation along a certain pathway, but also give rise to the synthesis and accumulation of similar inducer molecules in these cells (Lopashov, 1974, 1977, Lopashov and Zemchikhina, 1997).

Let us refrain from the analysis of this hypothesis and consider Lopashov's paper published in 1936. Ideas expressed in this paper are of considerable interest, especially with regard to the time of publication: (1) inducing substances influence target cells both qualitatively and quantitatively; (2) inducers have no direct effect on terminal cell differentiation but are responsible for specifying cell determination in a certain direction; (3) inducers lack any strict species-specificity, and the same agents can induce eye and lens formation in different species; (4) "cell constitution" is no less important than induction, and response to induction largely depends on the pre-existing morphogenetic fields and constitution of target cells; (5) all the possible directions of cell differentiation are "encoded" in the genotype, and inducers determine the choice of a certain direction; (6) the role of inducers consists in creating the general body plan (pattern) of an embryo, i.e., in determining the spatial arrangements of individual embryonic rudiments; and (7) inducing agents appear to be products of the genes.

In essence, the term "cell constitution" used in this paper is similar to the term "cell competence" introduced by Conrad Waddington (1932). Waddington understood competence as the ability of a tissue to respond to the signal coming from the inducer, as is explicitly formulated in his paper dealing with experiments on the lens-forming capacity of the embryonic ectoderm in amphibians. These experiments showed that the isolated newt ectoderm retains this capacity until the moment when control tadpoles reach the tail-bud stage (Waddington, 1936).

Consonant data were obtained by Lopashov (1941) in experiments with newt and axolotl embryos. He removed the entire neural plate with developing neural folds and replaced it with the ventral ectoderm of late gastrula – mid neurula-stage embryos. In such implants, he observed the formation of free lenses, nasal placodes, and ear vesicles in the absence of neural or eye tissues. Such a result suggested that the amphibian embryonic ectoderm retains the lens-forming capacity during neurulation. In recent experiments with *R. palustris* embryos, the non-lens head ectoderm at the neural-tube stage proved to be capable of forming lentoids under the effect of optic cup-inducing signals (Graiger *et al.,* 1997).

Lopashov's experiments (1941) also suggested that the anterior head endo-mesoderm has a certain lens-inducing activity, and this possibility was subsequently confirmed by fairly strong experimental evidence (see Kawakami, 1952; Jacobson, 1958, 1966; Mangold, 1961). On this basis, the anterior head mesoderm can be regarded as a source of signals determining the initial stages of lens determination (see Lopashov, 1961; Lopashov and Stroeva, 1964). In normal amphibian development, however, these hypothetical mesoderm-derived inducing signals do not lead to any signs of lens differentiation. Moreover, if the neurula-stage lens-forming ectoderm was explanted, no lenses develop in the cultures (Jacobson, 1958)*****. The exception was *Rhacophorus schlegelii*: in this species, the prospective lens epidermis at the neurula stage could transform into the lens upon explantation (Tahara, 1962).

In due course, the aggregate of these and other data provided the basis for the following two suppositions (Mikhailov, 1978): (1) early lens-inducing signals from the mesoderm predispose the

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***Footnote**: *Some time ago (Mikhailov, 1978), we supposed that the prospective lens ectoderm may synthesize some specific factors preventing "premature" realization of lens determination at the tissue level. In this context, of interest are data on the gene Xlens1 (a novel X. laevis member of the forkhead gene family) in amphibian embryos. During normal development, this gene is expressed in the prospective lens ectoderm, but its hyperexpression suppresses lens differentiation, suggesting that a possible function of Xlens1 is to maintain the committed lens ectoderm in an undifferentiated state (Kenyon et al., 1999).*

ectoderm to subsequent influences of the eye rudiment, which determine the place and time of lens formation, and (2) the mesoderm acts upon cells of the prospective eye rudiments (anterolateral portions of the neural plate) to initiate the synthesis of lens-inducing factors. The concentration of the latter gradually increases in the course of development, reaching the level sufficient for inducing lens formation in the ectoderm. These conjectures allowed us to explain why the embryonic ectoderm of some amphibian species is capable of free lens formation, whereas in other species it needs the eye-derived signal to develop the lens (Mikhailov, 1978).

When the early stages of lens determination of the amphibian embryonic ectoderm were subsequently reinvestigated, the results showed that both the mesoderm and the neural plate can participate in the corresponding processes. However, individual contributions of these tissues to progressive determination of lens ectoderm have not yet been evaluated comprehensively (see Granger, 1992; Graiger *et al.,* 1997; Servetnick *et al.,* 1996; Zygar *et al.,* 1998).

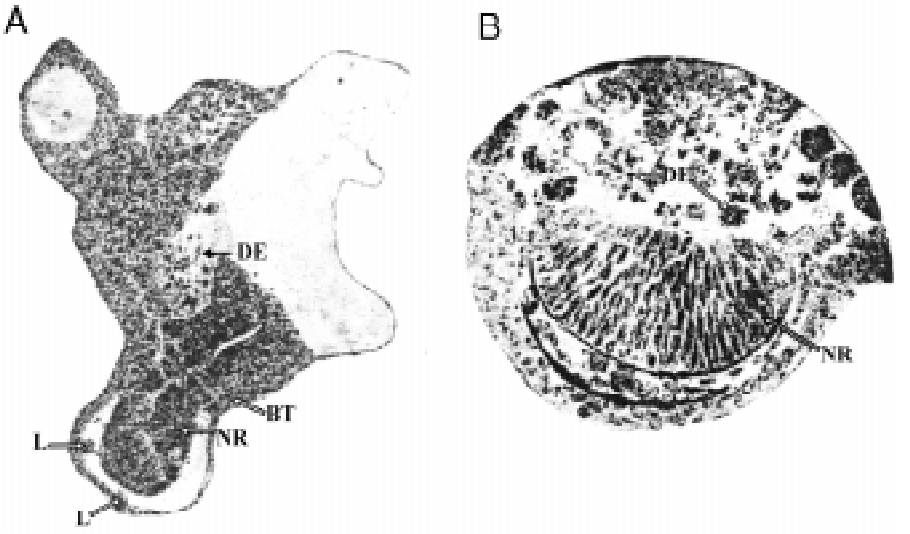

Fig. 3. Examples illustrating the results of experiments on the induction of eyes in amphibian gastrula ectoderm (Lopashov, 1936). **(A)** *Formation of free lentoids.* **(B)** *Eye-cup development in ectodermal sandwiches. L, lentoids. NR, neural retina. BT, brain tissue. DE, devitalized eye rudiments.*

In the 1950s and 1960s, interest in the processes of lens induction revived again, with the increasing number of studies performed on chick and mouse embryos. Boris Vladimirovitch Konyukhov was among the first to employ the genetic approach to the analysis of lens induction. Analyzing the developmental effects of different mutant genes in mice, he obtained additional evidence for the important role of the eye rudiment in lens induction. Thus, some mutations retard the growth and differentiation of the eye rudiment in mouse embryos, including differentiation of the neural retina at later stages. Studies on such mutants demonstrated that the optic vesicle is an essential source of lens-inducing signals that activate the synthesis of specific lens proteins (crystallins) in the lens ectoderm (Konyukhov and Sazhina, 1962; Konyukhov and Vakhrusheva, 1969; see also Konyukhov, 1980). Other researchers aimed to obtain data on the nature of possible lens-inducing molecules indirectly, separating the optic vesicle and the prospective lens ectoderm by membranes with different properties. The results showed that thin agar slices or membrane filters do not prevent induction, whereas a cellophane membrane placed between the interacting tissues completely blocks the development of the lens (McKeehan, 1958; Muthukkaruppan, 1965). Eventually, Marketta Karkinen-Jääskeläinen (1978 a,b) demonstrated that substances with a molecular weight of about 12 kDa or slightly higher can be involved in the process of lens induction in chick embryos.

These data stimulated us to perform a series of studies on the protein spectrum of eye rudiment in chick embryos (Mikhailov and Gorgolyuk, 1976). Using a micro-modification of polyacrylamide gel electrophoresis, we analyzed protein spectra at different stages of eye development, from the formation of the optic vesicles to early stages of tissue-specific differentiation in the neural retina. As the result, we identified several minor low-molecular fractions that were characteristic of the optic vesicle and the early optic cup but disappeared from the eye rudiments of embryos at later stages. By that time, it had already been shown that the lens-inducing activity of the eye rudiment in chick embryos reaches its peak at the stage of optic vesicle, gradually decreases as the eye cup is formed, and disappears with the onset of neural retina histogenesis (see Wedlock and McCallion, 1968). Hence, it was logical to assume that the identified "transitory" fractions of the optic cup may account for its lens-inducing activity (Mikhailov and Gorgolyuk, 1976; Mikhailov, 1978).

Experimental verification of this assumption was impeded by the fact that we could not use the prospective lens ectoderm of chick embryos as a test system: this tissue can form a lens even when isolated from the eye rudiment before reaching the stage of close morphological association with the latter. Moreover, not only the prospective lens region, but also the lateral ectoderm at the midbrain level and the oral ectoderm proved to be capable of spontaneous lens formation when explanted *in vitro* (see Fedtsova and Barabanov, 1978, 1990; Barabanov and Fedtsova, 1982). Fortunately, we received information from the L. Saxén's laboratory that the trunk ectoderm of 2-day-old chick embryos shows no signs of spontaneous lens differentiation after explantation *in vitro* but does form lentoids upon transfilter contact with the optic vesicle (Karkinen-Jääskeläinen, 1978 a, b). After a preliminary discussion with Lauri Saxén and Marketta Karkinen-Jääskeläinen, we decided to join our efforts and test the protein fractions of the eye rudiment for inducing activity on explants of the trunk ectoderm.

Methodologically, experiments proceeded successfully: gel fragments containing protein fractions remained in contact with the ectoderm for at least one day and had no toxic or damaging effect on this tissue, as shown by electron-microscopic analysis performed by Jorma Wartiovaara. In control experiments (ectoderm + optic vesicles), cultures formed lentoids containing crystallins, and the presence of these lens-specific proteins was confirmed by the method of retrospective fluorescence (see Mikhailov and Gorgolyuk, 1979, 1980; Mikhailov, 1979). However, the lens-inducing activity of low-molecular (transitory) fractions of similar optic vesicles proved to be very low, only 10–15%. Note, however,

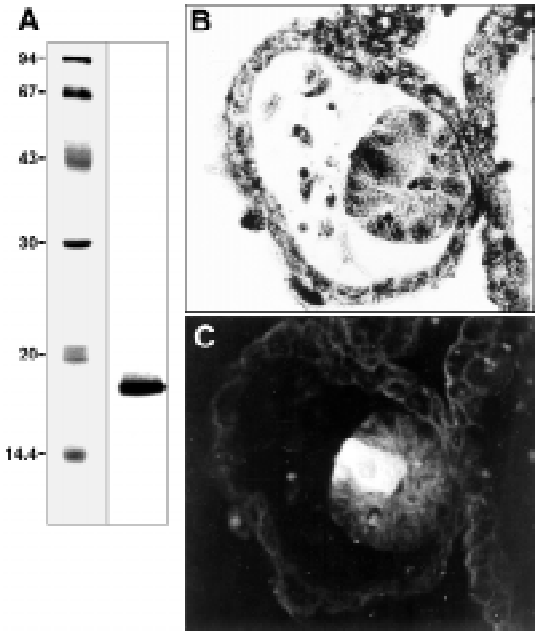

Fig. 4. Induction of free lentoids in explants of *R. temporaria* early gastrula ectoderm under the effect of protein fraction isolated from the neural retina of 8-day-old chick embryos (see Mikhailov and Gorgolyuk, 1987; Mikhailov, 1988). **(A)** *Protein pattern of the fraction in SDS-PAGE; only the 18-kDa band is detected (silver staining. 14-94 represent molecular weight markers in kDa).* **(B)** *A lentoid induced in the ectoderm by the fraction (section stained with azocarmine and Mallory's mixture).* **(C)** *The same section subjected to retrospective immunofluorescent analysis with rabbit antibodies against R. temporaria gamma-crystallins.*

that other fractions tested had no lens-inducing activity at all (Mikhailov *et al.,* 1983)*****.

Although these results agreed with the concept that the eye vesicle does contain some transitory lens-inducing proteins, it was apparent that the amount of the latter in tissues is so low that even their electrophoretic concentration into discrete fractions will not allow their molecular identification and assessment.

The simplest way out of this situation was to find the source of lens-inducing agents in tissues other than those of the early eye rudiment, including tissues of taxonomically different animals. Thus, there was ample evidence that several non-eye tissues brought in contact with the amphibian gastrula ectoderm can induce the development of lentoids in it (Toivonen, 1945; Kawakami, 1950; Becker, 1959). We decided to use the neural retina of chick embryos and tested the activity of retinal extracts in experiments with the explanted early gastrula ectoderm of *Rana temporaria* frogs (the animal cap assay). In this course, it was found that the extracts of 7- to 8-day-old chick embryo retina induced the formation of numerous free lentoids in 40–50% of explants (Mikhailov and Gorgolyuk, 1979 b).

Subsequent studies showed that the lens-inducing activity of these extracts is associated with their nondialyzable soluble components and is proteinase-sensitive. Electrophoretic separation of the extracts allowed us to identify fractions with an apparent molecular weight of about 18 kDa, which induced isolated lentoids (without brain and eye tissues) in the gastrula ectoderm (Mikhailov and Gorgolyuk, 1982, 1987; Mikhailov, 1988; see Fig. 4). However, we ran again into the same problem of the extremely low contents of these polypeptides in the embryonic nervous tissue, the more so that their lens-inducing activity proved to decrease significantly in the course of electrophoretic or chromatographic fractionation.

Although these experiments did not produce the desirable result (i.e., molecular identification and characterization of the lens-inducing agents), the aggregate of data obtained in their course showed that the hypothetical lens-inducing factors can be found among relatively small diffusible protein molecules, the concentration of which in embryonic tissues is very low.

In recent years, attention has been focused on the analysis of transcription factors involved in lens determination, induction, and differentiation (for reviews, see Kondoh, 1999; Francis *et al.,* 1999; McAvoy *et al.*, 1999). One of them is *Pax 6*, the factor mastering eye morphogenesis (Gehring and Ikeo, 1999; Chow *et al.,* 1999). This factor is expressed in the eye rudiment, beginning from the earliest stages of its morphogenesis, and in the prospective lens ectoderm (Li *et al.,* 1994, 1997; Hirsch *et al.,* 1996; Zygar *et al.,* 1998). It is noteworthy that the ectopic *Pax 6* expression in *X. laevis* embryos leads to the autonomous formation of numerous free lentoids in the ectoderm (Altmann *et al.,* 1997). In chick embryos, the ectopic expression of another transcription factor - *L-Maf* (lens-specific *Maf)* - is responsible for converting embryonic ectoderm cells into lens fibers (Ogino and Yasuda, 1998). The murine homeobox gene *Six 3* ectopically expressed in fish embryos promotes lens formation in the area of the otic vesicle (Oliver *et al.,* 1996). Note that the otic vesicle, in addition to the tapetum, is the source of signals that can stimulate lens differentiation (Dragomirov, 1929, 1932).

Thus, although research on lens inducers has history in experimental embryology, our knowledge of this problem is still rudimentary: the actual developmental roles of probable inducing agents and related molecules have not yet been elucidated. Fortunately, it is now possible to monitor the multiple stages of the lens determination process at the molecular level, and we are equipped with efficient methods for addressing the problem of lens inducers.

Neural induction

As noted above, studies performed by Spemann and his school on the induction of the axial complex, including neural tissue, gained much wider international acceptance than apparently contradictory data on lens determination during amphibian development (autonomous lens formation versus eye-dependent lens development). Several laboratories in Germany and other European countries began the search for neuralizing agents, using the amphibian embryonic ectoderm at late blastula–early gastrula stages as a responding tissue. Within a short time, a considerable amount of relevant experimental data were obtained. It soon became clear, however, that not only "normal" or killed embryonic organizer tissues, but also many adult animal tissues and the variety of obviously unrelated organic and even inorganic substances could induce neural differentiation in the ectoderm (Holtfreter, 1934a, 1945; Waddington, 1940; Needham, 1942; Brachet, 1944). Specialists had no rational explanation for this fact; moreover, it was found that ectoderm explants could differentiate into neural structures under

***Footnote**: *Unexpectedly, several optic vesicle fractions with the electrophoretic mobility similar to that of serum albumin exerted a neuralizing effect (50–60%) on the target tissue. Such a result indicated that the trunk ectoderm of 2-day-old chick embryos retains neuralization potential, notwithstanding its advancement along the epidermal differentiation pathway.*

inadequate cultivation conditions (Holtfreter, 1947). All these events created confusion among Spemann's followers and cast doubt on the very existence of specific neural inducers (see Saxén and Toivonen, 1962; Hamburger, 1988; Gilbert and Saxén, 1993; Grunz, 2000).

This attitude proved to be widespread and long-lived. As an example, consider comments on neural induction made by Pieter Nieuwkoop, an outstanding embryologist, as recently as in 1992 (sic!) and edited by Gurdon and Bjorklund (1999):

> *"... I am very skeptical about the possible identification of natural inducers, since fully competent "ectoderm" needs only a trigger to switch from its primary, epidermal, pathway into that for mesoendodermal or for neural development. ... The nature of the natural neural inducer is even more questionable than that of the mesodermal inducer, since very atypical stimuli, like e.g. high or low pH or Ca-free culture medium, are able to release neural differentiation in the highly competent gastrula ectoderm. This makes it much more difficult to analyze the nature of the natural inducing signal."*

Note that Niewkoop's skepticism did not prevent him from making a valuable contribution to the development of the theory of organizers: his studies on the role of endomesoderm and neural inductions in the embryo body patterning and morphogenesis are classic in this field (see Nieuwkoop, 1973; Nieuwkoop *et al.,* 1985; see also Gerhart, 1999).

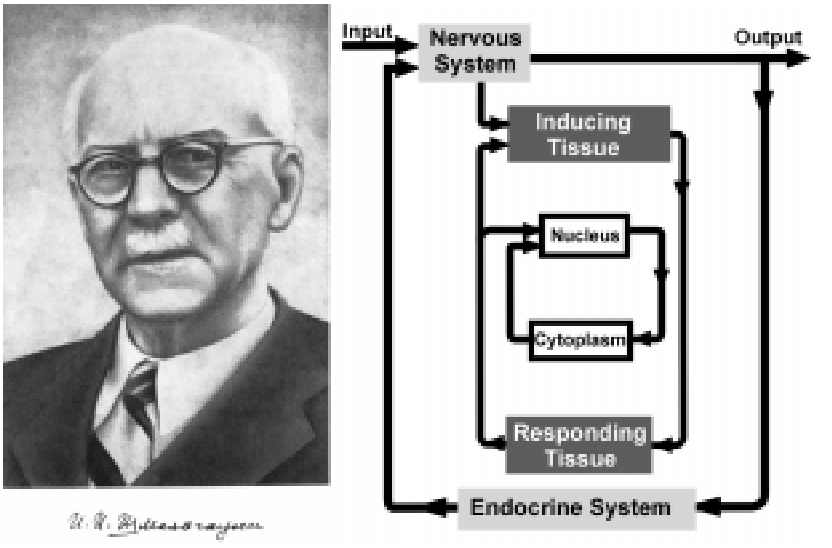

Fig. 5. Morphogenetic tissue interactions as an integrating factor of development. (Left) *I. Schmalhausen (Smal'hausen).* **(Right)** *The scheme on the right illustrates the three-circuit system of interdependencies in the developing organism (according to Schmalhausen, 1964, modified). Commenting on the scheme, Schmalhausen wrote: "The problem, however, concerns not only the analysis of feedback in individual morphogenetic systems. The latter are also interconnected, so that the output signals of one system are transmitted to the input of another system. The resulting structure of interdependencies is extremely complex. It should be noted that this system of regulatory influences is of the same hierarchical nature as organization of the embryo in general. The elementary regulatory systems of individual cells (nucleus–cytoplasm) establish connections and submit to more general systems of tissue- and organ-specific regulation, and the latter are integrated by regulation in the organism as a whole. Such a structure ensures the maximum stability of organization and perfect regulation."*

Accordingly, Russian scientists also understood the significance of studies on the phenomena of embryonic induction as one of the most important mechanisms of development at the cell, tissue, and organism levels, but identification of corresponding agents had been regarded as a secondary trend, an inevitable tribute to newly emerging biochemical and molecular embryology. For example, this situation is reflected in a series of studies by Ivan Ivanovich Schmalhausen (Smal'hausen; 1938, 1945, 1961), which are devoted to problems in the general theory of development. In the concluding manuscript *Regulation of Morphogenesis in Individual Development*, published posthumously in 1964, Schmalhausen noted:

> *"... The driving forces of individual development are created as the embryo differentiates owing to interactions between products of this differentiation. The interaction of different components leads to new differentiations and further interactions. The stability of organization is based on the complexity of the system of interactions (correlations) and their regulatory nature, rather than on the rigidity of certain structures. As a result of these interactions, the organism at any stage develops as an integral unity."*

Schmalhausen (1964; see Fig. 5), interested in the ideas of cybernetics, regarded inductive interactions as important but interdependent circuits of the general system of morphogenesis:

> *" ... In any interaction, one component proves to be more active and can be conventionally named inducer, or activator; the other one is less active, and can be named responder. However, the results of an interaction depend not only on the inductive influence, but also on competence of the responding component; in many cases, the reverse action of the responder on the inducer is observed. Morphogenetic systems are of the closed-cycle type. Unfortunately, feedback relationships in them have been studied insufficiently, and their analysis is now an urgent problem in embryology."*

Thus, in the Russian embryological milieu, embryonic inductions have been conventionally regarded from the general morphogenetic standpoint, without paying much attention to such "technicalities" as biochemical identification and characterization of inducing molecules (for instance, see Belousov, 1980, 1987, 1991). Lopashov and his colleagues appear to be a rare exception to this rule, as they regarded the analysis of inducing agents as the problem of primary importance (Lopashov, 1961; Lopashov and Stroeva, 1964; Lopashov and Hoperskaya, 1977).

We should also mention the problem of miserable state financing of embryological research in this country during the 1950s and 1960s. The revival of biology in the Soviet Union after the long period of social and scientific disaster known as the "Lysenko era" involved considerable investments in molecular biology and genetics, whereas embryology remained in the background. Under such conditions,

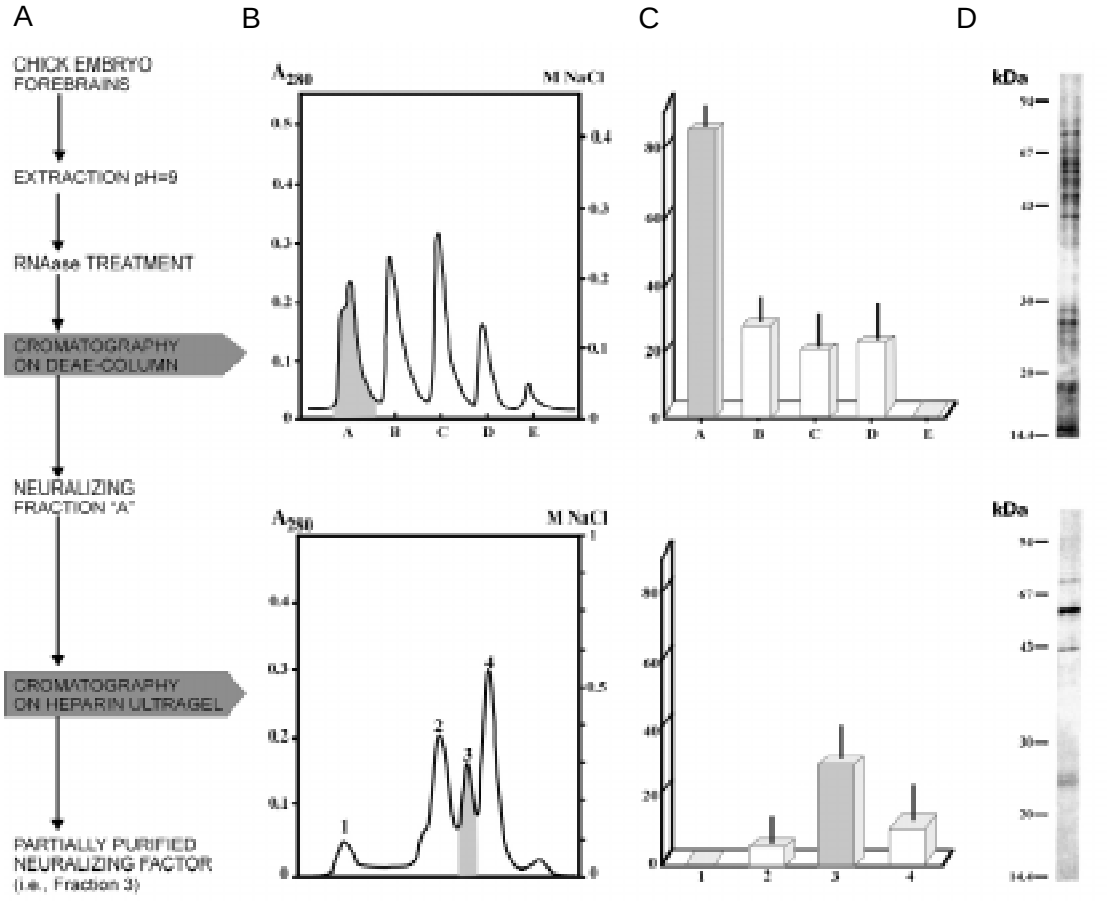

Fig. 6. Scheme of the procedure for partially purifying the neuralizing factor from the brain of 7- to 8-day-old chick embryos (Mikhailov *et al.*, 1993). (A) *Basic steps.* **(B)** *Chromatographic profiles; fractions with a high neuralizing activity are shown in grey.* **(C)** *neuralizing activity (frequencies of neural inductions, %) of chromatographic fractions as determined by the animal cap assay with R. temporaria early gastrula ectoderm.* **(D)** *SDS-PAGE analysis of fractions with the highest neuralizing activity (silver staining. 14–94 represent molecular weight markers in kDa).*

expensive and labor-consuming experiments on biochemical identification and characterization of embryonic inducers were virtually impossible. Embryonic inductions were mainly studied by traditional cytological and embryological methods, with preference given to the analysis of so-called secondary inductions, i.e., inductions of the inner ear (Ginzburg, 1950), cornea (Neyfakh, 1952), retina (Lopashov, 1960), and eye lens (Lopashov and Stroeva, 1964).

This is a brief (and, possibly, slightly exaggerated) account of the situation in which we developed our ideas concerning the search for neuralizing agents in embryonic tissues. Experiments began at the Kol'tsov Institute of Developmental Biology (see Mikhailov and Gorgolyuk, 1980), in the laboratory headed by Dr. G.V. Lopashov and soon gave rise to an independent line of research (see Mikhailov, 1984, 1988; Mikhailov and Gorgolyuk, 1987). By that time, Finnish scientists of Toivonen and Saxén's school and particularly of the German school founded by Heinz and Hildegard Tiedemann have already developed several identification and purification procedures for neuralizing factors, and it was clear that neural inducers are of protein nature (see Saxén and Toivonen, 1962; Saxén, 1980, 1989; Tiedemann, 1968, 1971, 1978, 1982)*****.

Instead of searching for natural neural inducers secreted by the Spemann-Mangold organizer, we decided to concentrate on their functional analogs, assuming that they are synthesized in the embryonic neural tissue at more advanced developmental stages (Mikhailov and Gorgolyuk, 1980). It is apparent that this approach was based on the concept of so-called *homoiogenetic* neural induction (Nieuwkoop, 1952) initially applied to the early neural rudiment (neural plate). In our experiments, however, the forebrain of chick embryos was used as the initial material for subsequent fractionation. This allowed us to obtain sufficient amounts of neural tissue with the minimal admixture of other tissue types. The neuralizing activity of brain extracts and their fractions was tested using the animal cap assay with *R. temporaria* early gastrula ectoderm.

Let us digress for a while to describe our attitude to the role of neural inducers at that time. The comparative biochemical analysis of water-soluble protein spectra of the early gastrula ectoderm and the neural plate showed that the normal process of neuralization in *R. temporaria* is accompanied by the drastic intensification of syntheses that had already occurred in ectoderm cells by the moment of their contact with the organizer (Mikhailov and Zakareishvili, 1983; Zakareishvili and Mikhailov, 1983). We interpreted this result as evidence for predetermination (labile determination) of some ectoderm cells toward differentiation into the neural tissue and, correspondingly, assumed that inducers identified with the aid of early gastrula ectoderm are responsible for the advanced stage in the process of neural determination (see Mikhailov, 1984, 1988; Mikhailov and Gorgolyuk, 1987).

***Footnote**: *It is our pleasant duty to note that scientific contacts with Professor L. Saxén and his colleagues from the Department of Pathology, University of Helsinki (Professors Eero Lehtonen, Irma Thesleff, Ismo Virtanen, Veli-Pekka Lehto, Jorma Wartiovaara), Professors Heinz and Hildegard Tiedemann, and, especially, Professor Horst Grunz were of great significance for our research on neural inducers. Their encouragement, support, and concern for our work provide an example of altruistic relationships that are now rarely observed between research teams dealing with similar problems.*

The results of fine molecular experiments performed by Sharpe *et al.* (1987) offered a convincing argument that cells of the amphibian gastrula ectoderm are somewhat predisposed to the future influence of the organizer (neural predisposition), which added credence to our viewpoint that neural inducers have the role of triggering factors in embryonic development. However, this viewpoint changed when Grunz and Tacke (1989) published the data on neuralization of amphibian ectoderm cells in disaggregated cultures. We understood that neuralizing factors do not activate neural differentiation but simply "lift the ban" against neural differentiation of ectoderm cells, which undergo predetermination before the onset of gastrulation (see Mikhailov and Gorgolyuk, 1992). We also believed that predetermination of these cells is associated with the presence of factors with a latent neuralizing activity (i.e., quiescent, or masked factors; see Holtfreter, 1934 b; Saxén and Toivonen, 1962; John *et al.,* 1984) and that the organizer discloses these intracellular factors in the course of normal development (Mikhailov, 1988, 1990).

These remarks are aimed to emphasize that we never ascribed the role of instructive signals to neuralizing factors identified in our experiments. Conversely, we regarded them as the additional permissive signals allowing the predetermined ectoderm cells to enter the neural differentiation pathway. The following quotation gives a summary of our views of that time on the process of neural determination:

> *"Long before coming in contact with the natural inducer (the dorsal blastopore lip), the ectoderm of early amphibian embryos becomes capable of forming the neural tissue upon explantation in vitro; this may occur either spontaneously (axolotl) or under unspecific influences (newts and frogs) ... It may be concluded that the process of neural determination in amphibian embryos begins at very early stages of development. In any case, part of the prospective dorsal ectoderm at the blastula stage is already predetermined toward neural differentiation, and we can reveal this predetermination under "provocative" experimental conditions. Final determination of the prospective neuroectoderm during normal development is achieved under the effect of signals from the dorsal blastopore lip"* (Mikhailov, 1990).

The work on isolating the neuralizing factor from chick embryonic brain progressed slowly, as we could test the activity of extracts and fractions only in spring, during the spawning season. After obtaining the first encouraging results (Mikhailov and Gorgolyuk, 1989), it took us about three years to develop an acceptable procedure allowing us to partially purify this factor (Mikhailov *et al.,* 1993). Its main steps are shown in the scheme (Fig. 6). In the final product (Fig. 6; fraction 3), SDS-PAGE revealed two major fractions with molecular weights of about 40 and 60 kDa. In the same year, the data was published that an individual secreted protein, named noggin, exerts a neuralizing influence on the explants of *X. laevis* gastrula ectoderm (Lamb *et al.,* 1993).

In the same period, we and Professor H. Grunz performed joint experiments in order to compare the effects of partially purified brain-derived factor on gastrula ectoderm of different amphibian species. The results showed that this factor could provoke neuralization of *R. temporaria* and *T. alpestris* ectoderm, whereas *X. laevis* early gastrula ectoderm proved to be unresponsive to its effect (Mikhailov *et al.,* 1995). This fact could be explained in two ways: either our factor has no relation to natural neural inducers or explants of the early gastrula ectoderm of amphibian species used in experiments differ in their sensitivity to neuralizing triggers. The latter is more likely, as the works of Tat'yana Antonovna Dettlaff (1983) provided evidence that amphibians, even belonging to the same genus (*R. temporaria, R. ridibunda, R. esculenta*), differ in the response of early gastrula ectoderm to implantation into the same tissue environment.

Note that affinity to heparin and Concanavalin A is among distinctive properties of the factor from embryonic chick brain (Mikhailov *et al.,* 1993, 1995). This circumstance suggested us an idea to analyze the extracellular matrix of gastrula ectoderm electrophoretically with the purpose to identify possible fractions capable of binding this factor. Unfortunately, a severe economic depression in Russia after "perestroika" made our project futile, regardless of support provided by Professors H. Grunz and H. Tiedemann.

The traditional concept owing its origin to Spemann's organizer experiments is that embryonic inducers should play an instructive role in the development of mesoderm and neural tissue. As concerns the latter, the actual situation proved to be much more complicated. Experimental studies showed that the Spemann-Mangold organizer synthesizes and releases a variety of proteins responsible for diverting nearby ectoderm to a neural fate (see Harland and Gerhart, 1997; Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997; Grunz, 1997, 1999; Weinstein and Hemmati-Brivanlou, 1999). Previously, we would have habitually interpreted the diversity of these factors as a reflection of their "unspecific" action on the ectoderm predetermined toward neural differentiation. At present, however, we know that the ectoderm enters the neural differentiation pathway owing to the interactions of molecules synthesized by the organizer, on the one hand, and the responding tissue, on the other. The factors released by the organizer, binding to the factors of epidermal differentiation (synthesized in the ectoderm) or "inactivating" their receptors, suppress the development of ectoderm into epidermis (Zimmerman *et al.*, 1996; Iemura *et al.*, 1998). As the result, ectoderm cells "escape" epidermal control and can choose another, neural developmental pathway, which appears to be also natural for these cells (for details, see the so-called *neural default model*; Wilson and Hemmati-Brivanlou, 1997).

Does this mean that, taking the epidermal pathway, embryonic ectoderm cells reserve the right to differentiate into another (neural) tissue autonomously, or they do need the additional external stimulus for entering neurogenesis? As noted above, the trunk ectoderm of 2-day-old chick embryos formed neural tubes under the effect of some protein fractions of the early eye rudiment. In this context, it is noteworthy that these tubes were often located near the foci of keratinization (the process typical for epidermal differentiation). At that time, we relied on the concept of so-called *homoiogenetic* neural induction in interpreting the results of these experiments (see Mikhailov *et al.,* 1983; Mikhailov, 1988). In any case, it is apparent that the development of neural tissues in such explants did not depend on the inhibition of epidermal differentiation. The well-known phenomenon of neural plate induction by a previously induced neural plate also poorly fits into the neural default model, as the previously induced neural plate itself does not express any of the antagonists of epidermal differentiation (Streit and Stern, 1999). It becomes increasingly apparent that mechanisms responsible for the early stages of neural determination of the embryonic ectoderm differ from mechanisms operating at the advanced stages (Harland, 2000; Streit *et al.,* 2000). Spemann's

discoveries provided a stimulus to the comprehensive analysis and molecular interpretation of only one period of neural determination, whereas the onset and many intermediate stages of this process have not yet been studied.

Concluding remarks

Several years ago, when three individual polypeptide molecules - noggin, follistatin, and fibroblast growth factor - have been shown to have neuralizing activity in amphibians, Richard Harland (1994) noted in the introduction to his review:

> *"The goal of isolating neural-inducing molecules has attracted biochemists for many years. ... The direct attempts to find neural inducers using biochemical purification have still not been successful and, as negative results do not much press, most of us will never know of the efforts that have been made."*

We hope that our paper will serve to partially fill this gap and to help the reader understand the ideas and pursuits of some Russian embryologists who happened to take the slippery path of the search for embryonic inducers. Unfortunately, the works of all the Russian scientists who addressed the problem of embryonic inductions were impossible to discuss in a short review, although many of them deserve serious attention. In particular, we refer the reader to the studies performed by Balinsky (1936), an outstanding specialist in experimental research and the theory of individual development. In addition, there have been many Russian embryologists who found the induction of amphibian axial structures useful for particular purposes. Notable among these at the moment is the Belousov's team at the Moscow State University (see Belousov and Luchinskaya, 1995; Belousov *et al.*, 1999).

Looking back, it becomes apparent that only a few candidates for the role of embryonic inducers had been isolated from the whole embryos by the late 1980s (see Tiedemann, 1982, 1984; Tiedemann *et al.,* 1998), and none of them was subsequently identified in the natural inducing tissues (this primarily applies to the neural and lens inducers). Today, we know that embryonic inducers exert their effect on responding cells at very low concentrations, which implies that their contents in inducing tissues are also low. Hence, it is not surprising that the first success was achieved in experiments with individual proteins isolated from large amounts of adult tissues (rather than natural organizers), the more so that these proteins fortunately proved to have a high and selective inducing effect on the cells of amphibian gastrula ectoderm (see Slack *et al.,* 1987; Smith, 1989; Dawid *et al.*, 1990).

Another aspect of the problem of embryonic inductions, no less important and interesting, concerns the phenomenon of competence, i.e., the ability of embryonic cells to respond to inducing signals, which they acquire and lose depending on their age and position in the embryonic space. As concerns the latter parameter, of particular interest are the data suggesting that the responding cells do not require contact with their neighbors for correctly assessing the concentration of an inducer; i.e. the single embryonic cell can identify its position in a morphogen gradient and can respond correspondingly to the concentration to which it is exposed (Gurdon *et al.,* 1999).

In our opinion, the term "inductive tissue interactions" implies that we deal with the formation of temporal and spatial developmental circuits within which intra- and intercellular signals can have a certain morphogenetic significance, giving rise to the phenomena habitually regarded as one-way processes (induction of the lens, induction of the neural tissue, etc.). Beyond these or in other circuits, the same signal molecules can perform quite different developmental functions. Moreover, the gradient of a single factor within an established "induction circuit" can produce several different effects in responding cells, or, conversely, the interaction of several developmental signals can evoke a single differentiation response (see Green *et al.*, 1997; McDowell and Gurdon, 1999; Niehrs, 1999; Piccolo *et al.*, 1999). Fraser and Harland (2000) justly noted that

> *"... any simple model that reduces a network of interacting factors to a linear set of players linked by arrows is destined to be incorrect. In the face of such complexity, computational tools must be employed as a tool for understanding. The purely theoretical attempts of a few years ago are now becoming increasingly constrained by data and may finally be gaining real utility to experimentalists."*

Thus, two model systems - lens and neural inductions - are of special significance to embryologists, as they allowed Spemann to make his famous discovery. Moreover, the analysis of lens induction actually gave rise to research on developmental mechanics in Russia. This is one of the reasons why we limited this review to these two systems. We happened to be among few Russian embryologists fascinated with the phenomenon of embryonic induction. Retrospectively, we understand that our interest in inducing factors had been permanently stimulated by new data from the laboratories headed by Professors S. Toivonen, L. Saxén, H. Tiedemann, and other distinguished scientists. We were also fortunate that this interest was supported by Professor O.E. Vyazov, head of laboratory at the Institute of Human Morphology (Russian Academy of Medical Sciences, Moscow), in which we planned our first experiments in the early 1970s, and by Dr. G.V. Lopashov, who invited us to his laboratory at the Kol'tsov Institute of Developmental Biology (Russian Academy of Sciences, Moscow) to perform these experiments. We are grateful to our colleagues, particularly to Professors A.A. Neyfakh, L.I. Korochkin, N.G. Khrushchov (Kol'tsov Institute of Developmental Biology), L.V. Belousov (Moscow State University), and B.V. Konyukhov (Institute of General Genetics), who, each in his own way, helped us in the work on identifying lens and neural inducers during the period when such investigations, both in Russia and abroad, were of no current interest to the majority of biologists.

Summary

The impact of the organizer concept on Russian experimental embryology is shortly reviewed. Attempts to study embryonic induction in Russia may be grouped into embryological and biochemical approaches. This paper provides a framework for, and overvalue of, the contributions of Russian biologists to the problem of embryonic induction. Two model systems - lens and neural inductions - are of special significance to modern developmental biologists. Moreover, the study of eye lens induction actually gave rise to research on developmental mechanics in Russia. This was one of the reasons why we limited this article to these two model systems. After retrospective consideration of the results of the search for possible lens-inducing factor candidates, the discussion

turns towards some of the examples of neural-inducing agents detected in embryonic tissues and the new questions raised by the progress that has been made in the analysis of the Spemann-Mangold organizer.

KEY WORDS: *lens induction, neural induction, Spemann-Mangold organizer.*

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