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CELL BIOPHYSICS

A "Micromere Model" of Cell–Cell Interactions in Sea Urchin Early Embryos

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Abstract—It has been shown that isolation of sea urchin blastomeres before the post-division adhesion leads mainly to the formation of equal blastomeres at the stage of 4th cleavage division, whereas isolation after adhesion results in the formation of micromeres simultaneous with that in intact embryos. Similar results were obtained in five sea urchin species. It has been concluded that there exists a critical point in the cleavage process, when blastomeres exchange information that determines the further cleavage pattern. It has been shown with this "micromere model" that serotonin and its analogs influence the cleavage pattern of half-embryos. These data have served as a basis for the hypothesis of "protosynapse," a bilaterally symmetric structure in which the blastomeres are not only source and target of the signal but also a passive obstacle to leakage of the signal substance from the interblastomere cleft to the milieu. Such a structure may also specify the primary asymmetry of the blastomeres. The micromere model may be useful in specific pharmacological screening.

Key words: sea urchin, half-embryo, cleavage division, blastomere isolation, micromere, serotonin, protosynapse

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INTRODUCTION

The discovery by Hans Driesch of the ability of isolated sea urchin blastomeres to form quasinormal plutei [1] has underlain the ideas on the regulation of early embryogenesis. According to Driesch's experiments, the blastomeres isolated during the first cleavage divisions possess totipotency that is, however, restrained by factors inherent in an intact embryo. This restraint might be caused by the presence of a sister cell, which, however, contradicts the classical data.

The ability to form full-fledged small plutei does not match the cleavage type corresponding to a half of the whole embryo, with formation of two micromeres [1, 2] (Fig. 1). These data made the basis of the "micromere clock" concept, whereby the moment of unequal division in sea urchin embryos is stipulated by the commencement of the fourth cell cycle, regardless of any external impact—embryo fragmentation or isolation of the blastomeres [2, 3]. Hence it follows that sea urchin development at the cleavage division stage is strictly deterministic, and the processes restituting the "integral" type of development are realized later. Indeed, the "open half-blastulae" formed of such half-embryos are capable of closure, but this process is apparently not associated with interaction between blastomeres during cleavage divisions, and there is no information on its mechanisms in the available literature.

From the contradiction between the "partial" type of cleavage of a half-embryo and the "integral" type of the larva arising from it, one had to admit either the

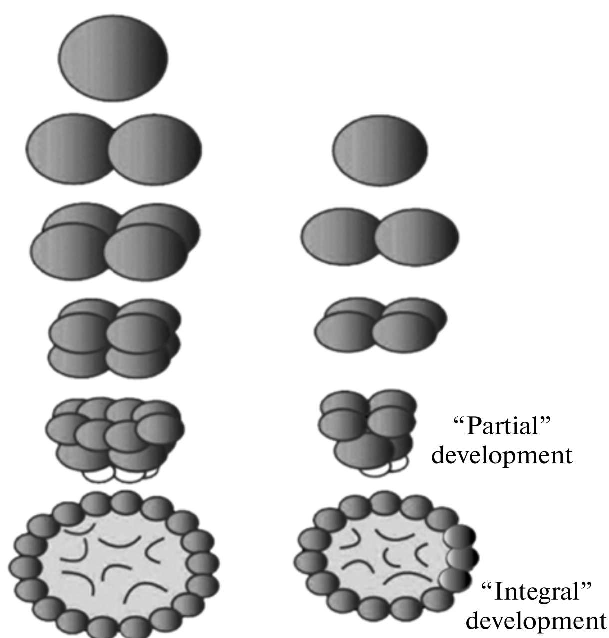

Fig. 1. Prospective fate of sea urchin isolated blastomere according to Driesch and Hörstadius.

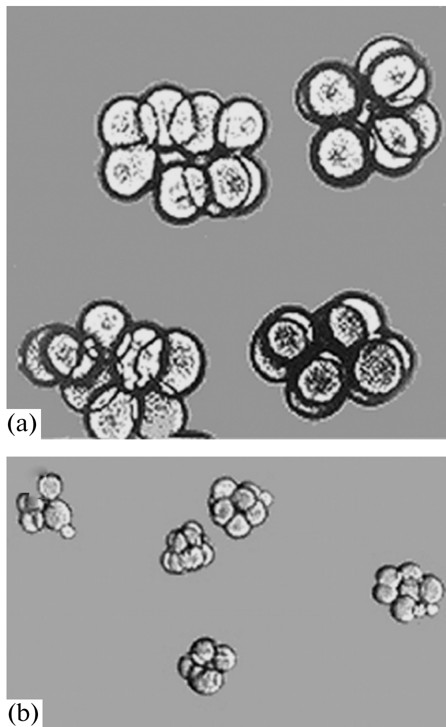

Fig. 2. Cleavage types for *S. mirabilis* half-embryos at 4th division: (a) isolated before adhesion in the 1st cleavage division; equal blastomeres formed; (b) isolated after adhesion in the 1st cleavage division.

incompleteness of these data or, as L.M. Chailakhyan put it, "the ability of embryos to count to four."

At the same time there were data that did not fit into the concept of rigorous temporal determinism of micromere formation [4, 5]. Again, our very first experiments on isolating *Strongylocentrotus nudus* and *Scaphechinus mirabilis* blastomeres at the stages of 1st or 2nd cleavage division revealed that half-embryos at the 4th cleavage division could form micromeres as well as equal blastomeres [6] (Fig. 2). Furthermore, we later succeeded in separating a number of *Sc. mirabilis* embryos by the 3rd cleavage furrow, and in 8 cases out of 100 we observed that both isolated blastomere quartets at the 4th cleavage division formed eight equal blastomeres [7].

Already the first experiments on blastomere isolation by shaking run with *St. nudus* embryos revealed a rule that the probability of micromere formation in a half-embryo was the higher the later was the isolation performed. Therefore, all further isolations were time-tagged. The first group of blastomeres were those isolated before their post-division adhesion [8], directly after completion of cytokinesis when the blastomere acquired a nearly spherical shape and the distance between sister blastomeres reached 10 μm. The second group of blastomeres were those isolated after adhesion, when the interblastomere cleft diminished drastically and the blastomeres became nearly hemispherical. In the half-embryos obtained, the moment of micromere formation was checked. The *Sc. mirabilis* embryos, from which blastomeres are easily isolated by microsurgery, are especially convenient for such experiments, because this removes the uncertainty caused by asynchronous development of the embryo population from blastomeres isolated by shaking.

This paper summarizes the experimental data on time-tagged isolation of blastomeres for sea urchins *St. nudus, St. intermedius, Sc. mirabilis, Echinocardium cordatum* (Sea of Japan: bays Vityaz and Troitsa, Russia) and *Paracentrotus lividus* (Mediterranean Sea: Kotor, Montenegro and Bay of Naples, Italy) that have been accumulated over years.

With *St. nudus, P. lividus* and especially *St. intermedius*, blastomere isolation was only possible upon washing the eggs thrice in artificial calcium-free sea water and keeping them in such water up to the commencement of the 1st cleavage division.

First with *St. nudus* and then with *Sc. mirabilis*, we found that blastomere isolation before (B) the post-division adhesion much more often yielded half-embryos consisting of eight equal blastomeres at the 4th cleavage division (stage when unequal division usually occurs in intact embryos), whereas isolation after (A) adhesion mostly led to unequal cleavage with formation of one or two micromeres (the half-embryos with 1 and 2 micromeres are pooled in Table 1; see also Fig. 3) [9]. The difference in the fractions of half-embryos forming micromeres at this stage between the 'Before' and 'After' groups was statistically significant (Table 2). Special experiments performed by T.Kh. Naidenko showed that in both cleavage types the half-embryos could form plutei I [9].

Analogous results were obtained with *P. lividus*, both in the Adriatic population, where micromeres were prevalently formed by intact embryos at the 3rd rather than 4th cleavage division, and in the Neapolitan population with a classical type of development [10, 11]. Much the same appears to apply to *E. cordatum*, where blastomere fragility, smallness and rapid adhesion practically precluded isolation after adhesion, but the results for blastomeres isolated before adhesion are similar to those described above. The only exception among the species studied was *St. intermedius*, where only few half-embryos cleaving equally at the 4th division were formed. Considering that one of our objects, *P. lividus*, has been studied in many classical works, we have grounds for supposing that equal division of half-embryos is a more or less universal phenomenon. In the course of the 1st, 2nd, and probably 3rd cleavage divisions in sea urchins, there perhaps are short periods (several minutes before completion of the post-division adhesion) when the type of subsequent cleavage is "redefined from integral to partial," i.e. the blastomere totipotency is eventually restrained. This is indirectly confirmed by experiments where *S. mirabilis* blastomeres were subjected to

Table 1. Influence of the moment of blastomere isolation on the type of cleavage of half-embryos

Species	Moment of isolation	Number of embryos	Fraction of half-embryos forming micromeres simultaneously with intact ones, %	p
Sc. 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
St. nudus	B_1	56	23.2 ± 5.6	<0.001
	A_1	26	92.3 ± 5.2	<0.001
St. intermrdius	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	<0.001
P. lividus	B_1	48	42.0 ± 4.1	<0.001
	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

Notes: * Adriatic population with overwhelming prevalence of embryos forming micromeres at the 3rd cleavage division. Half-embryos isolated (B) before or (A) after adhesion in (subscript) 1st or 2nd cleavage division.

Table 2. Difference in cleavage patterns for sea urchin half-embryos isolated (B) before or (A) after adhesion in the 1st or 2nd cleavage division

Species	Stages compared	Difference in fractions of embryos with the same cleavage pattern	p
Sc. mirabilis	B_1–A_1	33.5 ± 2.3	<0.001
	A_1–B_1	38.2 ± 6.2	<0.001
	B_2–A_2	51.1 ± 6.9	<0.001
St. nudus	B_1–A_1	69.1 ± 7.6	<0.001
St. intermrdius	B_1–A_1	3.9 ± 7.4	>0.05*
P. lividus	B_1–A_1	24.2 ± 6.2	<0.001
*P. lividus**	B_1–A_1	38.4 ± 1.7	<0.001

Note: * Difference not significant.

consecutive isolations at the 1st, 2nd, and 3rd cleavage divisions, which progressively reduced the fraction of small embryos cleaving unequally at the 4th division [12]. Upon double isolation (before adhesion in the 1st and 2nd divisions), micromeres formed simultaneously with the control in 21.0 ± 4.5% of embryos, and upon triple isolation, in 7.6 ± 0.7%. Again, half-embryos isolated in the same experiments after adhesion at the 1st division showed a usual frequency of micromere formation at the 4th cleavage division.

In analogous experiments with *P. lividus*, only 11.1 ± 6.0% of small embryos upon isolations at the 1st and 2nd cleavage divisions formed micromeres simultaneously with intact ones, which is 33.9 ± 6.2% ($p < 0.001$) less than in embryos upon isolation only before adhesion in the 1st cleavage division. Such a "cumulative effect" of abolishing normal interactions between blastomeres testifies, on the one hand, to their significance in determination of the type of development, and on the other, to repetition of these processes in early development.

The repetitive process considered here, besides being mediated by cell–cell interactions, is also reciprocal and asynchronous. Indeed, if the processes determining micromere formation are conditioned exclusively by intracellular factors and do not depend on interblastomere exchange of information, then upon blastomere isolation regardless of its moment we should have observed unequal 4th cleavage division in 100% of cases. However, we have monitored 142 cases of development of pairs of *Sc. mirabilis* half-embryos isolated at the 1st cleavage division from one

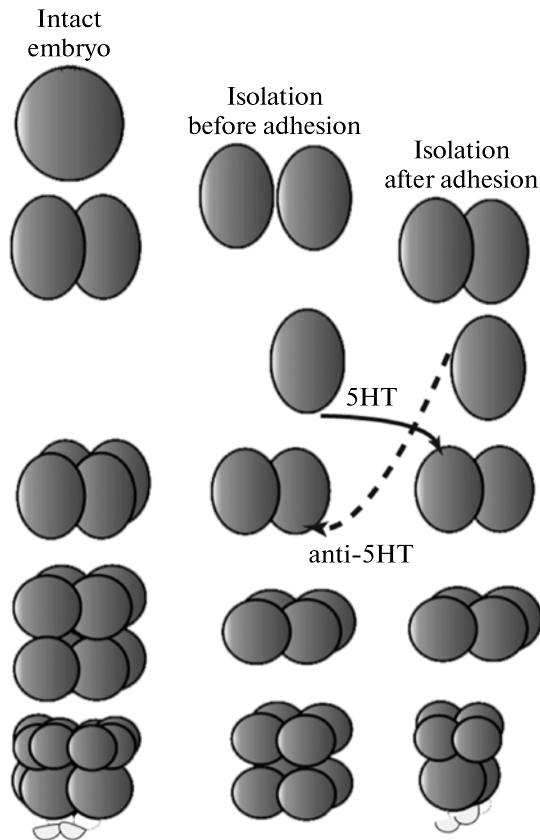

Fig. 3. Dependence of the cleavage type on the moment of isolation. Arrows show the effects of neuropharmaceuticals: 5HT, serotonin and its agonists; anti-5HT, serotonin antagonists.

"mother" embryo. In 97 cases (68.3%) such "twin embryos" showed the same cleavage type (equally or unequally dividing, in about the same amounts).

In this way, we have obtained evidence that the early development of sea urchins is not strictly deterministic, and the unequal cleavage—when cells of different types for the first time appear during development—is a result of a series of events taking place in blastomere adhesion at every cleavage division. By the way, it also becomes partly clear why the classical experiments lack a part of results. Indeed, if isolation is performed strictly after completion of division, then, according to the data above, the probability of detecting equally dividing half-embryos is very low. Another factor that could preclude obtaining a full picture in the classical experiments is associated with the peculiarity of *P. lividus* embryos; namely, for this species, prolonged manipulation in a calcium-free isolation medium results in complete destruction of blastomeres, and such attempts become successful only upon adhesion. Only recently this difficulty has been overcome by replacing calcium-free sea water with normal one directly before starting the isolation.

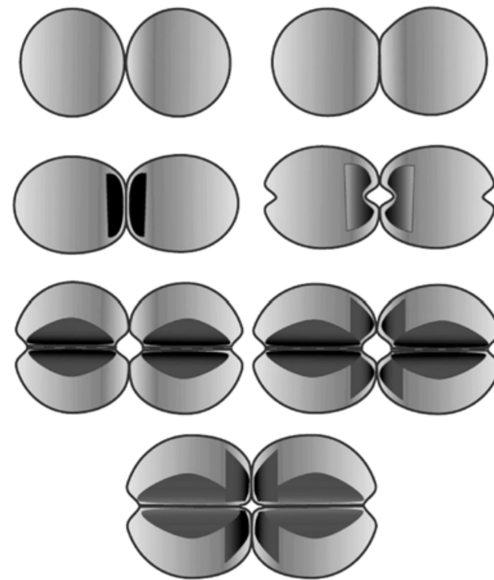

Fig. 4. "Re-adhesion" of blastomeres in the 2nd cleavage division of *St. nudus* (V.V. Smolyaninov, personal communication). The darker areas are the adhesion zones.

Establishment of the phenomenon missing from the classical data of Driesch and Hörstadius—that half-embryos at the 4th cleavage division can form eight equal blastomeres, i.e. a pattern of the 3rd cleavage division of an intact embryo—unequivocally refutes the rigorous "micromere clock" concept. Besides, this allows reconstructing the mechanism of normal development, whereby each successive division involves events that predetermine the position of the spindle and consequently of the plane of the next cleavage division, which saves the embryo the trouble of "counting to four". This happens (or does not happen upon isolation) in the course of post-division adhesion, when the interacting blastomeres change their shape and directly exchange signals. Such signaling was surmised by Vacquier and Mazia [8], who were the first to discern this critical period, and somewhat later confirmed in our experiments with neuropharmaceuticals [13]. Now the actual situation looks still more complex, inasmuch as time-lapse filming has shown (V.V. Smolyaninov, personal communication) that the 2nd cleavage division involves not just blastomere interaction at the new cleavage furrow, but also initial separation and then re-adhesion of blastomeres at the 1st division furrow (Fig. 4).

One can suppose that the geometry of blastomere interactions increasing in complexity with every division, the changes in their shape and in the properties of the cytocortex are the factors that eventually stipulate the exact succession of the division planes in normal development, and, after the 3rd cleavage furrow passes in the equatorial plane, the enactment of the cytoplasmic heterogeneity along the animal–vegetal axis, which leads to unequal cleavage. However, nor-

mally all these processes are realized owing to the presence of sister cells and certain cell–cell interactions. The regularity we have demonstrated in the sea urchin cleavage patterns upon time-tagged blastomere isolation, named the "micromere model", has proved highly productive in studying the mechanism of the disclosed interactions (see below).

It should be noted that isolation of a blastomere before adhesion does not uniquely define its fate but perhaps introduces an element of uncertainty, when the orientation of the next cleavage furrow, which is regular for an intact embryo, gets to be conditioned by more or less random factors. Therewith, from our studies on the effects of some chemicals on the cleavage pattern it follows that in the cytocortex there are some preferred places of initiation and completion of the cleavage furrow: in some cases the 4th division furrow started at the vegetal pole as in micromere formation, but then migrated to the middle and closed there to form two equal blastomeres.

So what can be the mechanism of blastomere interactions capable of such influence on the cleavage pattern? An earlier hypothesis implied that the cleavage pattern is conditioned exclusively by the change in blastomere shape [7], but no specialized structures in the contact region have been revealed by scanning electron microscopy [14]. Moreover, it has been emphasized that the blastomere contact surface does not appreciably differ at this level from the "outward" area. Anyway, this hypothesis has not been furthered, and there is no new evidence to discuss it.

At the same time, there are numerous data in favor of that blastomere interactions involve low-molecular regulators of prenervous development, such as compounds chemically identical to neurotransmitters, first of all serotonin. Already with intact embryos it was shown that serotonin antagonists introduced during cleavage division can cause effects indicative of impaired blastomere interaction [13]. It was therefore logical to check whether such neurotransmitters could be the interblastomere mediators, although at that time such a possibility had been rejected in principle [15].

Using the "micromere model" outlined above, we have examined the action of agonists and antagonists of neurotransmitter receptors, proceeding from the idea that transmitters can be the interblastomere signaling substance and can mimic the natural interactions, while their antagonists can abolish them. Control experiments have shown that serotonin has practically no influence on the type of cleavage of half-embryos isolated after adhesion, whereas its antagonists do not affect those isolated before adhesion. Therefore, serotonin and other transmitters were assayed with blastomeres isolated before adhesion, and the antagonists, with blastomeres isolated after adhesion. The results are presented in Table 3. One can see that serotonin in all cases significantly increased the fraction of half-embryos forming micromeres at the 4th cleavage division. A similar effect on *P. lividus* was observed with a serotonin agonist quipazine. Tryptamine had a serotonin-like effect on *Sc. mirabilis* while other transmitters had no influence on the cleavage type.

On the other hand, Table 3 shows that serotonin antagonists reliably influenced the half-embryos isolated after adhesion, significantly raising the fraction of embryos forming equal blastomeres at the 4th division, while antagonists of other transmitters did not appreciably affect the process. It is essential that, along with readily permeating antagonists that are tertiary amines, high efficiency in the "micromere model" was observed for their quaternary analogs. This suggested a surface localization of the corresponding receptors, and became one of the first physiological phenomena associated with the membrane reception in early embryos, rather than intracellular reception long held to be the specific feature of transmission in early embryogenesis [16].

In particular, 25 μM inmecarb or its methiodide significantly decreased the fraction of *Sc. mirabilis* half-embryos forming micromeres at 4th cleavage division. Inmecarb (50 μM) had no effect on *P. lividus*, while 40 μM inmecarb methiodide decreased the fraction of unequally cleaving embryos (Table 3). Similar results were obtained with other pairs of serotonin antagonists: KYuR-14 (an indole derivative) at 100 μM was without effect while its methiodide significantly decreased the micromere formation frequency. Likewise, tropanylindole carboxylate methiodide was twice more effective on *P. lividus* than its hydrochloride. Thus, drugs preferentially interacting with the membrane surface proved more effective than their analogs readily penetrating the cell, which suggests surface location of the receptive structures. Note that if the exposure to tertiary amines of these pharmaceuticals exceeded 15 min, their cytostatic action started to dominate, while their methiodides did not exert such an effect.

The effects of serotonergic drugs in the "micromere model" are specific, since simultaneous addition of serotonin (or its agonists) and its antagonists gave results very close to the controls without drug treatment.

Along with serotonergic drugs, the cleavage type of *S. mirabilis* half-embryos was very strongly affected by a phosphodiesterase blocker papaverine and dibutyryl-cAMP. Thus, the "micromere model" has proved quite productive and selective in searching for substances capable of influencing the early cleavage pattern, and has revealed that serotonin and its agonists can mimic the interblastomere signal and accordingly increase the fraction of half-embryos developing by the "partial" type, while the antagonists promote the type of half-sized "integer" embryo, as if "omitting" the division that involved blastomere isolation or chemical exposure mimicking it. One can sup-

Table 3. Effects of chemicals on cleavage patterns of sea urchin half-embryos

Species	Moment of isolation (number of half-embryos in the series)	Substance	Concentration, μM	Change in the fraction of half-embryos forming micromeres simultaneously with intact ones	p
Sc. 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)	Carbachol	275	−8 ± 8	>0.05*
	B_1 (53)	ATP	360	+3 ± 9	>0.05*
	B_1 (51)	Dopamine	260	+6 ± 10	>0.05*
	B_1 (84)	Papaverine	50	+34 ± 6	<0.001
	B_1 (101)	cAMP	270	+7 ± 5	>0.05*
	B_1 (107)	cGMP	270	−8 ± 6	>0.05*
	B_1 (92)	Dibutyryl-cAMP	210	+41 ± 6	<0.001
	A_1 (170)	Melipramine	5	−32 ± 5	<0.001
	A_1 (77)	Ciproheptadine	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	>0.05*
	A_1 (64)	Propranolol	135	+2 ± 7	>0.05*
	A_1 (96)	Ganglefene	32	−10 ± 7	>0.05*
	A_1 (48)	Quateron	400	−2 ± 9	>0.05*
	B_1 (86)	Valinomycin	5.4×10^{-3}	+22 ± 9	<0.05
	A_1 (89)	Ouabaine	1000	−25 ± 9	<0.01
	A_1 (70)	Trifluoperazine	49	−28 ± 13	<0.05
St. nudus	B_1 (36)	Serotonin	112	+24 ± 12	<0.05
P. lividus	A_1 (53)	Inmecarb	50	−1 ± 12	>0.05*
	A_1 (203)	Inmecarb methiodide	40	−30 ± 10	<0.05
	A_1 (27)	KYuR-14	100	0	>0.05*
	A_1 (43)	KYuR-14 methiodide	100	−17 ± 7	<0.05
	A_1 (137)	Melipramine	60	−34 ± 4	<0.001
	A_1 (97)	3-Tropanylindole-3-carboxylate methiodide	100	−25 ± 7	<0.001
	A_1 (96)	3-Tropanylindole-3-carboxylate hydrochloride	100	−12 ± 1	<0.001
	A_1 (82)	Quipazine	100	+24 ± 1	<0.001

Note: * Difference not significant. Designations B and A as in Table 1.

pose that these effects correspond to the action of serotonergic substances in the intact embryo [13, 17].

The particular mechanism of the action of serotonin as the interblastomere mediator in early development may be based on the following. It has been known that, because of the low monoamine oxidase activity, the main way of silencing the intracellular transmitters in early embryos is their discharge to the external medium [16]. Accordingly, sea urchin early embryos have a serotonin transporter, SERT [18], and by histochemical data, serotonin is elevated in the interblastomere space [19]; dissipation of the mediator from this small enclosed compartment is likely to be further impeded by adhesion contacts [9]. In this way the environs of a two-cell embryo become asymmetrical, and the same applies to the probability of the mediator interacting with presumed surface receptors even if the latter are spread uniformly over the blastomere. The transmitter–receptor interaction in the blastomere contact region can somehow alter the state of the cytocortex, and this subsequently determines the orientation of the next cleavage furrow. Quite con-

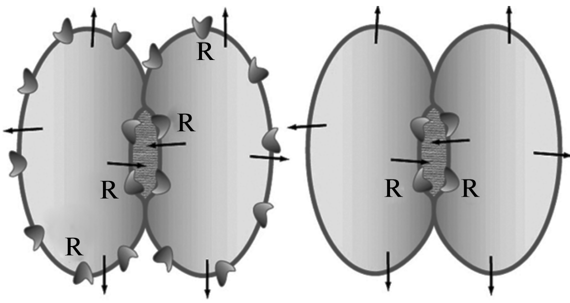

Fig. 5. Scheme of a protosynapse, with mediator receptors distributed uniformly (left) or localized in the blastomere contact area (right). R marks receptor, arrows denote mediator discharge to the milieu; the interblastomere compartment (hatched) accumulates the mediator.

sistent with this are the data on elevated adenylate cyclase activity in the blastomere contact region.

The pharmacological evidence for the existence of membrane serotonin receptors mentioned above was later confirmed both by measurements of intracellular calcium in *Lytechinus pictus* [20] and in voltage-clamp experiments with *P. lividus* [21]. All this has allowed formulating [10] and then refining the hypothesis of "protosynapse" as a bilaterally symmetrical structure where both blastomeres are (i) the sources of the signal (mediator), (ii) its targets, and (iii) a passive barrier to leakage of the mediator from the interblastomere compartment to the external medium (Fig. 5).

Recent whole-cell patch-clamp experiments have shown that local application of serotonin agonists to the interblastomere cleft of *P. lividus* embryos evokes specific inward currents, which have greater amplitude and, most importantly, a substantially shorter latent period than upon application onto the free surface of the blastomeres [11]. This has allowed us to suggest that the serotonin-receptive structures are localized in the interblastomere compartment, and to refine the notions on the protosynapse (Fig. 5). We have initiated a molecular-biological study of the receptor link of the process [22].

The protosynapse concept is productive inasmuch as it explains the aggregate of data concerning cell–cell interactions in early development. Particularly, we can suppose that Driesch in his classical experiment isolated the blastomeres already after their interaction, with all the above consequences. The still earlier Roux's experiment [23], which is still regarded as an artifact [24], does not disagree with the protosynapse concept: just admit that the in situ necrotized frog blastomere could still be a barrier to mediator leakage from the interblastomere space, so for the intact blastomere the situation was the same as in normal development.

Finally, the proposed protosynaptic mechanism may underlie the emergence of multicellularity as an aggregate of asymmetrical cells, with a simplification relative to Wolpert's concept [25] that there is no need to invoke a substrate as a prerequisite for establishment of cellular heterogeneity.

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