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POSSIBLE ROLE OF "PRENERVOUS" NEUROTRANSMITTERS IN CELLULAR INTERACTIONS OF EARLY EMBRYOGENESIS: A HYPOTHESIS

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Evidence is presented in support of the working hypothesis that "prenervous" neurotransmitters directly participate in cell–cell interactions occurring during the first several cleavage divisions of sea urchin embryos, a function which may occur during the early development of higher animals as well. This intercellular signaling could be a link in the evolutionary progression from the use of these substances as intracellular regulators to their participation in cell–cell interactions occurring during synaptic transmission.

INTRODUCTION

Acetylcholine, serotonin, catecholamines, and other physiologically active substances, often referred to as "neurotransmitters," have been found in animals without a nervous system, that is, at the prenervous stages of phylo- and ontogenesis. In various groups of Protozoa, cholinergic, adrenergic, and serotonergic systems have been found (4, 25, 27, 28, 40). The same systems are also present in early embryos of all vertebrates and invertebrates studied (Figure 1) (1, 6–9, 13, 18, 19, 23, 32, 41). Thus, the use of the term "prenervous neurotransmitters," strange as it sounds, is quite justified.

There are a number of specific features in "prenervous" neurotransmitter systems. The first consists of the presence of several neurotrans-

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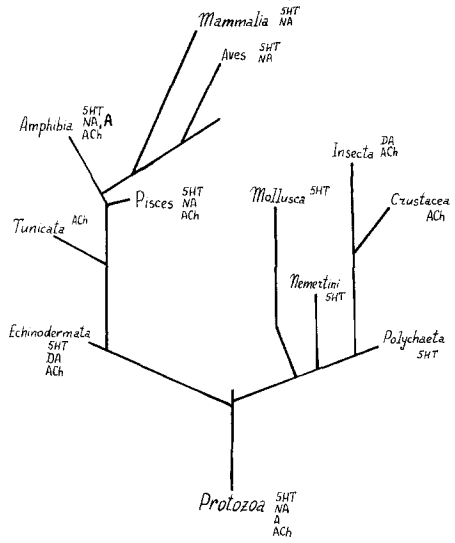

FIG. 1. Prenervous neurotransmitters in the animal kingdom. A, adrenaline; NA, noradrenaline; DA, dopamine; 5-HT, serotonin; ACh, acetylcholine.

mitters within a single cell. In the infusoria *Tetrahymena pyriformis*, for example, there have been found acetylcholine, serotonin, adrenaline, and noradrenaline, as well as cholino-, serotonino-, and adrenoreceptors (4, 27, 28, 40). At least three neurotransmitters—acetylcholine, dopamine, and a serotonin-like substance (presumably, a mixture of tryptamine with lesser amounts of serotonin) are found in sea urchin embryos at the one-cell stage and during the first cleavage divisions (first cell divisions following fertilization) (7, 13, 32, 41). At the same time, for the majority of the neurons of Metazoa studied, the "one transmitter–one neuron" rule holds true.

Another peculiarity of "prenervous" neurotransmitter systems is an intracellular localization of corresponding receptors or of their functional analogs. The existence of such receptors is suggested by the fact that many antagonists of neurotransmitters specifically block the cleavage divisions in sea urchin embryos and inhibit protein biosynthesis. These inhibitory effects manifest themselves only when the antagonists get into the cells. Moreover, the protective action of the transmitters administered in conjunction with these antagonists is also brought about at the intracellular level (10–12).

The prenervous neurotransmitters of embryos appear to participate in

the control over cleavage divisions and macromolecular synthesis (8, 9, 18). In the former case these substances may serve as triggers acting on certain elements of the apparatus of cell division (microtubules and microfilaments), or as regulators of intracellular transport of macromolecules involved in the induction of the cleavage furrow and in the formation of new cell membranes (1, 7–9, 18). In the latter case prenervous neurotransmitters have been presumed to participate in the control over the intracellular transport of macromolecules (8); this participation may also include an effect of neurotransmitters on microtubules and microfilaments.

The prenervous neurotransmitters were earlier assumed to fulfill intracellular functions only throughout early embryogenesis, i.e., up to the onset of gastrulation (8, 9). It was believed that only at the later developmental stages did these substances begin functioning as intercellular mediators participating in the regulation of embryonic motility and morphogenetic cell movements, i.e., in the processes of embryonic induction (8, 23, 33, 41). Now it seems necessary to broaden these ideas and to consider possible participation of prenervous neurotransmitters in early cellular (interblastomere) interactions. The existence of these interactions was established long ago. The main phenomenon pointing to their existence is the noncoincidence of the prospective fate and prospective potencies of single cells (blastomeres) which is most clearly pronounced in animals with so-called regulatory development (echinoderms, vertebrates, and others) (15, 16, 21). Isolation of one of the blastomeres produced in the course of the first cleavage divisions can result in the development of complete dwarf larva. In sea urchins the operation is possible up to the 8-cell stage, and even up to the 32-cell stage in starfishes (15, 16). Thus the blastomeres retain all the potencies of a fertilized egg, and their subsequent determination in the course of normal development can be accounted for only by functional cellular interactions (16).

Some characteristics of these interactions have been established. In sea urchins such interactions seem to be periodically repeated in every cell cycle. The blastomeres of the flat sea urchin, *Scaphechinus mirabilis*, when isolated from each other immediately after the completion of the 1st or 2nd cleavage division show, in most cases, the pattern of divisions typical for the fertilized egg, that is the production of cells of equal size (Figure 2A). With the blastomeres isolated 10–20 min later, however, the divisions proceed as if the cells were still together in the intact embryo (Figure 2B). In the former case the blastomeres seem to have been isolated before, and in the latter case after, the determining blastomere interaction which can be, consequently, considered as a relatively short discrete

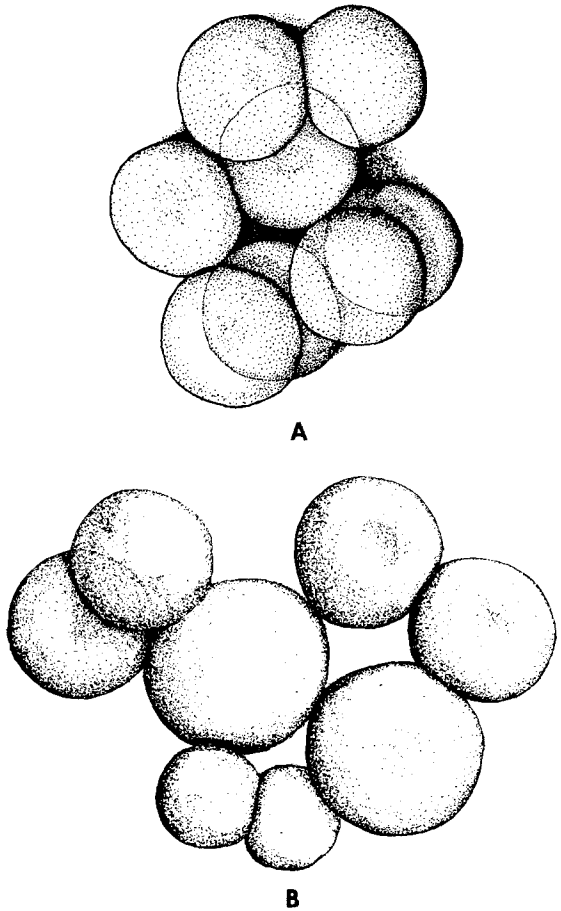

FIG. 2. The dwarf embryos of flat sea urchin *Scaphechinus mirabilis* from isolated blastomeres. (A) The blastomeres were isolated immediately after first cleavage division; all embryonic cells are the same size. (B) The blastomeres were isolated 10–20 min later; the embryo consists of a half set of micro-, meso-, and macromeres.

periodic signal. Such a repetition of blastomere interactions in sea urchins is a necessary condition of normal development.

It has been further shown, in experiments with *S. mirabilis*, that blastomere interactions are reciprocal, i.e., none of the blastomeres alone can be considered as the pacemaker. At the same time, the mutual influence of blastomeres upon each other is not necessarily synchronous and equal, since separation of blastomeres at the 2-cell stage in some 30% of cases results in the development of a half-sized whole embryo from one

of the blastomeres and of a half embryo of normal size from the other one.

It is still unknown, however, how the interblastomere signals affect further behavior of the blastomeres. In sea urchins, the first manifestation of the effect of these signals is the nonregulation of the fourth cleavage division, represented by a distinctive orientation of the mitotic spindles (microtubules) and contractile elements of the cell cortex (microfilaments) before the division begins. This, in turn, depends on the position of the contractile elements of the cells during the preceding cell cycles. Thus, it seems possible that the interblastomere signals act at the level of contractile elements of the cell or are at least somehow connected with these elements.

MODELS OF BLASTOMERE INTERACTIONS IN EARLY EMBRYOGENESIS

In principle, there are three possible variants of the transmission of interblastomere signals as shown below:

Cell A		Cell B
1. cell membrane	↔	cell membrane
2. cytoplasm	→	cell membrane
cell membrane	←	cytoplasm
3. cytoplasm	↔	cytoplasm

These may represent: (1) the direct interaction of the electroexcitable membranes in the zone of cell contact; (2) interaction by means of a chemical mediator acting on specific membrane receptors; (3) exchange of intracellularly received chemical or other signals through the zones of high permeability in the cell membranes or (4) through the cytoplasm of incompletely separated cells.

The first of these variants is noteworthy in view of the data on electroexcitability of the egg-cell membrane in all the animal groups studied—polychaetes, sea urchins, starfishes, amphibians, and mammals (24, 26, 29, 30, 34, 38). However, the persistence of this excitability has been reported only for polychaete embryos (24). As for newly formed membranes in the zone of interblastomere contacts, no electroexcitability has been found as yet, and at the present level of investigations this variant seems unlikely. However, other types of cell surface interactions are possible. With regard to the second variant, in the cell membranes of oocytes and eggs of polychetes, amphibians, and some other animals,

typical neurotransmitter receptors have been found (24, 29, 30), such as muscarinic cholinoreceptors in the oocytes of the clawed frog *Xenopus*. However, there are as yet no indications of the presence of neurotransmitter or hormonal receptors in the newly formed membranes of embryos, although they may still exist. Thus, the third and fourth variants remain as the strongest possibilities, i.e., an exchange of signals received intracellularly. Such an exchange might take place because of the continuity of the cytoplasm as a result of incomplete or slow formation of cleavage furrows. A high coefficient of electric connection between incompletely separated blastomeres has been found, and fluorescent label injected into one of such cells has been shown to pass to the remaining cells of the embryo (2, 3, 5, 35). Another possible method of intracellular signaling in early embryos is the transmission of chemical signals through gap junctions. In embryos of fishes and amphibians typical gap junctions appear at the blastula stage, and their permeability for substances of molecular weights up to 1000–1200 daltons has been demonstrated (2, 3, 20, 37, 39). Very early formation of gap junctions has been reported for mammals (17) as well, and in echinoderm embryos gap junctions have been found at the 32-cell stage (42), although one cannot exclude the possibility of their earlier formation (22, 44, 45).

An essential condition of intracellular signaling is the adhesion of interacting cells. It has been shown for the embryos of *Fundulus* that the adhesion of preliminarily dissociated cells results in very rapid establishment of intercellular connections because of the formation or recovery of the gap functions. In sea urchin embryos the blockade of cell adhesion leads to a complete impairment of intercellular interactions which produces effects similar to the isolation of blastomeres (44, 45).

The newly formed blastomeres of sea urchins are in contact at relatively small sites and the spacing between them is on the order of hundreds of angstroms. As development proceeds, however, the area of contact increases sharply and the cleft between cells narrows to 25–50 Å (so-called "adhesion after cleavage") (44, 45). To a considerable extent this adhesion is determined by a contraction of filopodia connecting the newly formed cells. An important role in this adhesion is also played by the hyaline layer closely adjoining the outer surface of the embryo (43–45). To suppress interblastomere connections in sand dollars, it is enough to treat embryos, at the moment of the formation of the next cleavage furrow, with substances damaging filopodia or interfering with their contraction (mercaptoethanol, dithiothreitol, detergents). In some regular sea urchins such treatment is effective after removal of the hyaline layer (14, 44, 45). In all these cases the blastomeres were observed to be functionally uncoupled, although they preserved their mechanical contact; as a result,

there develop complete dwarf embryos connected with each other (Figure 3).

In some cases (in sand dollars, for example) the interblastomere signals seem to be chemical rather than mechanical in nature. As a rule, these signals are transmitted during "adhesion after cleavage" (at the time when the mechanical contact is greatest), although they are occasionally transmitted before or after this time. Sometimes the blastomere interactions take place before the separation of the cell; that is, under conditions when a mechanical interblastomere signal is impossible. Moreover, it would be difficult for reciprocal interblastomere interactions of a mechanical nature to account for the frequent occurrence of asynchronous blastomere interactions. At the same time, asynchronous release of a transmitter substance by two equal interacting cells is not only possible, but rather seems more probable than strictly simultaneous release. One more argument in favor of the chemical nature of interblastomere con-

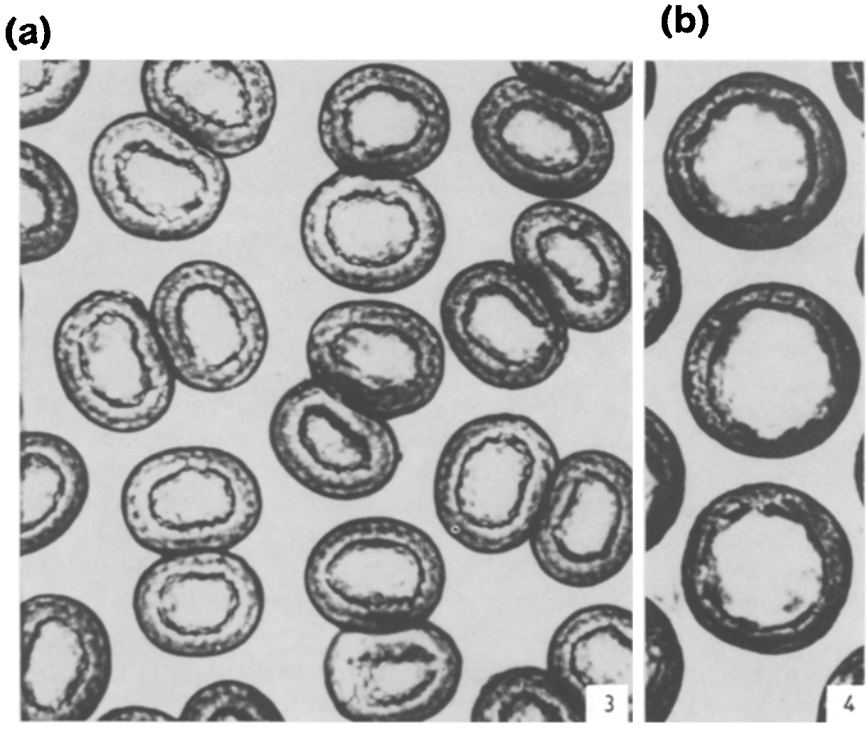

FIG. 3. Twin *Echinarachnius parma* embryos, which developed functional uncoupling of blastomeres with dithiothreitol (a); normal embryos (b) (from ref. 44).

nection is the presence of gap junctions and cytoplasmic intercellular bridges in early embryos of several animal groups.

CRITERIA FOR IDENTIFICATION OF INTERBLASTOMERE MEDIATORS AS NEUROTRANSMITTER-LIKE SUBSTANCES

Interblastomere mediators, if they do exist, must satisfy certain criteria similar to those used in neurotransmitter identification: (1) In the blastomeres a substance identified as a transmitter must be present. Moreover, it is probable, although not necessary, that its concentration may be changed synchronously with the rhythm of cleavage. (2) In the blastomeres there must be present intracellular structures with which the transmitter interacts (which might consist of contractile elements). (3) The functional interaction of blastomeres must coincide with interblastomere movements of the transmitter. (4) Under certain conditions the transmitter must mimic the interblastomere signal (e.g., eliminate the consequences of blastomere isolation). (5) On entering the blastomeres transmitter antagonists must induce the functional uncoupling of these cells and displace the transmitter from the structures of the cell sensitive to it. In addition, if the signal is transmitted through gap junctions, the molecular weight of the transmitter should not be more than 1000–1200 daltons. All of these conditions imply certain compartmentalization of the transmitter, especially if it fulfills some intracellular functions in addition to its action as an intercellular signal.

It should be noted that prenervous neurotransmitters found in early sea urchin embryos satisfy several of these criteria: they are present in all blastomeres, and their concentration changes synchronously with the rhythm of the first cleavage divisions (criterion 1) (13). There are also intracellular neurotransmitter receptors (or their functional analogs) in all the blastomeres, and these appear to be associated with microtubules and microfilaments (criterion 2) (unpublished results). The passage of the prenervous neurotransmitters from one blastomere to another has not yet been demonstrated (criterion 3), but data have been reported concerning the ability of biogenic monoamines to pass through intercellular contacts (36). As for criteria 4 and 5, these were tested in specially designed experiments with *S. mirabilis* as a subject and are described below.

EXPERIMENTS CONCERNING NEUROTRANSMITTER-LIKE NATURE OF INTERBLASTOMERE SIGNAL

In the first series of experiments, a study was made of the effect of tryptamine and serotonin on the blastomeres isolated immediately after

the first cleavage division (before the passage of interblastomere signal in most embryos) (Table I). It was observed that in the majority of embryos developing from the isolated blastomeres, the fourth cell division was regular, producing cells of equal size (Figure 2A, Table I). However, in some cases, a half set of micro-, meso-, and macromeres were produced rather than equal cells as in the normal irregular fourth cleavage (Figure 2B, Table I), indicating that these were normal-sized half embryos. In the presence of serotonin or tryptamine, the number of embryos with a normal (irregular) fourth cleavage division increased by about 100%. This effect disappeared when serotonin and melipramine (an antagonist of tryptamine and serotonin) were added together (Table I), indicating that this neurotransmitter action was specific. Therefore these prenervous transmitters appeared to act in the same way in which the interblastomere transmitter is supposed to act, thus complying with the fourth criterion.

In another series of experiments performed with intact embryos (unseparated blastomeres) treated at the moment of formation of the cleavage furrow with various antagonists of these "prenervous" transmitters (indole and azaindol derivatives as well as the tricyclic antidepressant melipramine), they effectively blocked the intercellular coupling of embryonic cells, causing the formation of twin embryos. The effect of these drugs was sometimes a prolonged one, being expressed in the formation not only of half-sized normal embryos, but smaller ones as well. The necessary condition for drug efficiency appeared to be their penetration

TABLE I

ACTION OF SEROTONIN, TRYPTAMINE, AND SOME NEUROCHEMICALS ON PATTERN OF 4TH CLEAVAGE DIVISION IN EMBRYOS DEVELOPED FROM BLASTOMERES AFTER ISOLATION AT 2-CELL STAGE

Experimental variants	Embryos with irregular (normal) 4th cleavage (%)
Blastomeres isolated before adhesion	
Control (sea water)	21.2 ± 1.9
Serotonin (22 μg/ml)	46.0 ± 4.2
Tryptamine (40 μg/ml)	40.2 ± 6.6
Serotonin (22 μg/ml) + melipramine (1.7 μg/ml)	26 ± 11
Blastomeres isolated after adhesion	
Control (sea water)	85 ± 3.5
Melipramine (1.7 μg/ml)	39 ± 6.5
Preparation NK-306 (25 μg/ml)	59 ± 12
Serotonin (22 μg/ml) + melipramine (1.7 μg/ml)	72 ± 10

into the cells (unpublished observations). Moreover, serotonin weakened or prevented the disruption of intercellular connections produced by some of the drugs studied. However, specificity of the observed effects has not been established. Similar results were produced with isolated blastomeres, separated after postcleavage adhesion (after the passage of interblastomere signal in most embryos). Such blastomeres usually develop into embryos consisting of half sets of micro-, meso-, and macromeres after the fourth cleavage (Figure 2B, Table I). However, when these isolated blastomeres were treated with neurotransmitter antagonists (melipramine or preparation NK-306), they developed into embryos consisting of equal-sized cells after the fourth cleavage (which might be due to blockade of second interblastomere signal). This effect was prevented by exogenous serotonin added with the antagonists (Table I).

From the results obtained it cannot be concluded whether the fifth criterion of functional uncoupling by antagonists and displacement of the interblastomere transmitter by such agents has been satisfied, since the antagonists tested might act directly to block cell adhesion (for example, by inhibiting filopodia contraction during a given cleavage division or at the subsequent divisions). However, as judged by these preliminary data, the neurotransmitter antagonists seem to specifically act on both blastomere adhesion and interblastomere signaling. These correlative findings now require further investigation.

HYPOTHETICAL MODEL FOR NEUROTRANSMITTER INVOLVEMENT IN INTERBLASTOMERE SIGNALING

From the results discussed thus far, the prenervous neurotransmitters (primarily tryptamine and serotonin) seem likely to act as mediators of interblastomere signaling. According to our hypothesis, blastomere interactions (at least in flat sea urchins) proceed in the following way (Figure 4): Prenervous neurotransmitters produce the shortening of filopodia connecting the newly formed blastomeres, which results in the postcleavage adhesion. This effect might occur at the intracellular level (most probably through the effect of the neurotransmitter on microfilaments) and could be considered as a continuation of the neurotransmitter's participation in the cleavage divisions. The second, and main, step of the process under consideration is the exchange of reciprocal interblastomere signals where these prenervous neurotransmitters would act as mediator substances by passing from one cell to another through specialized intercellular contacts. The exchange of signals would then result in a definite functional polarization of interacting cells determining their further development, an ef-

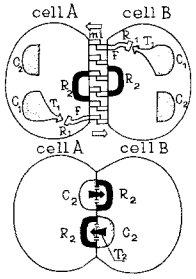

FIG. 4. A schematic representation of the supposed participation of prenervous neurotransmitters in cellular interactions of early embryos of flat sea urchins. (A) First stage (triggering of the adhesion after cleavage); (B) second stage (exchange of reciprocal intercellular signals). The arrows show the direction of cellular interactions (mi). F, microfilaments and filopodia (are shown only partially); C_1 and C_2, the first and second neurotransmitter pools; R_1 and R_2, recepros (or receptor analogs) interacting with, respectively, neurotransmitters of the 1st and 2nd pools; T_1 and T_2, release of neurotransmitters during the 1st and 2nd stages of cellular interactions.

fect which could also be due to the action of these neurotransmitters on intracellular contractile elements.

This hypothesis poses a number of additional problems which must be experimentally tested. For example, it is unclear which neurotransmitters actually participate in interblastomere signaling. In the present studies, tryptamine and serotonin appear to be involved. However, dopamine and acetylcholine, which are also present in sea urchin blastomeres, have not been tested. It is also unclear whether cyclic nucleotides may be involved in such processes, since these nucleotides are known to easily pass through gap junctions (31) and are presumed to be involved in embryonic cellular interaction at later developmental stages (33).

Still unknown are the mechanisms underlying the effects of neurotransmitters on interblastomere interactions; in particular, the relationship between intercellular and intracellular functions of these substances.

Finally, it is not yet clear whether our hypothesis is applicable to early embryos of all animal groups, although we assume that the process of interblastomere signaling must be rather universal and that the participation of prenervous neurotransmitters in this process may be a general transpecies phenomenon. At the same time the actual character of this participation may differ considerably, even in closely related animal groups.

Further testing of our hypothesis is important not only for understanding regulatory mechanisms of early embryogenesis, but also for the statement and solution of a number of important problems of evolutionary and developmental neurobiology. We know that the development of the def-

initive functions of neurotransmitters in the nervous system of adult metazoans was preceded by the appearance of intracellular regulatory functions of these substances in lower animal phyla. However, the manner in which this progression from intracellular to intercellular neurotransmitter functions was achieved during evolution remains unknown. The evidence presented here supports the view that one of the stages in this progression may have been the direct participation of prenervous neurotransmitters in cell–cell interaction during development in primitive multicellular organisms.

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