Comp. Biochem. Physiol. Vol. 106C, No. 1, pp. 269–273, 1993
Printed in Great Britain

0742-8413/93 $6.00 + 0.00

POSSIBILITY OF MEMBRANE RECEPTION OF NEUROTRANSMITTER IN SEA URCHIN EARLY EMBRYOS

YURI B. SHMUKLER

Laboratory of Embryophysiology, N. K. Koltzov Institute of Developmental Biology, Russian Acad. Sci., 26 Vavilov St., Moscow, 117808, Russia (Tel. 7 095 135-0052; Fax 135-8012, re electronic mail ybshm.@ibrran.msk.su)

(*Received* 8 *February* 1993; *accepted for publication* 19 *March* 1993)

Abstract—1. Methiodide derivatives of serotonin blockers—inmecarb and KYur-14 which poorly penetrate the cells specifically affect the pattern of cleavage division in half-embryos of *Paracentrotus lividus* and *Scaphechinus mirabilis*.

2. Specific [^{3}H]8-OH-DPAT binding under conditions strictly limiting penetration of the ligand into the cells of *Strongylocentrotus intermedius* was also shown ($K_d \sim 3 \times 10^{-10}$ M for a site with the greatest affinity).

3. On the basis of the data obtained it is concluded that neurotransmitter-specific membrane receptors may be present in sea urchin embryos during cleavage divisions. The "protosynapse" hypothesis is proposed which suggests the existence of a specific structure responsible for early blastomere interaction involving transmitters.

INTRODUCTION

A specific feature of embryonic processes involving neurotransmitters (Buznikov, 1967, 1987) is the intracellular localization of corresponding receptor structures. This has been demonstrated virtually in all cases studied up to the present time (Buznikov, 1987, 1989; Buznikov and Shmukler, 1978, 1981; Shmukler *et al.*, 1986). Thus, during ontogenesis, localization of receptors on the plasma membrane characteristic of adult cells (including oocytes (Kusano *et al.*, 1977; Dascal and Landau, 1980) which are also the cells of the maternal organism in this respect) changes in early embryos to the intracellular and are then localized to the membrane again. However, recent data cast doubt on the concept of exclusively intracellular transmitter reception in the embryo (Buznikov, 1989; Falugi and Prestipino, 1989).

One of the main arguments in favor of intracellular localization of the transmitter receptor structures in early embryos is the higher efficiency of tertiary indolylalkylamines which can penetrate the cells more easily (Landau *et al.*, 1981) than their quaternary analogs. This study is an attempt to determine the time when membrane receptors first appear during development, using the above-mentioned effect of neuropharmacological drugs on cell interactions in the early embryo. We have shown that serotonin and its antagonists specifically affect cellular interactions in early sea urchin embryos (Buznikov and Shmukler, 1978, 1981), including the control of micromere formation in half-embryos (moment of unequal cleavage division) (Buznikov and Shmukler, 1981; Shmukler, 1981; Shmukler *et al.*, 1981).

Brown and Shaver (1987) have demonstrated that [^{3}H]-serotonin is bound by all subcellular fractions of sea urchin embryos including surface membranes from the blastula stage. The purpose of this study is also to provide corresponding data for the period of cleavage divisions in the sea urchin embryos by the radioligand binding technique.

MATERIALS AND METHODS

Animals

Blastomeres of sea urchins *Scaphechinus mirabilis* (Japan sea, Troitza Bay, Russia), *Strongylocentrotus intermedius* (Japan sea, Popov Island, Russia) and *Paracentrotus lividus* (Adriatic sea, Kotor, Yugoslavia) were used for these experiments. Procedures for the collection of the sexual product and artificial fertilization were standard (Buznikov and Podmarev, 1991).

Ligand and drugs

[^{3}H]-labeled 8-OH-DPAT (189 Ci/mmol) was obtained from Amersham (England); serotonin (5-hydroxytryptamine), cyproheptadine, imipramine, desipramine and amitriptyline were obtained from Sigma (U.S.A.); serotonin blockers inmecarb and inmecarb methiodide (Buznikov, 1987) were kindly supplied by Prof. V. A. Zagorevsky (Institute of Pharmacology and Chemotherapy, Russian Acad.

Abbreviations: Ca^{2+}-FASW, Ca^{2+}-free artificial sea water; 8-Hydroxy-[^{3}H]DPAT, 2-(*N*,*N*-di[2,3(*n*)-^{3}H]propylamino)-8-hydroxy-1,2,3,4-tetrahydronaphthalene.

Med. Sci.), and KYuR-14 and its methiodide were prepared by Dr M. A. Yurovskaya, Moscow State University, Dept of Chemistry.

Embryophysiological experiments

Blastomeres of sea urchin embryos were isolated at the first cleavage division using a glass needle (Shmukler *et al.*, 1981). The vitelline membrane was removed by slightly shaking the suspension of embryos immediately after fertilization. Embryos of *P. lividus* and *S. intermedius* were washed three times with Ca^{2+}-free artificial seawater after fertilization to prevent the hyaline layer formation. Drugs were added to the medium at the beginning of isolation of blastomeres for 15 min. Concentrations of drugs were chosen on the basis of previous experiments (Shmukler, 1981; Shmukler and Grigoriev, 1984). The moment of micromere formation was monitored. In intact embryos of *P. lividus*, typical micromeres were often formed at the third rather than at the fourth cleavage division; therefore, both these stages of development were monitored during experiments. Experimental protocol for intact embryos was similar except for mechanical isolation of blastomeres. The form of developing blastulae was monitored.

Ligand binding experiments

Experiments were performed using embryos of *S. intermedius*. Freshly fertilized embryos were washed with Ca^{2+}-FASW, treated with 1 M urea to remove the vitelline membrane and hyaline layer and washed three times with Ca^{2+}-FASW (see Buznikov and Podmarev, 1991). The suspension of developing embryos was gently stirred using an electric stirrer equipped with a perspex blade (60 rpm, 20°C). Twenty minutes after fertilization or upon completion of the first cleavage division, embryos were chilled to 0°C in the ice bath. Samples (500 μl, about 20,000 embryos/ml, in triplicate) were incubated for 1 min in Ca^{2+}-FASW containing [^{3}H]8-OH-DPAT in parallel with the samples containing unlabeled ligand, transferred onto GF/B filters (Whatman, England), washed with 5 ml ice-cold Ca^{2+}-FASW and dried. Filters were placed in scintillation vials and radioactivity counted in a "Contron" (France) liquid scintillation counter with an average efficacy of 30% (1 min per sample). Dissociation constants were determined using "LIGAND" software (Munson and Rodbard, 1980).

RESULTS

Effects of serotonin blockers on the cleavage pattern of half-embryos of the sea urchins

Inmecarb (25 μM) decreased the frequency of unequal fourth cleavage division in *S. mirabilis* half-embryos by $33.6 \pm 7.9\%$ ($P < 0.001$); inmecarb methiodide at the same concentration also decreased micromere formation by $25.7 \pm 8.0\%$ ($P < 0.001$) compared with the control.

In contrast, isolation of blastomeres of *P. lividus* at the first cleavage division itself lowers the frequency of unequal third divisions on average from 70 to 46%, as compared with the intact embryos. Inmecarb (52 μM) did not affect the cleavage pattern at either the third or fourth division; however, inmecarb methiodide at the same concentration led to a decrease of micromere formation in half-embryos at the third division by $27.1 \pm 4.2\%$ ($P < 0.001$) compared with the control. The number of micromeres per embryo decreased by 0.5 ± 0.24 ($P < 0.05$). Serotonin (500 μM) added with inmecarb methiodide decreased the effect of the latter by $15.0 \pm 5.5\%$ ($P < 0.01$). When the incubation time exceeded 15 min, the cytostatic effect of tertiary indolylalkylamines prevailed, while their quaternary analogs were ineffective in this respect.

Similar results were obtained with another pair of serotonin blockers. KYuR-14 (100 μM) had no effect on the cleavage pattern of *P. lividus* half-embryos. Its methiodide derivative decreased micromere formation by $31.2 \pm 12.6\%$ ($P < 0.05$). It should be noted that iodemethylate derivatives of inmecarb and KYuR-14 also affected micromere formation in half-embryos of *P. lividus*, doing so at the fourth cleavage division: the number of micromeres per whole embryo was decreased by 0.9 and 1.1, respectively. Imipramine (60 μM) also decreased the incidence of unequal third cleavage divisions in half-embryos of *P. lividus* by $30.3 \pm 9.9\%$ ($P < 0.01$) and the number of micromeres per whole embryo by 0.9 ± 0.4 ($P < 0.05$). Preliminary experiments with *S. intermedius* embryos showed similar results.

Treatment of intact *P. lividus* embryos with neuropharmacological agents at the first cleavage division can alter the shape of the embryos. The embryos were dumbbell-shaped, and half-blastulae with an open blastocoel were formed, etc. This is evidence for the damage of cell interaction mechanisms. Such effects were produced by imipramine (30–60 μM), desipramine (64 μM), amitriptyline (66 μM), inmecarb (52 μM) and KYuR-14 (50–100 μM). At the same time, methiodide derivatives of inmecarb and KYuR-14 used at the same concentrations as their tertiary analogs had no effects on the shape of blastulae.

Binding of [^{3}H]8-OH-DPAT by early embryos of S. intermedius

The specific binding of [^{3}H]8-OH-DPAT by dehyalinized embryos of *S. intermedius* was demonstrated under the following conditions: 25 min after fertilization embryos were incubated with the ligand for 1 min at 0°C; KYuR-14 (100 μM) was used as the unlabeled ligand (Fig. 1). A Scatchard plot of the data obtained suggests the presence of more than one type of [^{3}H]8-OH-DPAT binding sites (Fig. 2). The dissociation constant for the sites with a higher affinity is approximately 3×10^{-10} M and their mean

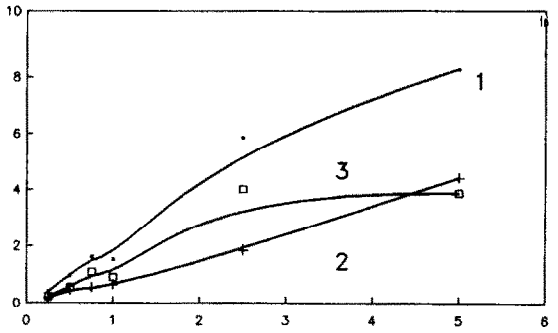

Fig. 1. Binding of [^{3}H]8-OH-DPAT by *S. intermedius* embryos after fertilization: (1) total binding; (2) unspecific binding; (3) specific binding. Abscissa: concentration of [^{3}H]8-OH-DPAT (nM); ordinate—binding of labeled ligand (cpm/mg protein). Unlabeled ligand—KYuR-4 (1×10^{-4} M).

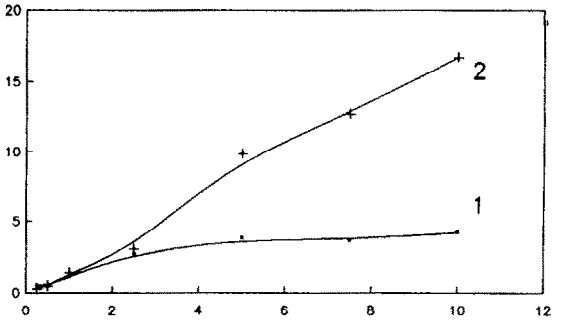

Fig. 3. Changes of [^{3}H]8-OH-DPAT specific binding during development of *S. intermedius* embryos: (1) 25 min after fertilization; (2) after the end of first cleavage division (co-ordinates as in Fig. 1). Unlabeled ligand—KYuR-14 (1×10^{-4} M).

capacity 1.8×10^{10} binding sites/mg protein (average of nine experiments). The affinity of the higher capacity binding sites is lower by at least 2 orders of magnitude. When embryos after the completion of the first cleavage division were studied, a tendency towards an increased binding capacity of a pool with lower affinity was observed (Fig. 3). Displacement of [^{3}H]8-OH-DPAT by unlabeled KYuR-14-methiodide was somewhat less pronounced (Fig. 4).

In experiments with samples incubated for 5 min at 0°C and 20°C in the presence of [^{3}H]8-OH-DPAT (5×10^{-9} M), specific binding at room temperature increased by about one-third. When the duration of incubation at 0°C was increased from 1 to 20 min, both the total and specific [^{3}H]8-OH-DPAT binding increased as well (by about 70% and by a factor of 1.5–2, respectively). In these experiments the dissociation constant was 1.6×10^{-9} M (approx. 7.2×10^{11} binding sites/mg protein), i.e. by an order of magnitude lower than with the exposure for 1 min (Fig. 5).

DISCUSSION

Quaternary indolylalkylamines specifically inhibit micromere formation in half-embryos of sea urchins *S. mirabilis* and *P. lividus*. The data obtained in the experiments with *P. lividus* embryos with generally unequal third cleavage division are used because of the persistence of the effects of quaternary drugs through fourth cleavage division. Premature micromere formation was also observed in *S. mirabilis* embryos, as well as the persisting effects of neuropharmacological agents (Shmukler *et al.*, 1981; Shmukler, 1981). The specificity of this effect is shown by the fact that serotonin weakens it. Since quaternary indoles poorly penetrate the cell (Buznikov, 1987; Landau *et al.*, 1981), this suggests that receptor structures are located on the plasma membrane of the embryo. The cytostatic action of tertiary inmecarb and KYuR-14 indicates that the absence of effects on the cleavage pattern of *P. lividus* is not related to the transport of molecules into the cell in Ca^{2+}-FASW.

Antagonists of 5-hydroxytryptamine are capable of suppressing blastomere adhesion after cell divisions (Buznikov and Shmukler, 1981) which results in

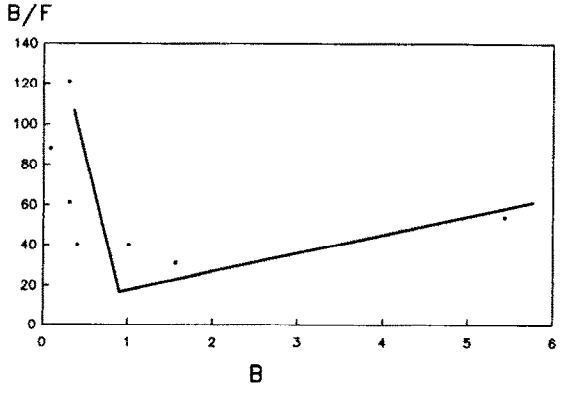

Fig. 2. Scatchard plot of the [^{3}H]8-OH-DPAT binding by *S. intermedius* embryos: B—bound ligand, F—unbound ligand.

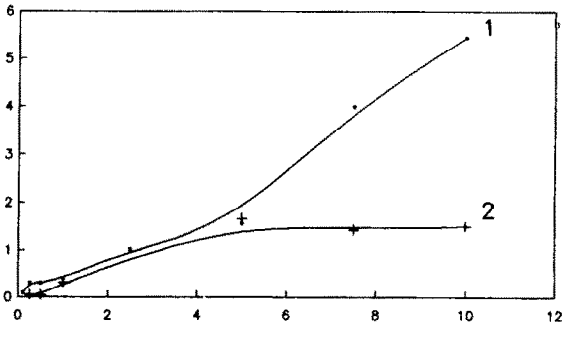

Fig. 4. Binding of [^{3}H]8-OH-DPAT by *S. intermedius* embryos in the presence of unlabeled ligand: (1) unlabeled KYuR-14 (1×10^{-4} M); (2) unlabeled KYuR-14 methiodide (1×10^{-4} M) (co-ordinates as in Fig. 1).

similar disturbances of development. The specific disturbances of the shape of blastula in experiments with the intact *P. lividus* embryos were caused only by drugs which easily penetrated the cells. This suggests that the receptor structures are located within the cells and are inaccessible for quaternized indolylalkylamines which were practically ineffective in these experiments.

Our experiments also demonstrate a distinct specific binding of [^{3}H]8-OH-DPAT in *S. intermedius* embryos. The following facts confirm that these receptor structures are located at the cell membrane. Binding and displacement of the labeled ligand were observed after (i) short exposure (1 min) and at low temperature of the medium (0°C) (this virtually prevents active transport; as a result, the total binding decreases 2–3-fold) and (ii) quick removal of the unbound labeled ligand. Penetration of the ligand into cells to a considerable extent and establishment of equilibrium at intracellular receptor structures are less probable under such conditions. The dissociation constant under these conditions characteristically points to the presence of binding sites with a higher affinity ($K_d \sim 1 \times 10^{-10}$ M) than those which are detected after prolonged exposure ($\sim 1 \times 10^{-9}$ M). The latter constant possibly refers to the intracellular binding sites. Moreover, the displacement of [^{3}H]8-OH-DPAT was also observed when KYuR-14-iodemethylate was used as the unlabeled ligand. This proves the possibility of the membrane localization of receptors that has been demonstrated earlier for [^{3}H]serotonin binding by the plasma membrane fraction of the sea urchin blastulae (Brown and Shaver, 1987). At the same time, it cannot be concluded from the data obtained that embryonic membrane receptors do really exist, since similar experiments with the plasma membrane fraction have not yet been performed.

Possible mechanism of embryonic cellular interaction

In view of the data obtained, the mechanism of cellular interaction can be described in the following way. It is known that the concentration of serotonin in the intercellular space of early sea urchin embryos increases (Markova *et al.*, 1985) due to leakage of the transmitter from the cells and possible barriers to its diffusion into external medium from the intercellular space. The probable presence of transmitter receptors on the contact membrane demonstrated in this paper suggests the existence of the "protosynapse" (Fig. 6) between the blastomeres beginning from the completion of the first cleavage division. Contacting blastomeres are equal and represent both pre- and post-synaptic cells; moreover, the presence of both blastomeres is a prerequisite for maintenance of transmitter concentration in the interblastomere gap at a considerably high level. Similarities with the classic synapse become more evident when taking into account the possible connection of the transmitter system with cyclic nucleotides (Shmukler, 1981; Shmukler and Grigoriev, 1984) and the membrane localization of adenylate cyclase activity in the contact area of blastomeres (Vorbrodt *et al.*, 1977; Rostomyan *et al.*, 1985). It may be suggested that activation of the transmitter receptors and of second messengers in the contact area of blastomeres leads to functional asymmetry of the cortex which determines the orientation of the next cleavage furrow and, consequently, the moment of micromere formation (Rappoport, 1969, 1982; Shmukler *et al.*, 1981); while at the free membrane surface, the concentration of the transmitter leaking from the cell is considerably lower which results in more uniform effects. This can explain disturbances in the cleavage pattern after blastomere isolation.

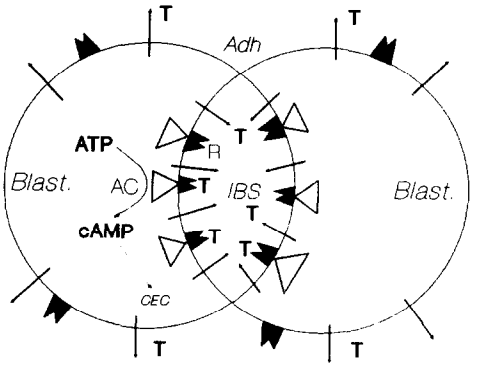

Fig. 6. Scheme of hypothetical "protosynapse": Blast.—blastomeres; IBS—interblastomere space; R—membrane transmitter receptors; AC—adenylate cyclase; CEC—contractile elements of cortex; Adh—cellular adhesive contacts; T—transmitter leakage; ATP—adenosine triphosphate; cAMP—cyclic adenosine monophosphate.

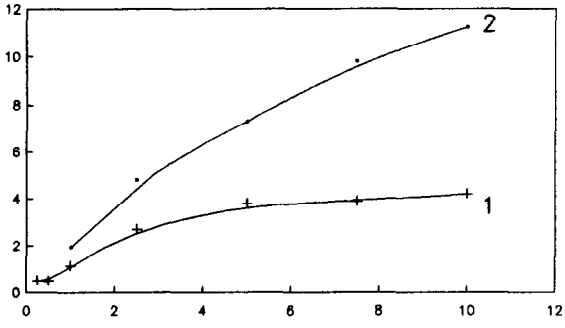

Fig. 5. Comparison of [^{3}H]8-OH-DPAT specific binding at prolonged and short exposition of *S. intermedius* embryos: (1) 1 min exposition; (2) 60 min exposition. Unlabeled ligand—KYuR-14 (1×10^{-4} M) (co-ordinates as in Fig. 1).

Acknowledgements—The author is grateful to Dr P. Zhadan (Pacific Institute of Oceanology, Far-Eastern Branch of Russian Acad. Sci., Vladivostok), Drs I. Deridovich, D. Kreimer and Yu. Khotimchenko (Institute of Marine Biology, Far Eastern Branch of Russian Acad. Sci., Vladivostok), Mrs Tatyana Barinskaya (Moscow Research Institute of Psychiatry) and to Academician Lj. Rakić (Beograd,

Yugoslavia) for their help in organizing this research, and to Dr B. Manukhin (N. K. Koltzov Institute of Developmental Biology, Russian Acad. Sci., Moscow) for his aid in the computer data analysis.

REFERENCES

Brown K. M. and Shaver J. R. (1987) Subcellular distribution of [^{3}H]serotonin binding sites in blastula, gastrula, prism and pluteus sea urchin embryos. *Comp. Biochem. Physiol.* **87C,** 139–148.

Buznikov G. A. (1967) *The Low Molecular Weight Regulators of Embryonic Development.* Nauka, Moscow.

Buznikov G. A. (1987) *Neurotransmitters in Embryogenesis.* Nauka, Moscow.

Buznikov G. A. (1989) Transmitters in early embryogenesis (new data). *Sov. J. Dev. Biol.* **20,** 637–646.

Buznikov G. A. and Podmarev V. I. (1991) The sea urchins. In: *Animal Species for Developmental Studies* (Edited by Detlaff T. A. and Vassetzky S. G.), Vol. 1, pp. 251–283. Consultants Bureau, New York/London.

Buznikov G. A. and Shmukler Yu. B. (1978) Influence of antitransmitter substances on cellular interactions in early embryos of sea urchin. *Sov. J. Develop. Biol.* **9,** 173–178.

Buznikov G. A. and Shmukler Yu. B. (1981) The possible role of "prenervous" neurotransmitters in cellular interactions of early embryogenesis: a hypothesis. *Neurochem. Res.* **6,** 55–69.

Dascal N. and Landau E. M. (1980) Types of muscarinic response in *Xenopus* oocytes. *Life Sci.* **27,** 1423–1428.

Falugi C. and Prestipino G. (1989) Localization of putative nicotinic cholinoreceptors in the early development of *Paracentrotus lividus. Cell. Mol. Biol.* **35,** 147–161.

Kusano K., Miledi R. and Stinnakre J. (1977) Acetylcholine receptors in the oocyte membrane. *Nature* **270,** 739–741.

Landau M. A., Buznikov G. A., Kabankin A. S., Teplitz N. A. and Chernilovskaya P. E. (1981) The sensitivity of sea urchin embryos to cytotoxic neuropharmacological drugs; the correlations between activity and lipophylity of indole and benzole derivatives. *Comp. Biochem. Physiol.* **69C,** 359–366.

Markova L. N., Buznikov G. A., Kovacevic N., Rakic L., Salimova N. B. and Volina E. V. (1985) Histochemical study of biogenic monoamines in early ("prenervous") and late embryos of sea urchins. *Int. J. Dev. Neurosci.* **3,** 493–500.

Munson P. J. and Rodbard D. (1980) LIGAND: a Versatile computerized approach for characterization of ligand-binding systems. *Analyt. Biochem.* **107,** 202–239.

Rappoport R. (1969) Aster-equatorial surface relations and furrow establishment. *J. exp. Zool.* **171,** 59–67.

Rappoport R. (1982) Cleavage of geometrically altered cells. *Cell Diff.* **11,** 359–361.

Rostomyan M. A., Abramyan K. S., Buznikov G. A. and Gusareva E. V. (1985) Electron cytochemical finding of adenylate cyclase in early embryos of sea urchin. *Tsytologiya* **27,** 877–881.

Shmukler Yu. B. (1981) Cellular interactions in early embryos of sea urchins. III. The influence of neuropharmacological substances on the cleavage pattern of *Scaphechinus mirabilis* half embryos. *Sov. J. Dev. Biol.* **12,** 404–409.

Shmukler Yu. B., Chailakhyan L. M., Smolyaninov V. V., Bliokh Zh. M., Karpovich A. L., Gusareva E. V., Naidenko T. H., Hashaev Z. H.-M. and Medvedeva T. D. (1981) Cellular interactions in early embryos of sea urchins. II. Dated mechanic separation of blastomeres. *Sov. J. Dev. Biol.* **12,** 398–403.

Shmukler Yu. B. and Grigoriev N. G. (1984) Cellular interactions in early embryos of sea urchins. V. New data on the regulatory mechanism of micromere formation. *Sov. J. Dev. Biol.* **15,** 308–310.

Shmukler Yu. B., Grigoriev N. G., Buznikov G. A. and Turpaev T. M. (1986) Regulation of cleavage divisions: participation of "prenervous" neurotransmitters coupled with second messengers. *Comp. Biochem. Physiol.* **83C,** 423–427.

Vorbrodt A., Konwinski M., Solter D. and Koprowski H. (1977) Ultrastructural cytochemistry of membrane-bound phosphatases in preimplantation mouse embryos. *Dev. Biol.* **55,** 117–134.