Chapter 2

Transmitters in Blastomere Interactions

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Additional information is available at the end of the chapter

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1. Introduction

Normal multicellular organism develops from fertilized egg cell, although the latter may give birth for twins too. Mechanisms that lead to the realization of these variants of the development may differ greatly, however, it naturally suggests itself that such deviation as twin formation is conditioned by this or that disturbance of cellular interactions. But the very fact of the existence of such interactions after classic experiments remained disputable until now. Naturally, far less is known on the suggested mechanisms of such interactions that provide the formation of integral organism or, correspondingly, their distortion that lead to the twins formation.

Neurotransmitters that are known as the mediators of cellular interactions in adult organisms are involved also in the regulation of various processes of embryonic development. This field remain little bit exotic for a majority of biologists although the researches in this field were started more than 50 years ago and brought a number of interesting facts and hypotheses. It was logically to suggest that neurotransmitters may take part in the embryonic cellular interactions together with the regulation of cleavage divisions and other processes of embryonic development.

2. Problem of the existence of early cellular interactions

Since the beginning the studies of cellular interactions at the early stages of embryonic development were hindered by the fact that very existence of those interactions was doubted. Really, August Weismann suggested his **germ plasm theory** in 1883 [1] that chromosome determinants are distributed nonuniformly and it in turn determines distinct prospective fates of embryonic cells. In particular, according to this concept already first division predetermines the fates of blastomeres as “left” and “right”. Really, soon after Wilhelm Roux has carried out his pioneer experiment that became the starting point of experimental embryology [2]. Roux has denaturated one of two blastomeres of the frog *Rana*

fusca by hot needle, as the result the intact blastomere formed half set of larval structures (Fig. 1). The conclusion was made that the development of embryonic cells is predetermined. Later such type of the development (mosaic) was found in a number of taxons where each blastomere forms the specific part of the definitive organism. Ideal example of such development is favorite subject of various researches the nematode *Caenorhabditis elegans*, where the fates of all more then 900 cells were traced [3]. Evidently that at least in such embryos substantial cellular interactions are absent at the early stages of the development.

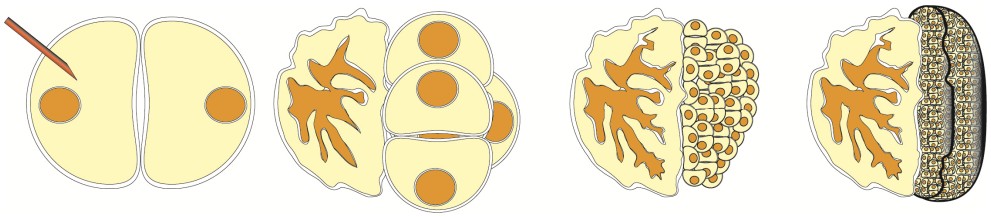

Figure 1. Roux's experiment. Denaturation of frog blastomere by hot needle. Left blastomere was killed by hot needle but right one prolong to cleave and form half set of structures

However, soon the death-blow to the universality of Weismann's theory was dealt by other Founding Father of experimental embryology Hans Driesch [4]. He demonstrated the ability of sea urchin isolated blastomeres to form quasi-normal half-size larvae (Fig. 2). Later McClendon carried out similar experiment in amphibian embryos with the same result [5]. On one hand, it supported Driesch's data, on the other it finally compromised Roux' result. Moreover the experiment similar to Roux' one was performed in sea urchin embryos at the end of XX century. In contempt to Roux' data it was shown that intact blastomere is able to form the diminished quasi-normal larval in spite of the death of sister blastomere [6]. So the data of Wilhelm Roux is believed artifact of defect experiment in all contemporary handbooks of embryology and developmental biology – *"Something in or on the dead blastomere still informed the live cells that it existed"* [1]. We shall return to the evaluation of this statement at the end of the present work.

Thus, amphibians and echinoderms as some other taxons, as distinct from mosaic ones, are able to form whole organisms from the blastomeres isolated at cleavage divisions. In particular, sea urchins preserve such ability, with some reserves, until 8 blastomere stage [7] whereas starfishes – until 32 cell stage [8]. Nevertheless prospective potencies of the blastomeres in the intact embryo are limited and there are no explanation for it except cellular interactions.

At the first glance classical Hans Driesch' experiment, that is the base of contemporary concept of the regulation of the development, proves this idea, however, it contain serious internal contradiction. On one hand, the result of Driesch' experiment evidences in favor of the existence of substantial cellular interactions, although, on the other, half-embryos at 4th cleavage division in his work showed the cleavage pattern of the half of whole embryo – 4 meso-, 2 macro-, and 2 micromeres (Fig. 2, [4]). As the matter of fact **such result principally**

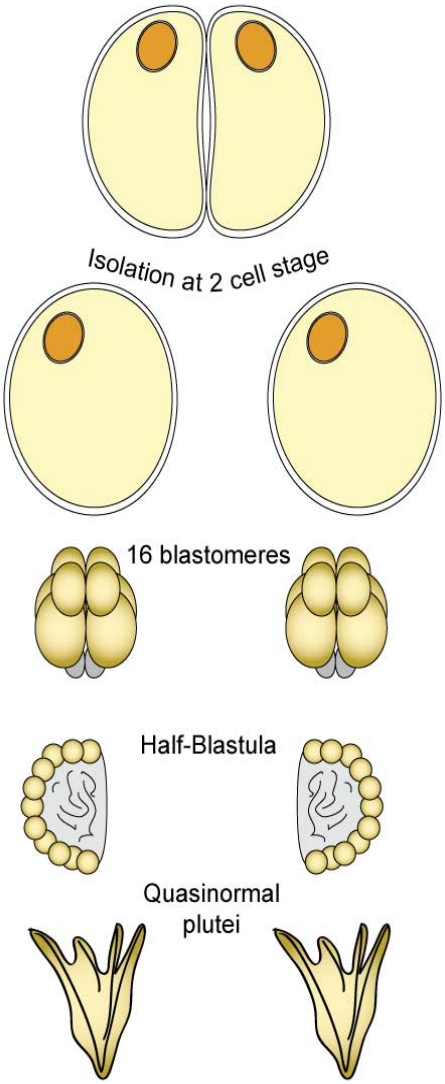

Figure 2. *Hans Driesch experiment* Isolated blastomere forms at 4th cleavage division half-set of cells – 4 meso-, 2 macro- and 2 micromere. Later they form half-size blastula with the blastocoel opened to outer medium. Then half-embryos undergoes "regulation" and form quasi-normal half-size plutei

do not differ from Roux' one except single difference – the ability of sea urchin embryos for the "regulation of the development" at the later stages. Really, such half-embryos first formed "opened half-blastulae" – hemisphere with the blastocoel opened to the outer medium that then closed and became indistinguishable from intact one. It was noted yet by Driesch [4]: "After the isolation one of two first *Echinus microtuberculatus* blastomeres it cleaved as half-embryo [Halbbildung], but form whole half-size organism. Recently it was

confirmed by the experiments with blastomere isolation [9] and ricin microinjection [6]. After our own observations half-embryos may show first "partial" cleavage pattern, then form opened hemiblastulae, later (early blastula 2 – midblastula [10]) close the blastocoel in about 10 minutes and further develop into normal half-size embryos and plutei I [9].

Driesch' experiments in all possible variants were reproduces by a number of researchers. On the base of wide and scrupulous experiments on the isolation of the blastomeres and the fragmentation of fertilized eggs of sea urchin Swedish researcher Sven Hörstadius came to the conclusion on the independence of their cleavage pattern on any influence. He suggested the idea of the "micromere clock" – the micromere formation precisely at the moment of 4th cleavage division in the intact embryo independently on any experimental intervention [11, 12]. Properly speaking it means that every embryo is able "to count to four" that is quite strange. Nevertheless this concept persists in unaltered form until now in handbooks of embryology [13, 14] instead of a number of publications that deny it (see below).

In fact such result ruled out the role of direct blastomere interactions during cleavage divisions that would be able to limit the prospective potencies and "shift" the process of development regulation to later developmental stages. Mechanism of such "late regulation" is out of scope of present work, however, we need note that irreversible restriction of prospective potencies of sea urchin blastomeres occur at 4th cleavage division. In particular, micromeres further form the primary mesenchyme and then larval skeleton spicules [15]. Moreover, one of micromere quartets formed at 5th cleavage division stop the divisions at all and, probably, works as the pacemaker of further development [16]. Already at the next division (60 cell stage) the determination of prospective embryonic territories occur [17] and it is too short time for "regulation of the development". No publications of the possible mechanisms of such regulation was found in scientific literature.

In the meantime the facts that contradict the canonic concept were accumulated in parallel with the process of its consolidation. When Plough [18] reproduced Driesch' experiments he could obtain the regulation in the part of embryos only. The author explained this discrepancy with classic data by his imperfect technique as compare to Driesch' one. Many years later Marcus performed statistically assured study and confirmed Plough' data not Driesch' [19]. Harvey in her original work reported on the possibility of equal 4th cleavage division in sea urchin [20] but later, probably under the pressure of "public opinion", she specially stressed in her masterwork "American Arbacia" that half embryos **always** form 2 micromeres at 4th cleavage division [21]. So Driesch-Hörstadius concept preserved its dominating position at least until the end of sixties of XX century.

However the process of the revision of classic knowledge in this field was finally reinitiated. Katzuma Dan and his co-workers disproved the micromere clock concept when they have demonstrated that the suppression of any one cycle of changes of free sulfhydryl groups in sea urchin embryos leads to the delay of micromere formation to the next cell cycle [22-24]. Soon after the data were obtained on the possibility to evoke the functional isolation of blastomeres using short application of chemical substances during 1st or 2nd cleavage divisions (without mechanical isolation of the blastomeres) that lead to the formation of the

specific aberrations of blastulae such as half-size blastulae, "Siamese twins" blastulae, 8-form embryos etc [25-27].

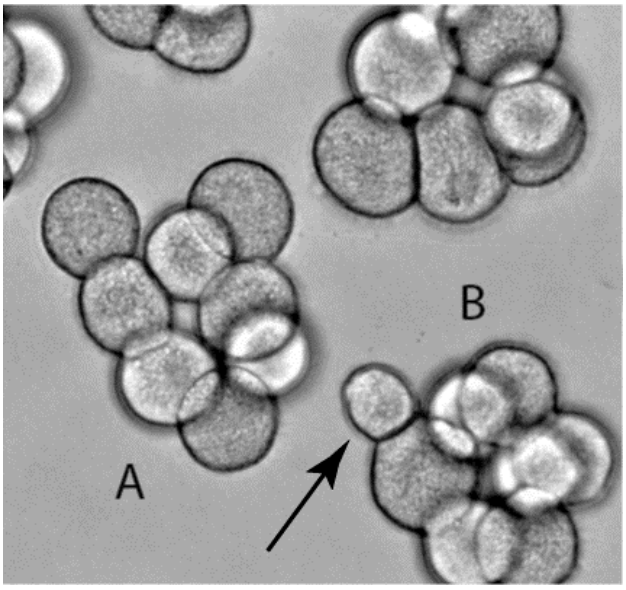

Figure 3. Half-embryos of P.lividus, isolated by glass needle
A – half-embryos, consisting of equal blastomeres at 4th cleavage division
B – unequal cleavage, the micromere is marked by the arrow

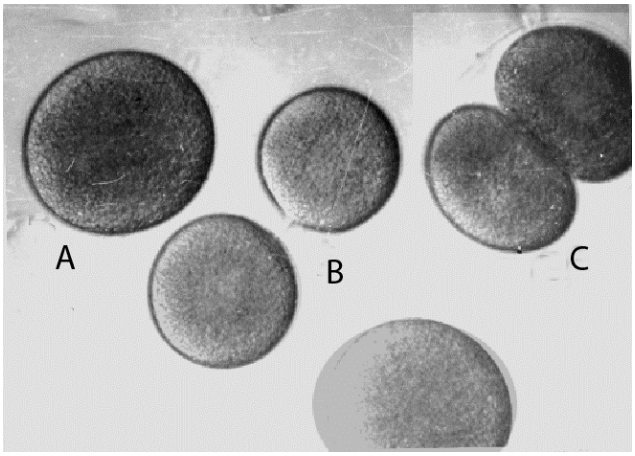

Figure 4. Figure 4 Adhesion of the *S.nudus* blastomeres
A – undivided egg cell; B - two blastomeres before adhesion; C – 2-cell embryo after adhesion

The problem of the existence of blastomere interactions was finally solved in the series of studies of blastomere isolation. More than 80 years after Driesch pioneer work it was carried out by the group of researchers who was not indoctrinated by old dogmas, probably, because there were no embryologists among them. In the very beginning of the work it was found that the isolation of sea urchin *Strongylocentrotus nudus* blastomeres (Japan sea) during 1st or 2nd cleavage division leads to the formation of half-embryos with two different cleavage patterns: partial, previously described by Driesch, and integral, when the embryo formed 8 equal blastomeres at the 4th cleavage division (Fig. 3), i.e. their cleavage pattern coincided with the whole embryo pattern but of a half size but not to half of intact embryo [9, 28]. Moreover in many cases when 4th cleavage division was unequal one micromere was formed only in contrast to classic results. The majority of half-embryos with equal 4th cleavage division could form the micromeres at the next stage.

During further studies in the embryos of the sea urchin *S.nudus* and sand dollar *Scaphechinus mirabilis* some logic was found in the formation of this or that cleavage pattern: equal 4th cleavage divisions predominantly occur in half-embryos isolated before "post-division blastomere adhesion" [25] (Fig.4), but unequal (with micormere formation) in half-embryos isolated some 10 – 15 minutes later when the adhesion was completed. The presence of this rule was then confirmed in a variety of sea urchin species (Tables 1 and 2)[1].

This rule was also confirmed by the experiments with multiple consecutive isolations of the blastomeres from the same *Sc.mirabilis* embryo. Twofold blastomere isolation lead to the increase of portion of half-embryos with equal 4th cleavage division by 13% as compare to single isolation and threefold – by 26% [29]. Analogous experiments in the embryos of *P.lividus* have shown that only 11,1% of twofold isolated blastomeres formed the micromeres simultaneously with intact ones, i.e. by 33,9% less then embryos isolated once before adhesion in 1st cleavage division. Such "accumulation" of the effect of the elimination of normal blastomere interactions, on one hand, evidences in favor of their importance in the determination of the pattern of early development and, on the other, on their **repeatability** during cleavage divisions. As concerns 3rd cleavage divisions there are some reservations because the isolation of the blastomere quartets at this moment is technically difficult because of spatial structure of the embryos. Rare cases of equal cleavage of such half-embryos (about 8%) at 4th division may be explained also by the specific peculiarity of this stage (see below).

Thus, the critical periods exist in the cleavage division cell cycles of sea urchin embryos when the processes limiting the prospective potencies of the blastomeres are realized. Evidently, these processes need to be mediated by the cellular interactions.

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[1] It need be specified that three groups of sea urchin species could be distinguished by the relative contribution into cellular interactions of passive mechanical component (hyaline layer) and direct blastomere interaction [25]. Logic change of cleavage patterns of half-embryos was characteristic for the group of *S.nudus* also as sand-dollar *Scaphechinis mirabilis*. In the embryos of *Strongylocentrotus intermedius*, where the hyaline layer is far more important for the integrity of the embryo, half-embryos with equal 4th cleavage division were substantially less numerous. But equal 4th cleavage divisions were observed regularly even in these species.

Species	Moment of isolation	Number of embryos	Portion of half-embryos forming micromeres simultaneously with intact ones (%%)	Significance
S mirabilis	B_1	818	34,7±1,7	<0,001
	A_1	865	68,2±1,6	<0,001
	B_2	60	30,0±6,0	<0,001
	A_2	127	81,1±3,5	<0,001
S.nudus	B_1	56	23,2±5,6	<0,001
	A_1	26	92,3±5,2	<0,001
S.intermedius	B_1	62	79,4±5,1	<0,001
	A_1	48	83,3±5,4	<0,001
E.cordatum	B_1	22	27,3±9,5	<0,01
P.lividus	B_1	48	42,0±4,1	<0,01
	A_1	42	76,2± 5,3	<0,001
*P.lividus**	B_1	309	45,0±1,4	<0,001
	A_1	214	93,4±1,0	<0,001

Table 1. Influence of the moment of blastomere isolation on the cleavage pattern of half-embryos
* Adriatic population with absolute predominance of intact embryos prematurely forming micromeres (at 3rd cleavage division), therefore in this experiments the formation of the micromeres was controlled in 3rd but not 4th cleavage division
B – half-embryos, isolated before adhesion in 1st or 2nd cleavage division, A - half-embryos, isolated after adhesion in 1st or 2nd cleavage division

Species	Stages compared	Difference in portions of embryos with the same cleavage pattern (%%)	Significance
S. mirabilis	B_1 - A_1	33,5±2,3	<0,001
	A_1 – B_2	38,2±6,2	<0,001
	B_2 – A_2	51,1±6,9	<0,001
S. nudus	B_1 – A_1	69,1±7,6	<0,001
S. intermedius	B_1 – A_1	3,9±7,4	n.s.*
P.lividus	B_1 – A_1	24,2±6,2	<0,01
*P.lividus**	B_1 – A_1	38,4±1,7	<0,001

Table 2. Differences of cleavage patterns of sea urchin half embryos, isolated before or after blastomere adhesion in the 1st or 2nd cleavage divison
* not significant
Designation B and A as in Table 1

It can be added that real pattern of blastomere interactions are more sophisticated than simple consecutive signal exchange in the freshly formed contact zone. Time-lapse recordings of the development of sea urchin *S.nudus* embryos have shown that during 2nd cleavage division blastomeres first become rounded, zone of the tight adhesion in the furrow of 1st cleavage diminished, and then blastomere adheres along both 2nd and 1st cleavage furrows (Fig. 5), i.e. the interaction process repeats at the same place much times

and the aggregated result is the consequence of the formation of three-dimensional structure of contacts.

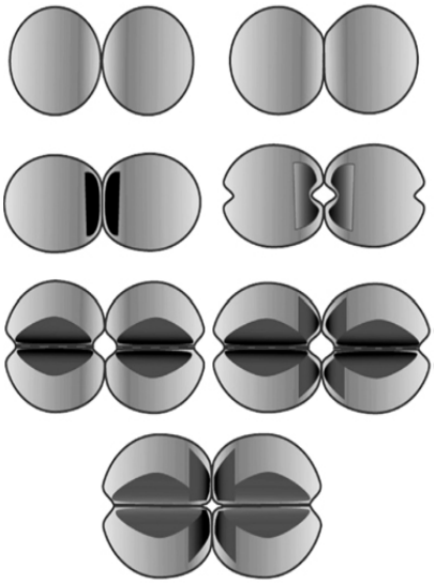

Figure 5. "Re-adhesion" of blastomeres during the 2nd cleavage division of *S. nudus* [29]. The dark areas are the adhesion zones.

Further studies have shown also that blastomeres isolated from the same embryo may have both the same or different cleavage patterns, and coinciding pattern may be both partial and integral. It follows thence, that no blastomere are "pacemaker" in the signal exchange and their interactions are **equal** but also **nonsynchronous**, at least their consequences.

A propos, on the base of above mentioned results it is possible to explain the reasons why Hans Driesch discovered only one variant of the development of half embryos – partial pattern. First, if the classic of experimental embryology has exerted scrupulousness and always performed isolation of blastomere after the full completion of their adhesion he **might** obtain exclusively partial pattern. Second, the results of Driesch' studies were influenced by more or less occasional choice of the species for the experiments (*Echinus microtuberculatus* and *Paracentrotus lividus*). These species have the specific feature: the blastomere isolation using Ca^{2+}-free sea water is impossible before the full completion of cleavage because it leads to the cell death and only after adhesion the isolation brings viable blastomeres. Discovery of the second cleavage pattern happened because experimenters did not wait for the completion of adhesion of blastomeres and that species used (S.nudus и Sc. mirabilis) allowed easy isolation both using Ca^{2+}-free sea water or simple mechanical isolation. Only recently the way to isolate blastomeres of *P.lividus* was found: the replacement of Ca^{2+}-free sea water for normal sea water just before isolation preserves the viability of the blastomeres, isolated before adhesion [30].

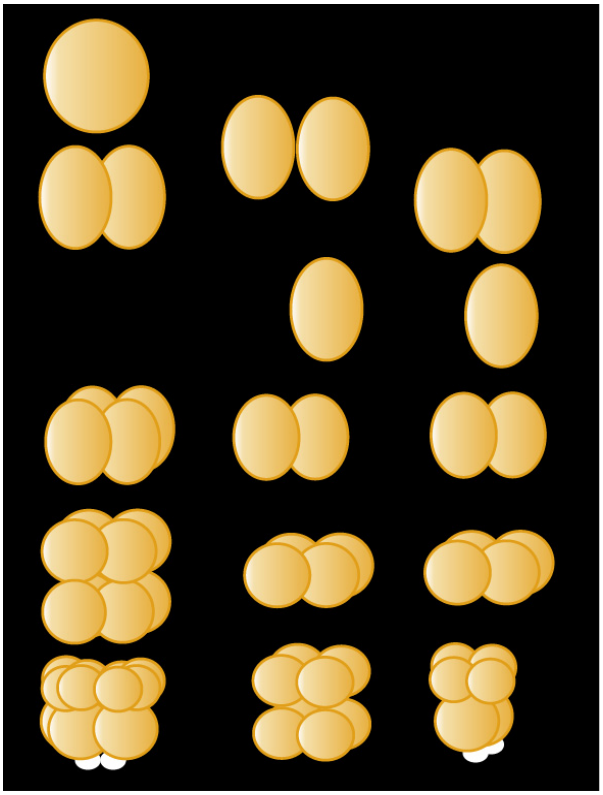

Figure 6. "Micromere model" of blastomere interactions
Left column – normal development of sea urchin, middle – cleavage pattern of blastomere, isolated before adhesion with equal 4th cleavage division, right - cleavage pattern of blastomere, isolated after adhesion with micromere formation simultaneously with intact embryos

Amusingly, the classical Driesch's statement on the ability of early sea urchin half-embryos to regulate their development was right but based on incomplete data concerning the cleavage pattern, disregard of internal contradiction, and specific phenomenon of late regulation of the blastula form (closing of opened half-blastula). So the correct conclusion was made on the basis of wrong premises. Now on the base of our own results we can suggest new and more complicated but more adequate "micromere model" that take into account the existence of substantial blastomere interactions instead of "micromere clock" (Fig. 6).

3. Possible mechanisms of blastomere interactions

Among the hyaline layer, providing the mechanical integrity of the embryo, the holistic development is grounded on the "direct blastomere signaling" [25]. Abstracting away from the nature of such signal for a time we can reconstruct the sequence of the events, leading to

the formation of the micromeres as follows. The position of the furrow of the 1st cleavage division is predetermined by animal-vegetal axis, then the position of next cleavage furrow is determined by internal asymmetry of the blastomere (see for review [31]) formed under the influence of local intercellular signal (other word – Sax - Hertwig rule works). The above mentioned asymmetry is re-determined at the each next cleavage, including repeated signals from the contact zones of previous divisions (Fig. 7). Finally, after the 3rd cleavage division whose furrow is normally formed the at the equatorial plane the "critical mass" of vegetal cytoplasm evoke the asymmetric anchoring of the contractile ring of the 4th cleavage division. Even this process is situational and dynamic because the surprising observation which was made in *S.mirabilis* embryos: the furrow of the 4th cleavage division initially formed asymmetrically (as micromere will form there) but then the contractile ring migrated to about the middle of the blastomere and closed there [32]. Probably, there are some preferred sites of the furrow anchoring that are selected in dependence of cytocortex configuration. Let us recall that the processes of cellular interactions forming this or that cleavage pattern are multiple, non-synchronous and have own geometry, therefore the cleavage pattern of half-embryos is more or less stochastic but not unambiguous.

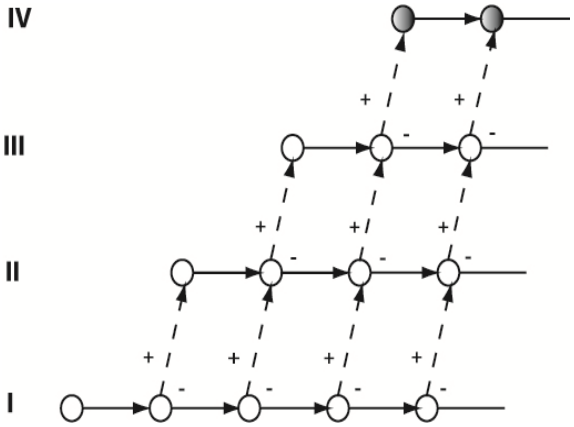

Figure 7. Scheme of the events, leading to the formation of the micromeres (from [9])
I – IV – numbers of cleavage divisions
"+" - realization of intercellular signal
"-" - absence of normal signal

Thus the sea urchin embryo really **is not able to "count to four"** but the micromere formation is determined by the realization of, at least, three consecutive intercellular signals (Fig. 7 [9, 29]).

One of the concepts, explaining the observed phenomena, was grounded on the geometric considerations, i.e. on the blastomere shape changes exclusively [33]. The cleavage patterns of half-embryos were scrupulously studied using the labeling of the cell surface with carbon particles and great diversity of the blastomere constellations was found. Nevertheless all this diversity resolves into three main variants: formation by half-embryos at the 4th

cleavage division of one or two micromeres, or equal blastomeres only. However, this line of research got no further development, moreover no specialized structures were found in the contact zone using scanning electron microscopy [34] and no any new facts allow to discuss this approach.

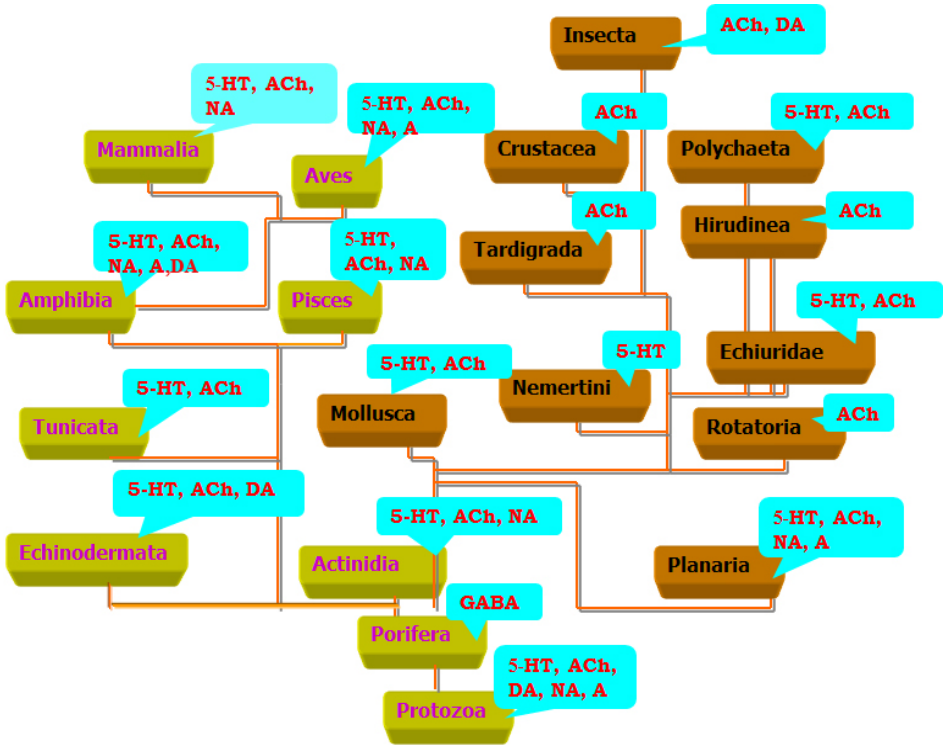

Figure 8. Transmitters in early embryos and Protozoa
5-HT – serotonin, DA – dopamine, NA – noradrenaline, A- adrenaline, ACh - acetylcholine

The idea on the possibility of interblastomere signaling via gap junction [35] failed because of that simple fact that such structures first occur in sea urchin embryos at 16-cell stage only [36, 37].

The investigations of the transfer of chemical signals between blastomeres occur more perspective and developed better. The possibility of the participation of prenervous transmitters in these processes became evident after the demonstration that transmitter antagonists are able to evoke the functional disturbances of cellular interactions [38]. Transient (10 – 15 minutes) action of serotonin antagonists before the end of post-division adhesion lead to the formation of Siamese Twins, half-embryos (including "opened half-blastulae") and 8-shaped embryos. This findings served as the base for studies of the effects of such substances in above mentioned "micromere model", although it was clear that at least in part their effects are due to the blockage of "post-division adhesion" [27].

4. Usual transmitters in unusual situation

Why the idea appeared on the participation of the transmitters such as serotonin, catecholamines and acetylcholine in embryonic cellular interactions far before the formation of nervous cells and even their precursors?

After Otto Loewi's discovery of neurotransmitter function of acetylcholine [38] the researches in this field became avalanche-type and brought immense new knowledge on the intercellular signal substances (now more than 40) and their intracellular transduction pathways. It lead to the revolution in the understanding of the mechanisms of various pathologies and, on the other hand, new pharmacology and therapy appeared, based on the knowledge on the chemistry of neurotransmitter processes. For a long time the nervous or nervous-muscular function of the transmitters were believed as unique that became sacrosanct paradigm.

As always in parallel to the neurotransmitter concept formation the facts were accumulated that not fitted into it. First of all, it is the discovery of the presence of acetylcholine in gonads and early embryos of sea urchins [39-42]. The only author's explanation was that it is "the supply for future use in nervous system". Later a lot of data was accumulated on the presence of the transmitters in the early (prenervous) embryos of all species studied also as in protozoans [43-46] (Fig. 8).

Figure 9. Principal scheme for first step of evolutionary transmitter origin (after [54])
1 – stream of substantial aminoacids into the cell; 2 – high threshold of key transmitter-synthesizing enzyme; 3 – transmitter-synthesizing enzymatic system; 4 – protein synthesis; 5 – newly synthesized transmitter; 6 – transmitter receptor, involved in the control of protein synthesis; 7 - flower

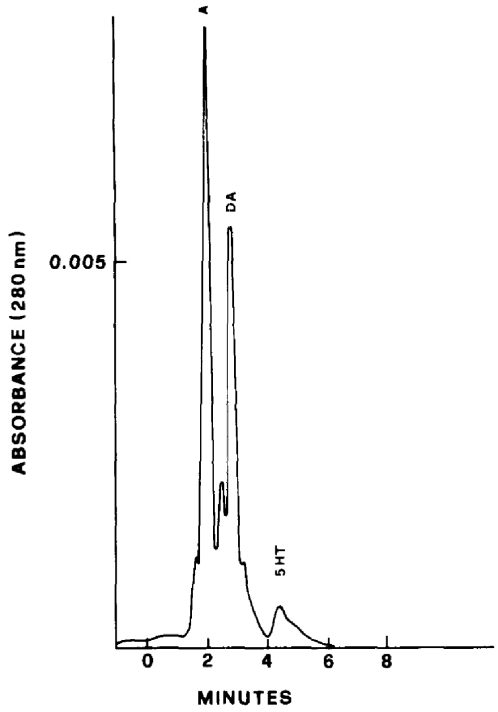

Figure 10. HPLC of the transmitters in unfertilized Paracentrotis lividus eggs
A – adrenaline, DA – dopamine, 5-HT – serotonin (from [56])

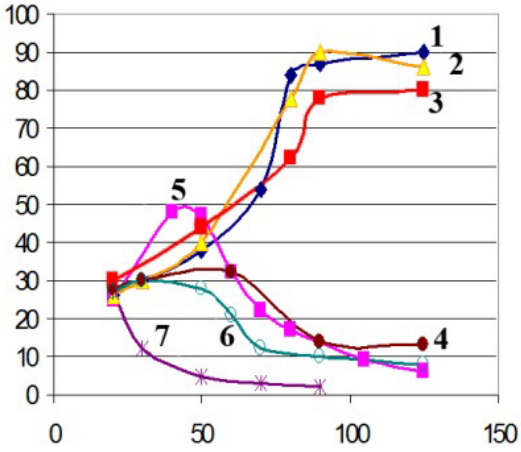

Figure 11. Effects of β-adrenergic (1-3) and serotonergic antagonists on the rigidity of sea urchin Paracentrotus lividus early enbryos (from [46]).
1 – alprenolol (400 μM), 2 – propranolol (200 μM, 3 – dichloroisoproterenol (500 μM),
4 – cyproheptadine (35 μM), 5 – inmecarb (20 μM), 6 - DPTC (75 μM), 7 – cytochalasine B
(10 μM, for comparison). Abscissa: time from fertilization (min); ordinate: rigidity (din x $cm^2/\mu m$)

Components of transmitter system such as receptors and corresponding enzymes were also found everywhere in animal kingdom [44-49] even in Prokaryota [50, 51] although it is impossible to exclude secondary origin of such receptors as the result of the interactions with highly developed host organisms.

First attempt to elaborate the concept that could connect all the data on the transmitters was shot in the middle of XX century by outstanding comparative physiologist Khachatur Koshtoyantz [52]. He advanced the idea that neurotransmitter function is the result of evolution of original intracellular mechanisms of the metabolism regulation which can persist in any changed forms in the embryos of the contemporary species. Pioneer experiments of Buznikov, former student of Koshtoyantz, have shown the serotonin regulation of nudibranch velliger ciliary motility [53] and the ability of transmitter antagonists to block specifically sea urchin cleavage divisions [54] that confirmed the functionality of embryonic transmitters. Later this concept was developed on the base of Koshtoyantz original idea and the data accumulated [55], coming from the fact that some classic transmitters are the metabolites of the substantial aminoacids. Such aminoacids as phenylalanine and tryptophan cannot be synthesized by the animal cells and, thus, are the limiting point in the process of protein synthesis. High threshold enzyme that transforms aminoacid residues into the form that, on one hand, cannot be used in the protein synthesis and, on the other hand, could be recognized as the signal molecule together with the receptor molecule form the intracellular probe of the levels of the component, limiting the protein synthesis (Fig. 9). If both threshold concentrations triggering the enzyme, transforming the aminoacid, and the sensitivity of proteins (prospective receptor) to such transformed aminoacid (prospective transmitter) are sufficiently high, this offer the possibility of control over intracellular levels of substantial aminoacids in the cell. In other words, it is easier for cell to detect even a few of transmitter molecules (transformed aminoacid) then measure the absolute levels of regular aminoacids. An increase in the concentration of certain transmitter (transformed aminoacid) to the threshold levels would then indicate that total aminoacid concentration attained the level sufficient for successful protein synthesis. According to the number of substantial aminoacids there are corresponding number of their derivatives, performing the functions of the transmitters (phenylalanine – dopamine, catecholamines; tryptophan – tryptamine, serotonin, histidine – histamine etc). It is noteworthy that just in Protozoa and early embryos of multicellular organisms Dale principle: "one neuron – one transmitter" does not work. It was shown that protists and early embryonic cells may contain more than one, up to four, transmitter simultaneously (Fig. 10, see also for review [44]). Our recent study has shown the simultaneous presence of dopamine, noradrenaline and serotonin in zygotes and cleaving embryos of *Xenopus laevis* (Shmukler, Nikishin, unpublished data). Such "metabolic hypothesis" can also explain the multiplicity of neurotransmitters and finally solve the problem quoted by Kandel and a number of authors [57-60]: «Why do neurons have different transmitters when any one transmitter could in fact mediate all the required electrical signals?» All previous attempts to answer this question were limited to various features of the process of nervous signaling organization but ignored evolutionary aspect of the problem.

5. Embryonic transmitters – functions and specific features

Soon after discovery of the serotonin ability to regulate embryonic ciliary motility, the specific effects of transmitter antagonists onto cleavage divisions in various species, first of all echinoderms, were found then confirmed in all taxons studied [43, 44, 61, 62]. Antagonists of serotonin, catecholamines and acetylcholine added soon after the fertilization blocked the cleavage divisions and their effect could be prevented or weakened by the addition of specific transmitter. Most probably, transmitter antagonists have multiple effects onto cleaving embryos, in particular the triggering of cell cycle is influenced (44, 46, 55, 63] and the state of cytoskeleton [64], interestingly, in the latter serotonin and catecholamines worked as antagonists of each other (Fig. 11). Probably, serotonin also takes part in the control of closing of the contractile ring [65] of cleavage furrow and further adhesion of blastomeres [27]. At the later stages transmitters takes part in the control of left-right asymmetry formation, larval ciliary motility, gastrulation, cranio-facial and heart morphogenesis etc [46, 87, 88, 100]. These transmitter functions are realized simultaneously and/or consecutively all over ontogenesis (Fig. 12) [46]. Thus, neurotransmitter function itself is *ultimus inter pares* only.

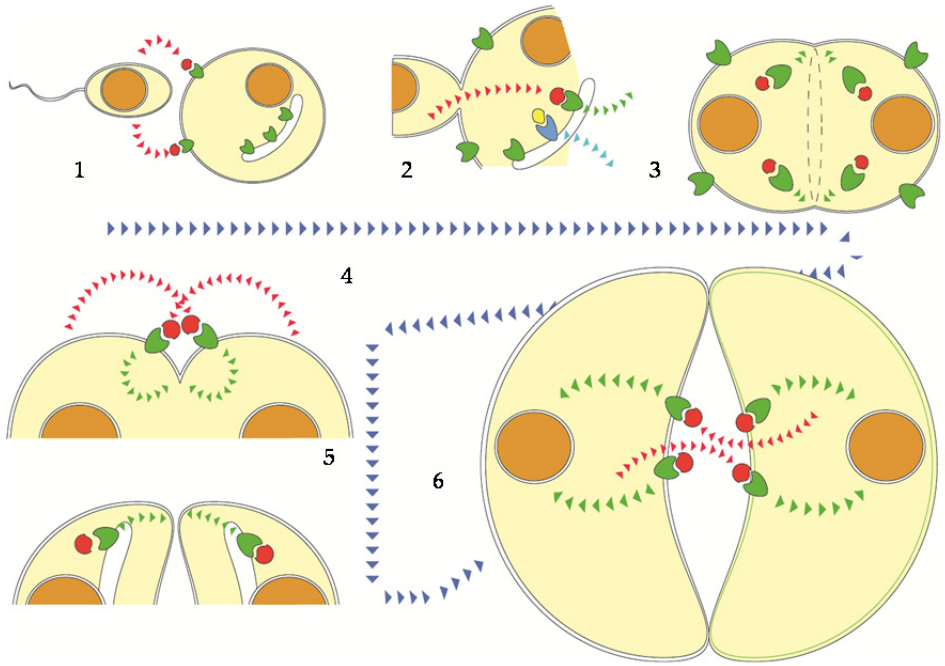

Figure 12. Transmitter control of the 1st cell cycle of sea urchin embryo.
1 – interaction with surface membrane receptors at fertilization; 2 - interaction with intracellular receptor at fertilization and triggering of cell cycle; 3 – control of the state of cytocortex via intracellular receptors; 4 – control of the completion of cleavage furrow via surface membrane receptors; 5 – control of post-division adhesion of blastomeres via intracellular receptors; 6 – direct exchange with interblastomere signals via surface membrane receptors.
Red and yellow signs – transmitters, green and blue signs - receptors

Many transmitter-related effects in embryogenesis are coupled to **intracellular** receptors [43, 44, 63, 66]. For the first time ever it was marked occasionally during the studies of the effects of transmitter antagonists, especially serotonin, it was found that the embryostatic activity depends on their ability to penetrate the cytoplasm from the medium [27, 44]. First the difference was noted between tertiary and quaternary serotonin analogues [43] but then the direct dependence was found between the embryostatic effect of indole derivatives and their lipophily [67]. On the base of these data Buznikov suggested non-trivial idea on intracellular localization of receptor link of embryonic transmitter process [43] that remain strange for physiologists until now despite the results of direct experiments with microinjection of transmitter receptor ligands into the cells of early *Xenopus* embryos [63, 66]. Microinjection of propranolol (antagonist of β-adrenoreceptors) and atropine (antagonist of m-cholinoreceptors) evoked transient block of cleavage divisions in *Xenopus* embryos that could be weakened by the addition of corresponding transmitters. Specific binding of radiolabeled ligands by microsomal fraction of *Xenopus* embryos were also demonstrated [68]. Data on the intracellular localization of transmitter process were obtain also in other subjects [69-71]. Recently the expression of the components of embryonic serotonergic system was shown that allow us to suggest the scheme of such intracellular receptor mechanism that includes receptor and transporters of the transmitter (Fig. 13). For a time the intracellular localization of embryonic transmitter mechanisms became new paradigm for the researchers in this field until new data forced to withdraw from it.

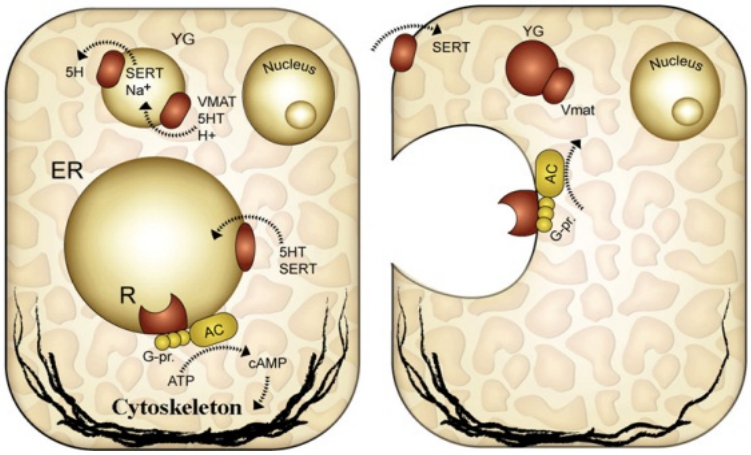

Figure 13. Hypothetic scheme of intracellular embryonic receptor mechanisms
Left – transmitter receptor is localized on the inner surface of endoplasmic reticulum (ER) being coupled to G-protein and adenylate cyclase (AC). Transport of the transmitter to ER and out there into cytoplasm is supplied by the transporters SERT and VMAT. Supposed place of transmitter synthesis (serotonin in present case) is yolk granule (YG). Right – imbedding of receptor structure from the left into the surface membrane gives usual membrane receptor complex

In spite of such non-trivial localization the embryonic transmitter mechanisms show the features similar to the classic ones. Effects of serotonin can be imitated by cyclic nucleotides,

i.e. they weaken embryostatic effects of serotonin antagonists [72-74] and evoke the increase in cAMP levels in embryonic cells [75]. At the same time the ability of transmitter ligands to influence the activity of protein kinase C and intracellular free calcium ion levels was also shown in early sea embryos [65, 76, 77].

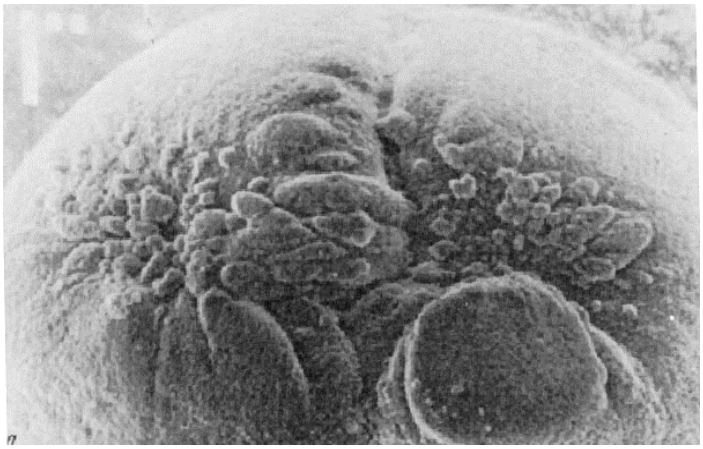

Figure 14. "Bubbling" of blastomere surface of X. laevis after cAMP microinjection (scanning electron microscopy) (from [80])

The presence and functional activity of both transmitters and second messengers inside the embryonic cells may seem excessive but only at a superficial glance. First, second messengers are effective at relatively short distances (in case of IP3, no more than 20 μm and about 3 μm for calcium ions) whereas the diameter of, for example, sea urchin egg is about 100 μm or even greater [78, 79]. Moreover microinjection of cAMP and calcium ions caused diffuse "surface bubbling" (Fig. 14, [80]) whereas microinjection of adrenaline into the blastomeres of Xenopus laevis merely accelerated cleavage furrow formation [63]. Thus transmitter, at least in this case, is more "targeted" messenger as compare to second ones that are able to activate a number of the effectors.

We should note that intracellular localization of embryonic transmitter receptors is in a good agreement with original Koshtoyantz' idea that "cell-keeping" function of the transmitters is evolutionary archetypal. Nevertheless, the fate of this paradigm is the same as the fates of other paradigms which seemed unbreakable. At the early nineties of XX century first facts were found that not fitted into previous paradigm. Some phenomena such as effects of neuropharmaca in "micromere model" (see below) and "phorbol syndrome" in sea urchin early embryos in contrast to previously studied effects on cleavage division were evoked by transmitter ligands poorly penetrating embryonic cells [81]. Correspondingly, the specific binding of radiolabeled ligand of serotonin receptor 8-OH-DPAT in the conditions that maximally restrict the penetration of ligand into the cytoplasm (0°C, short incubation) was shown [82, 83]. Later another effects of serotonergic ligands, poorly penetrating the cells of sea urchin embryos, in particular the influence of ligands on the levels of intracellular free

Ca^{2+} and inward currents [30, 65, 84]. Thus, most probably embryonic cells contain not only few transmitters but also multiple receptors that differ in their localization. In the next section the complexity of this system will be additionally sophisticated by the diversity and multiplicity of the receptor types.

6. Expression of the components of transmitter system in embryogenesis

For a long time the studies of embryonic transmitter systems developed using mainly physiological, biochemical and rarely cyto- or immunocytochemical approaches, whereas molecular biology data were sparse and rare. Only during last decade some studies appeared that confirmed by these methods the presence of the expression of the components of transmitter systems, first of all transmitter receptors in early embryos.

It was shown that already during cleavage divisions embryos of various species expressed mRNAs of transmitter receptors (Fig. 15, Table 3). Several types of the receptors to the same transmitter can be expressed simultaneously besides receptors to other transmitters. In particular, the expression of serotonin receptor type 4 also as several n-cholinoreceptor subunit was shown in early sea urchin embryo whereas in clawed frog embryo – serotonin receptors type 2 and 7 along with β-adrenoreceptor. Together with the data on the specific binding of transmitter ligands (44, 68, 79, 83) it suggests the presence of corresponding receptor proteins too. Identity of transmitter receptors' sequences in embryos and in adults is the indirect argument in favor of genetic unity of embryonic and definitive transmitter mechanisms, including cellular interactions. The expression of other components of serotonergic system such as transporters SERT and VMAT and enzymes of serotonin synthesis was found in clawed frog Xenopus and mammalian embryos [62, 85-87].

Species	Gene	Reference
Mouse Mus musculus	HTR1D	[90]
	HTR5	[91]
	HTR7	[92]
	β-AdR	[93]
Caenorhabditis elegans	HTR2C	[94]
Danio rerio	HTR1A	[95]
Sea urchin Paracentrotus lividus	HTR4	[97]
	nAChR α6	
	nAChR α10	
	nAChR α7	[96]
Clawed frog Xenopus laevis	HTR2C	[85]
	HTR7	
	β-AdR	[98]

Table 3. Accumulated molecular biology data on the expression of transmitter receptors during the early embryogensis of various species (from [85]).

Our study of other serotonin receptors in *Xenopus* embryos has shown that HTR1A is detectable beginning at late blastula stage only, HTR3A and HTR4 – at the beginning at the neurula stage, and HTR2B after hatching. The entire set of serotonin receptors is expressed at the tadpole stage only. Pharmacological and immunohistochemical data support the possible participation of 5-HT3 and 5-HT4 receptors in establishing the left-right asymmetry in *Xenopus* [88]. The 5-HT_3 receptor is a ligand-dependent ionic channel whose functional activity is associated with the presence of HTR_{3A}-subunits [89]. Therefore, the absence of HTR3A expression during early embryogenesis results in the absence of *de novo* formation of the functional 5-HT_3 receptors at this stage. The controversy between our data and those reported by Fukumoto et al. [88] can be explained by the difference in the sensitivity between mammalian and amphibian serotonin receptors to the same ligands. Fukumoto et al. [88] also reported the presence of HTR4 transcripts during the early stages of the development using *in situ* hybridization (ISH). Taking into account the higher specificity and sensitivity of RT-PCR [85] compared with ISH, a false-positive result of the ISH is more probable than a false-negative RT-PCR result.

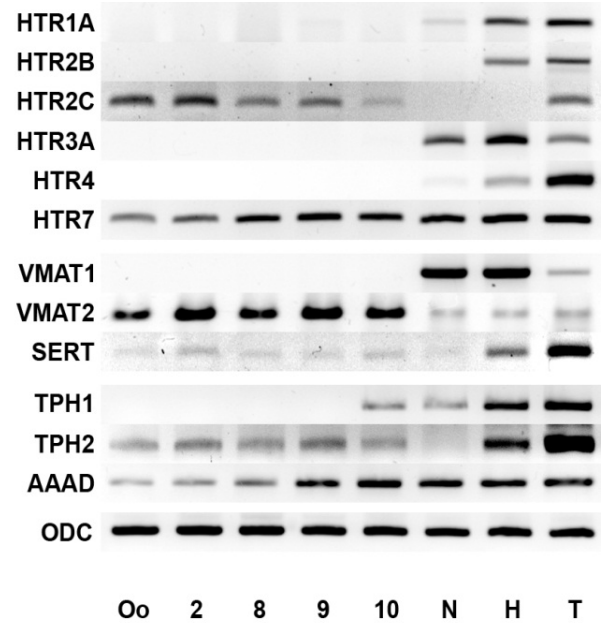

Figure 15. Temporal expression of serotonergic system components during Xenopus laevis development (from [82].
Oo – oocyte, 2 – 2-cell embryo (stage 2), 8 – midblastula (stage 8), 9 – late blastula (stage 9), 10 – early gastrula (stage 10), N – neurula (stage 15), H – hatched larvae (stage 33-35) and T – tadpoles (stage 57). ODC is the endogenous control. The serotonin receptors HTR2C and HTR7, vesicular transporter VMAT2, sodium-dependent transporter SERT, enzymes of synthesis tryptophan hydroxylase (TPH2) and aromatic aminoacid decarboxylase (AAAD) are expressed during the early stages of development (from [85])

It is intriguing that we found a wide diversity of serotonin receptor types that are expressed during early development (Table 1) when summarizing both our data and the literature data, with a few coincidences – the 5-HT2C in *Xenopus* and *C. elegans*, and the 5-HT7 in *Xenopus* and mouse. It is possible to reliably predict that serotonin receptors are also expressed in the embryos of species not yet studied in this regard, similarly to serotonin and other transmitters that were discovered in embryos of all species investigated. However, the types of serotonin receptor evidently are not strictly determined, although their mechanisms would be highly conserved.

Thus the transmitters and all main components of their systems which are characteristic for definitive organisms are present in early embryos and are functionally active all over early development (all over whole ontogenesis too).

7. Again to embryonic cellular interactions

Interestingly, prenervous transmitter mechanisms were not considered as the candidate to the role in embryonic cellular interactions at the start of these researches although it evidently offered. Probably, the paradigm of intracellular functions of embryonic transmitters influenced the approach of Founding Father of this scientific field Prof Buznikov who suggested that "*transmitters may take part in early cellular interactions by certain way but it is improbable that they are embryonic intercellular signal substances itself*" [99]. The ability of the serotonin antagonists to suppress blastomere adhesion (that lead to the functional isolation of the blatomeres) did not contradict this concept. Nevertheless it was highly inviting to check the ability of the prenervous transmitters to participate in the process named by Vacqueir and Mazia [25] "direct interblastomere signal exchange" that really influence the prospective fates of the cells of embryo.

8. The transmitter effects in micromere model

Addition of the serotonin to blastomeres, isolated before post-division adhesion, significantly increased the portion of half embryo with partial cleavage pattern, when unequal 4th cleavage occur (Table 4), meaning serotonin imitates the interblastomere signal in intact embryo. It is noteworthy that it was the first early embryonic model that allowed to obtain own effect of the transmitter but not its antagonists since original experiment in nudibranch veliger. The same concentrations of serotonin onto the blastomeres isolated after adhesion did not influence the cleavage pattern of half-embryos since, probably, it added nothing to natural signal already received by blastomere. In turn, serotonin antagonists had no effect in half-embryos, isolated before adhesion but after adhesion significantly increased the portion of half-embryos with integral cleavage pattern (with equal 4th cleavage division), i.e. imitated the avoid of interblastomere signal (Fig. 16). Along with serotonin antagonists the blocker of serotonin and catecholamine reuptake imipramine occur highly effective (Table 4). It is important that the delay of micromere formation under the action of neuropharmaca was found also in intact embryos in the special experiments [27].

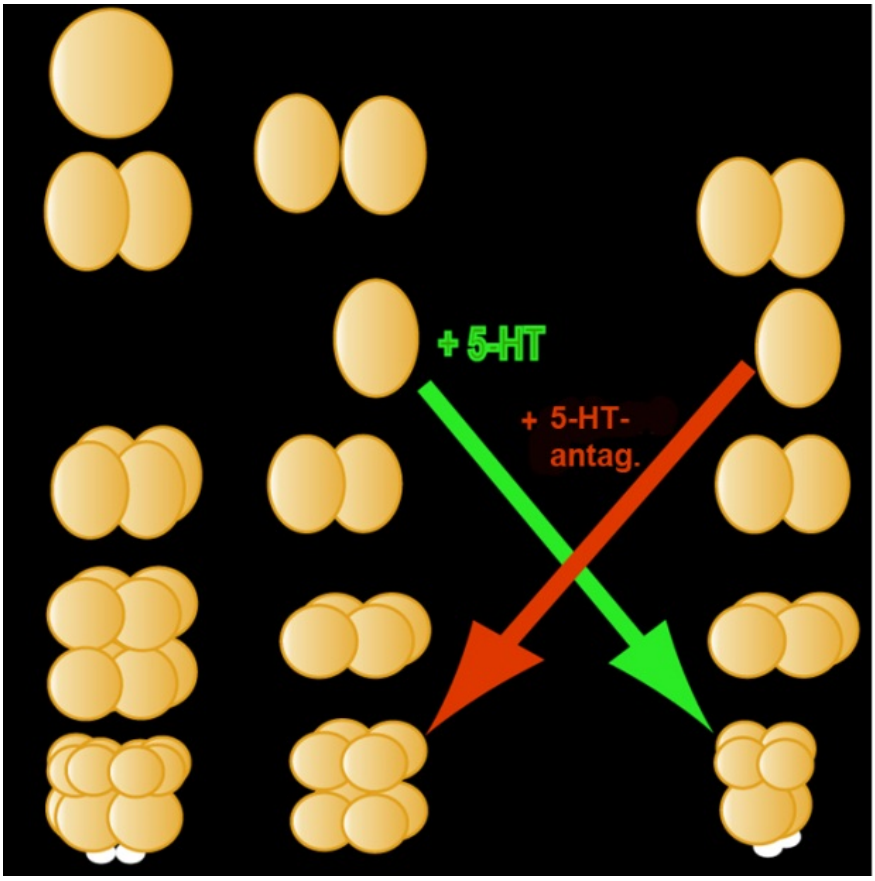

Figure 16. Effects of serotonin and its antagonists in "micromere model"
Left column – cleavage pattern of intact embryo; middle – prevailing pattern of half-embryos, isolated before adhesion, right – prevailing pattern of half-embryos, isolated after adhesion. Arrows show the possibility to influence the pattern type by serotonergics

The works with "micromere model" were started at the time when neither contemporary ligands nor transmitter classification existed [32], so recently we needed to repeat some experiments using new ligand (mainly belonging to agonists of 5-HT_3-receptors) in classic subject – sea urchin *Paracentrotus lividus* embryos [30]. These experiments confirmed that in this model ligands, poorly penetrating the cells, are equally or more effective than their lipophilic analogues [82, 83], i.e. the receptors involved are localized on the surface membrane in contrast to that regulating cleavage divisions and blastomere adhesion ones. This statement is supported also by the ability of surfactants to disturb the cellular interactions and blastomere formation in intact embryos of sea urchins [27, 32] and by the results of serotonin ligand binding assays in the conditions maximally restricting the penetration of ligand into the cell [82].

Species	Moment of isolation	Substance	Concentration (μM)	Change of portion of half-embryos, forming micromeres simultaneously with intact embryos (%%)	Significance
S. mirabilis	B_1 (317)	Serotonin	55	+14 ± 4	<0,001
	B_{12} (180)		55	+12 ± 6	<0,05
	B_{123} (86)		55	+14 ± 4	<0,01
	B_1 (115)	Tryptamine	250	+13 ± 6	<0,05
	B_1 (73)	Carbacholine	275	- 8 ± 8	n.s.*
	B_1 (53)	ATP	360	+ 3 ± 9	n.s.
	B_1 (51)	Dopamine	260	+ 6 ± 10	n.s.
	B_1 (84)	Papaverine	50	+34 ± 6	<0,001
	B_1 (101)	cAMP	270	+ 7 ± 5	n.s.
	B_1 (107)	cGMP	270	- 8 ± 6	n.s.
	B_1 (92)	dibutyryl-cAMP	210	+ 41 ± 6	<0,001
	A_1 (170)	Imipramine	5	-32 ± 5	<0,001
	A_1 (77)	Cyproheptadine	60	-21 ± 6	<0,05
	A_1 (85)	Inmecarb	25	-34 ± 8	<0,001
	A_1 (62)	Inmecarb methiodide	25	-26 ± 8	<0,001
	A_1 (71)	Aminazine	15	- 3 ± 8	n.s.
	A_1 (64)	Propranolol	135	+ 2 ± 7	n.s.
	A_1 (96)	Gangleron	32	-10 ± 7	n.s.
	A_1 (48)	Quatelerone	400	- 2 ± 9	n.s.
	B_1(86)	Valinomycine	$5{,}4 \times 10^{-3}$	+22 ± 9	<0,05
	A_1(89)	Ouabaine	1000	-25 ± 9	<0,01
	A_1(70)	Triftazine	49	-28 ± 13	<0,05
S.nudus	B_1 (36)	Serotonin	112	+24 ± 12	<0,05
P.lividus	A_1 (53)	Inmecarb	50	- 1± 12	n.s.
	A_1 (203)	Inmecarb methiodide	40	-30 ± 10	<0,05
	A_1 (27)	KYuR-14	100	0	n.s.
	A_1 (43)	KYuR-14 methiodide	100	-17 ± 7	<0,05
	A_1 (137)	Imipramine	60	−34 ± 4	<0,001
	A_1 (97)	3-Tropanylindole carboxylate methiodide	100	−25 ± 7	<0.001
	A_1 (96)	3-Tropanylindole carboxylate hydrochloride	100	−12 ± 1	<0.001
	A_1 (82)	Quipazine	100	+24 ± 1	<0.001

Table 4. Effects of chemical substances on the cleavage patterns of sea urchin half-embryos
* not significant
Designation B and A as in Tables 1 and 2. B_{12} – embryos, isolated before adhesions in 1st and 2nd cleavage divisions. B123 - embryos, isolated before adhesions in 1st – 3rd cleavage divisions. Number of embryos in parenthesis Inmecarb and KYuR-14 also as their quaternary analogues are the originally synthesized indole derivatives

Ligands of acetylcholine- and adrenoreceptors had no significant effects in this model of blastomere interactions, although, as was mentioned above, we detected the expression of the subunits of nicotinic cholinoreceptor in the early sea urchin *P.lividus* embryos [97]. At the same time in the whole-cell patch-clump experiments in cleaving *P.lividus* embryos nicotine and n-AChR-agonists epibatidine and methylcarbamylcholine evoked small inward currents in a few cases only, whereas 5-HT_3-receptor agonists (5HTQ, SR 57277A, quipazine, methylquipazine) stably induced inward currents that were maximal during the formation of furrows of 1st and 2nd cleavage divisions both at microapplication or addition into experimental chamber [30]. Such discrepancy may be because of two circumstances: first, receptor ligand were elaborated and tested in mammalian models and their specificity in echinoderm embryos is insufficient, especially if take into account high homology between nicotinic acetylcholinoreceptor and serotonin receptor type 3. The similarity of 5-HT_3- and n-AChR is quite considerable, in particular, aminoacid sequence of rat 5-HT_{3A}-receptor (P35563) coincides to rat α_{10}-n-AChR subunit sequence (NP_072161) by 89%, and by 96% - to the sequence of n-AChR α_6-subunit precursor (NP_476532) [97]. Second, it is surprising but ligands of 5-HT_3-receptor are highly effective in 5-HT_4-receptor although former is ligand-dependent ionic channel whereas latter is metabotropic receptor [101, 102], whose expression in early sea urchin embryo was recently shown [97].

The suggestion that the effects of serotonin agonists are mediated by 5-HT_4-receptor is supported by the data as follows. 5-HT4-receptor is known to activate adenylate cyclase [101]. Papaverine (blocker of phosphodiesterases) and dibutyryl-cAMP have similar and even more pronounced effects as serotonin in “micromere model”, i.e. it is possible that in the present case serotonin triggers the signal transduction pathway via adenylate cyclase. Moreover serotonin activates adenylate cyclase in sea urchin embryos [73] Furthermore, the adenylate cyclase activity in the early sea urchin embryos which first was localized at membrane of endoplasmic reticulum then transferred to the microvilli in the contact zone and after adhesion increased greatly at the places of the closest contact of the membranes of sister blastomere [103]. Similar data were obtained in mammalian embryos [104].

9. Concepts of chemical blastomere interactions

The first attempt to form the concept on the transmitter-based mechanism of embryonic cell-cell interactions was made yet in 1981 but ruling paradigm of intracellular localization of embryonic transmitters lead to the formation of pretentious construct that included isolation of the signal molecules from the receptors in the same cell [35] and involvement of gap junctions. Soon occur this concept is totally insufficient because it was found that gap junctions first appear in sea urchin embryo at 16-cell stage only [105]. It forced us to revise the concept especially taking into account the data on the existence of surface membrane transmitter receptors obtained to date.

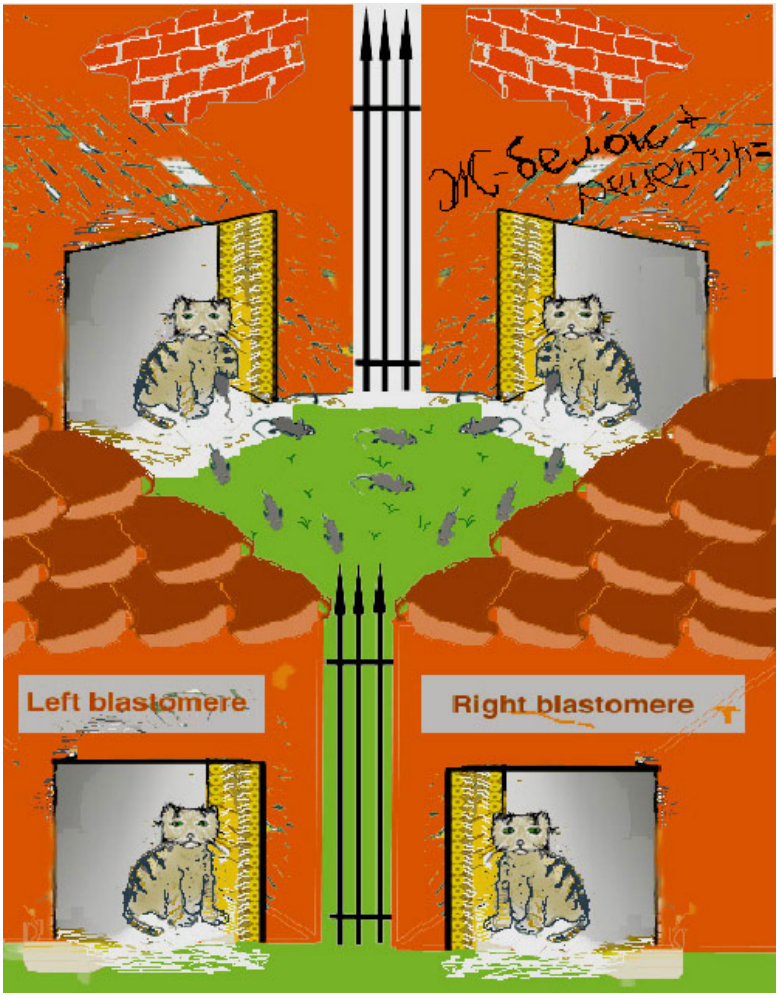

Figure 17. Allegory of protosynapse
Tomcat – receptor; mouse – transmitter; green lawn – interblastomere cleft; bars – adhesion contact, isolating interblastomere cleft from outer medium
The probability of transmitter–receptor interaction is higher in the interblastomere cleft then at free blastomere surface.

10. Protosynapse

The idea on the exchange with chemical signals between blastomeres suggested itself because of accumulation of various data on unusual concentrations of transmitters and related substances as gangliosides [109, 110] and products of adenylate cyclase activity [103] in the contact area of blastomeres. Suggestion on the localization of transmitter receptors at the surface membrane of blastomeres [81-83] became impulse to elaborate the new concept.

Similar situation is suggested in case of cholinergic interaction of gametes that both contain acetylcholine and corresponding receptors, taking part in the fertilization [96, 107]. The second was the astonishing fact that the main way of transmitter inactivation in the embryonic cells are the transport of the transmitter molecules to outer medium because of low activity or absence of MAO (enzyme of serotonin and catcholamine degradation) [96, 108]. Recently the absence of the expression of MAO A at early stages of *Xenopus* development was shown [85].

Transmitter-driven blastomere adhesion shorten the distance between blastomeres and creates the interblastomere space which is the prerequisite for further intercellular signal exchange. The leakage of the transmitters from the interblastomere compartment is restricted by adhesive contacts and the concentration of transmitter in the interblastomere cleft remain increased as compare to free blastomere surface.

Coming from above mentioned and the fact that both blastomeres of regulative embryos are equal in properties and prospective potencies the suggestion was made on the existence of **double-side symmetric structure of signal exchange**. The presence of transmitter receptors in blastomere surface membrane, including interblastomere contact zone, make possible such structure, where both blastomeres are: i) the source of signal substance, ii) its target, and iii) the obstacle for leakage of the transmitter, named **"protosynapse"** [82, 83] (Fig. 17).

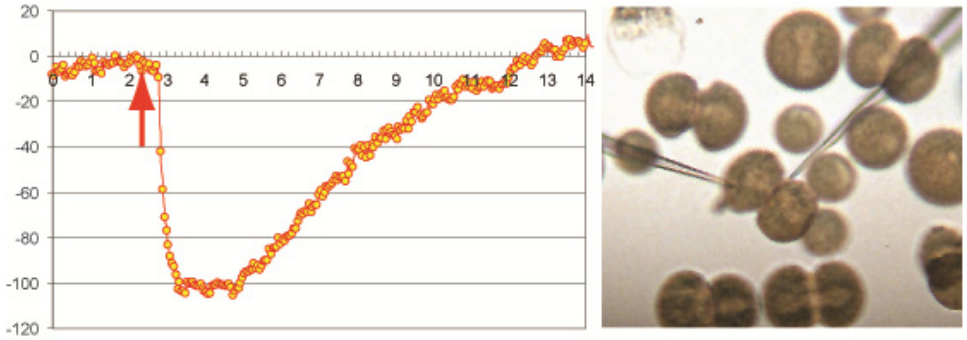

Figure 18. Effect of 5-HTQ microapplication into contact area of 2-cell *P.lividus* (from [30])
Left – current, evoked by single pulse of 5-HTQ (serotonin agonist), red arrow - the moment of application; abscissa - current (pA), ordinate – time (sec); right – experimental arrangement, left pipette is for whole cell patch, right pipette – for application

Coming from such point of view it is not significant whether transmitter receptors are equally distributed over the blastomere surface or they are concentrated at the contact area because the physiological response is due to the difference of transmitter concentrations in the contact zone and at the free blastomere surface. However, this problem was solved too using microapplication of serotonin agonists to the contact area and to the free surface of whole-cell patch-clumped sea urchin blastomeres [30]. It was shown that the application of the agonist into the interblasomere cleft before the end of adhesion evoked significantly more pronounced inward currents with substantially shorter latent period, then the

application to the free surface of blastomere or after the end of adhesion (Fig. 18). Thus, localization of the transmitter receptor in the interblastomere cleft is more probable (Fig. 19), although such localization can be the result of secondary specialization of contact blastomere surface.

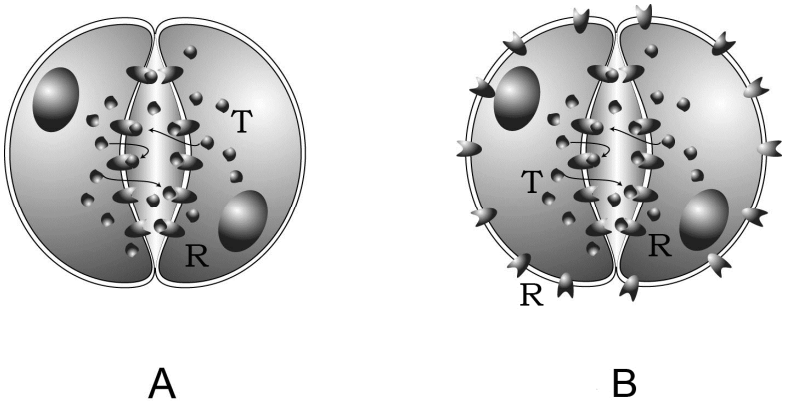

Figure 19. Serious protosynapse scheme (A – variant with transmitter receptors localized in interblastomere cleft, B – with equal receptors distribution over whole cell surface); R – receptor, T- transmitter (from [30])

Increased transmitter concentration in the interblastomere cleft and concentration of the receptors here can be the base for the formation of the primary cellular asymmetry which may thus determine the position of further cleavage plane orientation.

The existence of such structure together with the data on the genetic identity of embryonic transmitter receptors to those from adult organisms allows us to suggest that protosynapse is evolutionary predecessor of definitive synaptic structures. As the matter of fact, it is quite to remove the transmitter from one cell and the receptor – from another in protosynapse scheme to get classic synapse.

Protosynapse concept allows the analysis of all previous data on the blastomere interactions, including classic ones, and eliminate some historical injustice. As was already mentioned above, there are the complex of roots that Hans Driesch could find only one type of cleavage pattern after blastomere isolation. From the point of view of the concept under consideration late isolation, most probable in Driesch's experiments, means that blastomeres quite long remained in contact with each other, i.e. **had time to receive normal intercellular signal** and then developed with the partial cleavage pattern (Fig. 20A). Later development of such half embryos into opened half-blastula and then to closed one and quasinormal larva are the result of yet unstudied processes, not directly coupled to early blastomere interactions.

Really the same situation is reproduced in Roux experiment because although denaturated blastomere cannot be nether source, nor target of the transmitter it remains the obstacle for

the leakage of transmitter from interblastomere cleft, thus the situation for intact blastomere remain unchaged as compare to intact embryo (Fig. 20B). Therefore, it is time to rehabilitate the experiment of one of founders of experimental embryology and exonerate Roux from guilt in artifact experiment.

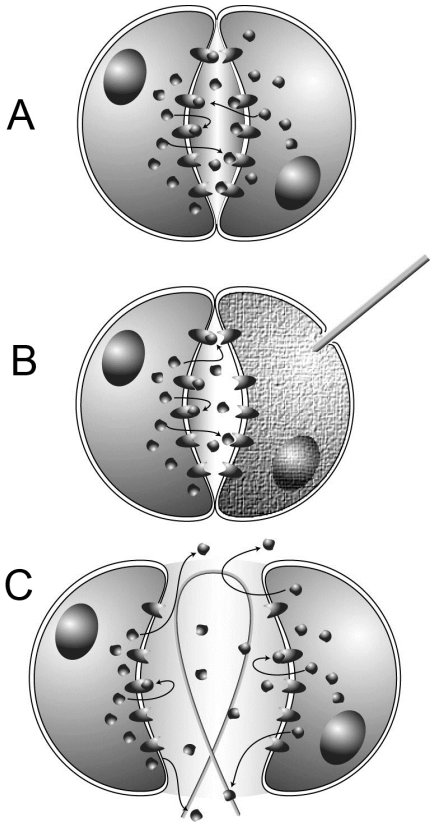

Figure 20. Analysis of various experiments on the blastomere isolation
A - Normal development and Driesch experiment; B – Roux' experiment;
C – early blastomere isolation (after [32])

In frame of the protosynapse concept it is possible to explain the fact earlier never considered. The formation of only one micromere was quite frequent. Taking into account non-synchrony of interblastomere signal realization and specificity of the geometry of interblastomere space and transmitter receptors' distribution there it is clear that adequate signal, changing the state of cytocortex, could be received by part of contact blastomere surface only that lead to the pattern formation with only one micromere.

Finally, the most easy explainable is the pattern of equal 4th cleavage division because in this case the blastomere is isolated before accumulation of enough transmitter in the interblastomere space and, correspondingly, the receiving of the adequate signal (Fig. 20C).

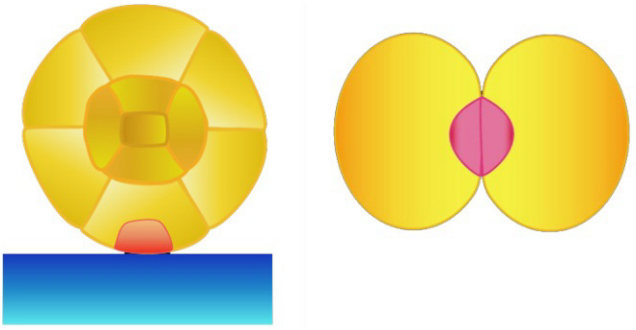

Figure 21. Origin of cell asymmetry
Left - Wolpert' concept of cell interaction with the substrate; right – protosynapse, forming internal asymmetry of the cell

The protosynapse concept also is in a good agreement with Wolpert's idea on the origin of multicellularity [111] and allow to simplify it. After Wolpert the base of origin of the cell asymmetry, needed for their specialization is the contact of part of cell with the substrate, where the zone is formed with the specific conditions that could differ from other cell surface, in particular, various regulatory substances may accumulate. The protosynapse concept allows to exclude the substrate because its role can be played by sister blastomere (Fig. 21).

11. Conclusion

So, we hope that problem of the existence of blastomere interactions is finally solved and all historic misunderstandings in this field are eliminated. Wilhelm Roux and Hans Driesch were both great researchers but they could not be irreproachable because they were first and they have created new science, invented new methods, and discovered amazing facts. It was far easier to correct their impreciseness some 90 years later.

The role of the transmitters in early development, especially in the embryonic cellular interactions, is proved too, although it attracted relatively low attention of biologists. Maybe it is because this field is "too physiological for embryologists and too embryological for physiologists". Anyway great insight of Koshtoyantz and immense Buznikov's work are still developing by their students and followers. At the same time readers can observe that great deal of the data in this field were obtained quite long ago and although they did not lose their value need in revision and renovation.

New pharmacology and molecular biology have brought new knowledge that sometimes contradicts original ideas of the researchers who carried out pioneer experiments in this field. Now studies of transmitters in the development looks like the battlefield after tank breakthrough, when fighting front got far ahead, new strategic targets appeared but a lot of enemies' firing point remained far in the rear that needs in wide and scrupulous further works.

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