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Expression and functional activity of neurotransmitter system components in sea urchins' early development[†]

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Summary

Reverse-transcription polymerase chain reaction (RT-PCR) investigation of the expression of the components supposedly taking part in serotonin regulation of the early development of *Paracentrotus lividus* has shown the presence of transcripts of five receptors, one of which has conservative amino acid residues characteristic of monoaminergic receptors. At the early stages of embryogenesis the expressions of serotonin transporter (SERT) and noradrenaline transporter (NET) were also recognized. The activities of the enzymes of serotonin synthesis and serotonin transporter were shown using immunohistochemistry and incubation with para-chlorophenylalanine (PCPA) and 5-hydroxytryptophan (HTP). Pharmacological experiments have shown a preferential cytostatic activity of ligands characterized as mammalian 5-hydroxytryptamine $(5\text{-HT})_1$-antagonists. On the basis of the sum of the data from molecular biology and embryo physiological experiments, it is suggested that metabotropic serotonin receptors and membrane transporters take part in the regulatory processes of early sea urchin embryogenesis.

Keywords: Embryo, Receptor, Sea urchin, Serotonin, Transporter

Introduction

Early sea urchin embryos were among the first subjects of pre-nervous neurotransmitter function research (Buznikov *et al.*, 1964; Buznikov, 1989). During the next decades a wealth of embryo physiological data was obtained on the participation of the transmitters, in particular in the control of cleavage divisions (Buznikov, 1989), state of the cytoskeleton (Buznikov & Grigoriev, 1990), blastomere interactions (Shmukler, 1981, 2010) and ciliary motility (Doran *et al.*, 2004). Maternal serotonin was also shown to participate in morphogenesis regulation (Yavarone *et al.*, 1993; Buznikov *et al.*, 1996; Côté *et al.*, 2007). The majority of embryo physiological data was accumulated quite a long period ago when neither a modern panel of ligands nor transmitter receptor classification existed (Hoyer *et al.*, 1994). In many cases, questions arose concerning the receptor specificity of ligands used in earlier works on sea urchin embryos (Landau *et al.*, 1977; Buznikov, 1989), which were based on chemical structure only, and when sea urchin transmitter receptors were not characterized adequately. In the context of modern techniques available, a revision of previous data using contemporary approaches, in

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[†]In memory of Prof. Gennady A. Buznikov, pioneer of embryonic transmitter research.

particular receptor classification elaborated in the last decades and newly synthesized and characterized ligands, is now required. Such an investigation, mainly focusing on the relatively late effects of serotonergic ligands, has been undertaken in embryos and the larvae of sea urchins (Buznikov *et al.*, 2005). We carried out similar experiments on cleaving sea urchin *Paracentrotus lividus* and *Arbacia lixula* together with molecular biology research on the expression of the components of serotonergic system. Whereas a wide molecular biological investigation of the expression of the components of the serotonergic mechanism had been carried out in amphibian embryos (Nikishin *et al.*, 2012a), only sparse data on the serotonergic system (Nikishin *et al.*, 2012b) and GABAergic system (Kaeser *et al.*, 2011) of sea urchins existed. The present work is devoted to the characterization of the pre-nervous serotonergic system of the sea urchin *P. lividus*, and investigation of the expression and functional activity of the components taking part in the synthesis, vesicular transport and reception of serotonin.

Materials and methods

Experiments were performed in the embryos of sea urchins *Paracentrotus lividus* (Lamarck, 1816) and *Arbacia lixula* (Linnaeus, 1758) at the Institute of Marine Biology (Adriatic Sea, Kotor, Montenegro). Embryos were obtained using artificial fertilization according to a standard protocol (Buznikov & Podmarev, 1975).

Sea urchin embryos and larvae were fixed in a 10-fold volume of RNALater (Ambion) for molecular genetic research, and the total RNA was isolated using TRIReagent (Sigma), according to the manufacturer's instructions, and treated with DNase I (Fermentas) to remove the genomic DNA; 1 mg of RNA, M-MLV reverse transcriptase (Evrogen) and random hexanucleotides (Silex) were used for cDNA synthesis. PCR was performed on an MJ Mini thermal cycler (BioRad) using colored *Taq* polymerase (Silex) and specific oligonucleotides (Lytech) using the parameters that were selected considering the sequence of the primers and the length of the product (Table 1). To exclude false-positive results, negative controls were included (PCR without reverse transcription and PCR without cDNA). The PCR products were analysed by 1.5% agarose gel electrophoresis with ethidium bromide (0.5 mg/ml). Primers were designed by Lasergene PrimerSelect (DNASTAR) using sequences from the NCBI GenBank database (Table 2). Prediction of transmembrane helices and topology of proteins were performed using open access online service HMMTOP (Tusnády & Simon, 1998, 2001). PCR products of serotonin transporter (SERT)-like, dopamine active transporter (DAT)-like and vesicular monoamine transporter (VMAT)-like genes were obtained from *P. lividus* embryos using specific oligonucleotides selected on the basis of mRNA sequences of *S. purpuratus* (Table 2). Products were isolated, cloned, sequenced (Evrogen) and deposited into GenBank with the following accession numbers: KC599202 (SERT-like) and KC599201 (VMAT-like).

Embryos were incubated for immunohistochemistry in artificial sea water, containing substances under investigation – serotonin hydrochloride (Tocris Bioscience #3547), 5-hydroxy-L-tryptophan (Sigma-Aldrich #H9772), 4-chloro-L-phenylalanine (Sigma-Aldrich #C8655), for 3 h at room temperature (20°C), and then fixed in 4% paraformaldehyde, transferred into methanol and stored at –20°C. Immunohistochemical staining was performed with primary polyclonal anti-serotonin rabbit antibodies (Chemicon #AB938) and anti-rabbit IgG-Atto 633 antibody produced in goat (Sigma-Aldrich #41176). Preparations were viewed under confocal microscopes either an Olympus FluoView FV10i (Confocal Microscopy Laboratory, Lomonosov Moscow State University) or a Leica TCS SP (Optical Research Group, Koltzov Institute of Developmental Biology) at equal laser intensity and detector sensitivity. The intensity of immunofluorescence was measured using ImageJ software (NIH), and statistical analysis was performed using STATISTICA software (StatSoft).

In embryo physiological experiments substances were added to the medium after elevation of the fertilization envelope and evaluation of the percentage of fertilization (more than 90%). Embryos were incubated at room temperature and the percentage of embryos that successfully passed first cleavage division was recorded. Substances used in the experiments are as follows: (S)-WAY 100135 dihydrochloride (Tocris Bioscience #1253), methiothepin maleate (Tocris Bioscience #0582), cyproheptadine hydrochloride sesquihydrate (Sigma-Aldrich #C6022), GR 55562 dihydrochloride (Tocris Bioscience #1054), SB 242084 (Tocris Bioscience #2901), spiperone (Sigma-Aldrich #S7395), 5-nonyl-oxytryptamine oxalate (Tocris Bioscience #0901), NAN-190 hydrobromide (Tocris Bioscience #0553), 3-tropanylindole-3-carboxylate methiodide (Sigma-Aldrich #T113), mianserin hydrochloride (Tocris Bioscience #0997), clozapine (Sigma-Aldrich #C6305), BW 723C86 hydrochloride (Tocris Bioscience #1059). Arachidonoyl serotonin and arachidonoyl-dopamine were synthesized in the Laboratory of Oxylipins, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry as described (Bisogno *et al.*, 1998; Bezuglov *et al.*, 2001).

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Table 1 Specific oligonucleotides used in the present work

	Sequences of oligonucleotides	T_{ann} (°C)	Product length (bp)
Actin	GAGACGAGGCCCAGAGCAAGAGA; CAGCGGTGGTGGTGAAAGAGTAGC	60	450
HTR-like1	TCATCCTACGAACGCCCAGTCTAC; TGCTGCCGTCAAGGATGTT	56	329
HTR-like2	TATGGCACACGGTCGGGATTCTCT; GTCTTTTCGCGGCGTTTCAGC	58	409
HTR2B-like	GTGTGGCGCCCTCTGTCTGAT; TATTGGGTGCGGCTGGTTTATG	58	509
HTR4-like	ATGCCGGATGAGACCAATACCA; GGCCAGGCTGACCACGAA	56	219
D2-like	GTCCTGCCTTTTTCGATTCACAT; ATCAGCGCCATTCTTTTCGTCAT	55	203
TyrR2-like	GTGGAAATGGAACTGGAAATAATG; AAAGAGGTTGACTAAAAATCCACTGT	53	273
SERT-like	ACGGTGGTTTGGGTGACAGCTAC; GTAATGATCGATTCCAATCCACC	55	504
DAT-like	ATTTTCTCCTATCCGTCATTGG; TCCCTCCGATCAGTAGAAACAAC	53	129
NET-like1	TCAGAAACGATACAGACCCTCAC; TCCCTGAATCCATTGTTACTT	51	443
NET-like2	CTCGCTGTCGTTGTTTCTTTCTT; AACCCGTTACCCCAATCAGGA	54	332
VMAT-like	TTGTATAACATTTGGTAATATGGGCATAG; GCTATGGCGTAGACACTACCATATAC	55	378

Results

PCR analysis

To identify the components of transmitter systems possibly involved in the regulation of early development of sea urchins, the screening of *P. lividus* genes homologous to transmitter receptors and transporters was performed, also as a temporal analysis of their expression using reverse transcription PCR. There are several genes that encode G protein coupled receptors (GPCR), predicted in the GNOMON project as serotonin receptor homologues in the completely sequenced genome of the purple sea urchin *Strongylocentrotus purpuratus*. A BLAST search for homologues of these genes in the Expressed Sequence Tags (EST) database of *P. lividus* was performed. EST clones homologous to four HTR-like genes of *S. purpuratus* were identified (Table 2). PCR analysis of mRNA expression of these receptors during embryogenesis was performed and showed that all four genes are expressed in all stages of *P. lividus* development, including the earliest ones (Fig. 1). At the same time, the analysis of amino acid sequences of these receptors was performed using the online service HMMTOP, and revealed that the third transmembrane domain of all four receptors lacks the aspartate residue (Fig. 2*A*) that is highly conservative in metabotropic serotonin receptors (Padayatti *et al.*, 2013).

Another approach was therefore used to detect transmitter receptors expressed during early *P. lividus* embryogenesis. A BLAST search for sequences homologous to human serotonin receptor *HTR1A* and those obtained from early *P. lividus* embryos (EST clones tagged EGG, CLEB or MBSB) was undertaken in the EST database. Sequences found in this way were searched for using BLAST analysis for corresponding homologous *S. purpuratus* genes, whose amino acid sequences in turn were analysed using the HMMTOP service to check for the presence of an aspartate residue at the third transmembrane domain. Thus, the sequences homologous to *S. purpuratus* genes GNOMON predicted as *TyrR2-like* and *D2-like* were detected (Table 2). PCR analysis of mRNA expression of these receptors has shown that a *D2-like* receptor is expressed during whole embryonic development whereas the *TyrR2-like* receptor starts to be expressed from the gastrula stage. It needs to be taken into an account that these are monoaminergic receptors, whose specificity was determined formally and may be imprecise, as in the case of the abovementioned receptors erroneously attributed as serotonergic ones.

There are genes in the *S. purpuratus* genome that are annotated as homologous to monoamine

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Table 2 GenBank sequences used in the work

	S. purpuratus, mRNA		*P. lividus*, EST	
	Accession number	Definition	Accession number	Developmental stage
HTR-like1	XM_001194088	5-Hydroxytryptamine receptor-like (LOC756233)	AM567784	Gastrula and pluteus
			AM569570	Gastrula and pluteus
			AM570763	Gastrula and pluteus
HTR-like2	XM_003726952	5-Hydroxytryptamine receptor-like (LOC579893)	AM535868	Cleavage and early blastula
HTR2B-like	XM_003728148	5-Hydroxytryptamine receptor 2B-like (LOC100888030)	AM576175	Gastrula and pluteus
			AM583751	Gastrula and pluteus
			AM592724	Gastrula and pluteus
HTR4-like	XM_003727648	5-Hydroxytryptamine receptor 4-like (LOC589531)	AM600436	Unfertilised egg
			AM543314	Cleavage and early blastula
D2-like	XM_003725921	Dopamine receptor 2-like (LOC100893289)	AM527359	Cleavage and early blastula
TyrR2-like	XM_003725105	Putative tyramine receptor 2-like (LOC581142)	AM577686	Gastrula and pluteus
			AM553528	Mesenchyme and swimming blastula
			AM587067	Gastrula and pluteus
			AM562929	Gastrula and pluteus
			AM590833	Gastrula and pluteus
			AM576359	Gastrula and pluteus
SERT-like	XM_785856	Sodium-dependent serotonin transporter-like (LOC586058)	–	–
DAT-like	XM_779389	Sodium-dependent dopamine transporter-like (LOC579263)	–	–
NET-like1	XM_784053	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2 (slc6a2)	AM218772	Unfertilised egg
			AM218169	Unfertilised egg
			AM227036	Unfertilised egg
			AM600533	Unfertilised egg
NET-like2	XM_784934	Sodium-dependent noradrenaline transporter-like (LOC585094)	AM574035	Gastrula and pluteus
VMAT-like	XM_780128	Synaptic vesicular amine transporter-like (LOC580049)	–	–

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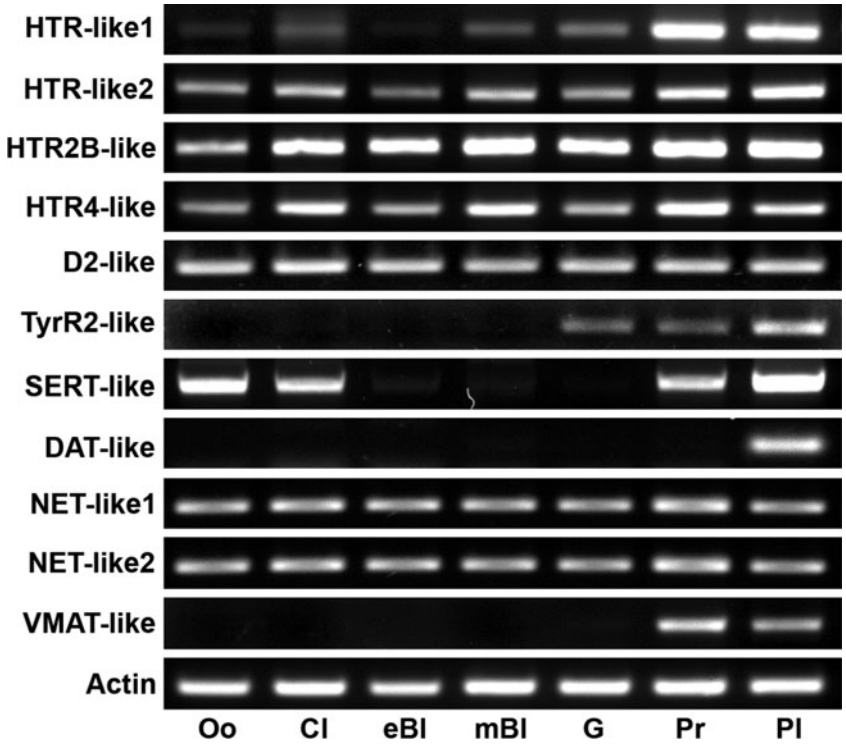

Figure 1 PCR analysis of the mRNA expression of homologues of monoaminergic transmitter system components during *P. lividus* embryonic development. Cl, cleavage stage (16–32 blastomeres); eBl, early blastula; G, gastrula; mBl, mesenchyme blastula; Oo, oocyte; Pl, pluteus; Pr, prism.

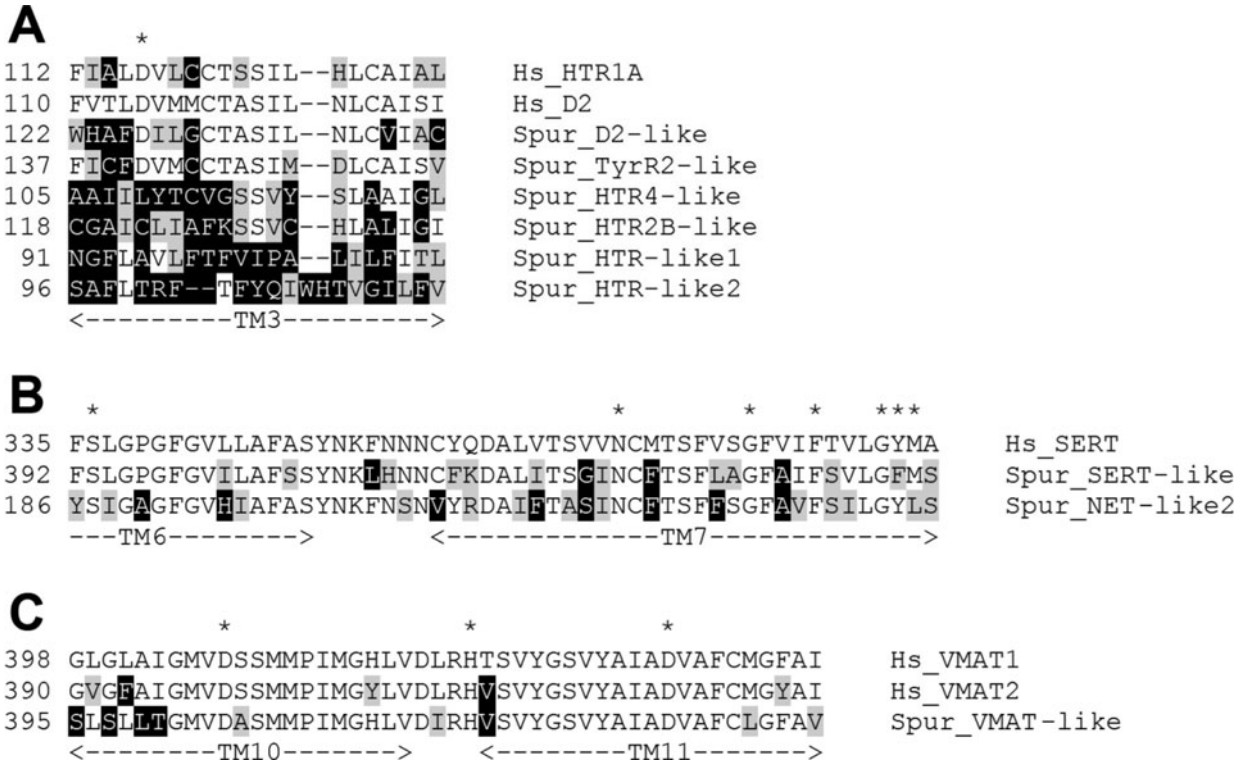

Figure 2 Alignment of amino acid sequences of monoamine receptors (*A*), membrane transporters (*B*) and vesicular transporters (*C*) of human (Hs) and sea urchin *S. purpuratus* (Spur). Highly conserved amino acid residues are marked by asterisks. Amino acids mismatched with the reference human sequence are marked in black, and conserved amino acid substitutions are marked in grey. The boundaries of the transmembrane domains are marked under the alignments.

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transporters: membrane transporters of serotonin *SERT-like*, and norepinephrine *NET-like1* and *NET-like2*, active dopamine transporter *DAT-like*, and vesicular monoamine transporter *VMAT-like* (Table 2). A search for homologous sequences in the *P. lividus* EST database was successful in cases of *NET-like1* and *NET-like2* only. Specific oligonucleotides for the investigation of the expression of the rest transporters were selected to the most conservative regions of *S. purpuratus* mRNA sequences of *SERT-like*, *DAT-like* and *VMAT-like.* Results of PCR analysis are presented in Fig. 1. The gene homologous to *SERT-like* was expressed in all of the developmental stages investigated. The most pronounced expression was found in the earliest stages such as oocyte and cleavage divisions, then decreased during early development gradually until zero at the gastrula stage and increased again at the neural stages (prism, pluteus). The expression of the gene homologous to *DAT-like* was detected at the pluteus stage only. At the same time, two homologues of the norepinephrine transporter *NET-like1* and *NET-like2* are expressed at all developmental stages. Computational analysis of amino acid sequences of transporters under investigation have shown that conservative residues characteristic for membrane monoamine transporters (Penado *et al.*, 1998) are present in *SERT-like* and *NET2-like* (Fig. 2*B*). A gene that is homologous to *VMAT-like* starts to be expressed at the prism stage only, and its amino acid sequence contains amino acid residues that are conservative for vesicular monoamine transporters (Schuldiner *et al.*, 1995) (see Fig. 2*C*).

Immunohistochemical experiments

Serotonin was detected immunohistochemically in all blastomeres of *P. lividus* (Fig. 3*A*). Blastomere cytoplasm was stained uniformly as close grains. With uniform cytoplasm staining there were large grains distributed over the blastomere volume but they were concentrated at the surface membrane of the embryos. The negative control (without first antibodies) showed staining neither of the cytoplasm nor of large grains, and therefore the staining in the experimental samples was due to the anti-serotonin antibody immune reaction. To check the activity of serotonin reuptake, the incubation of sea urchin embryos at the cleavage division and blastula stages in the presence of serotonin (100 μM) was carried out. Subsequent immunostaining of incubated embryos have shown an increase in its intensity in the cytoplasm (Fig. 3*B*) up to 188% as compared with the control at the cleavage stage (Fig. 3*E*) and to 131% at the early blastula stage (Fig. 3*F*). To check the activity of aromatic L-amino acid decarboxylase (AADC) embryos were incubated with serotonin precursor 5-hydroxytryptophan (HTP) (100 μM). Immunohistochemical staining elicited an increase in serotonin levels in the cytoplasm of embryos incubated in HTP (Fig. 3C) to 297% as compared with the control at the cleavage stage (Fig. 3*E*) and to 190% at the early blastula stage (Fig. 3*F*). Immunohistochemical staining of the embryos incubated with tryptophan hydroxylase (TPH) inhibitor PCPA (100 μM) showed a decrease in cytoplasmic serotonin (Fig. 3*D*) to 56% as compared with the control at the cleavage stage (Fig. 3*E*) and to 22% at the early blastula stage (Fig. 3F). The number and staining intensity of large granules in the cytoplasm and at the surface of blastomeres were not changed with incubation in 5-hydroxytryptamine (5-HT), HTP or PCPA (Fig. 3*B–D*).

Embryo physiological experiments

The sensitivity of embryos to serotonergic and dopaminergic ligands was studied by their ability to block the first cleavage division of *P. lividus.* The minimum concentrations of ligands that blocked first cleavage division are presented in Table 3 and show that the most effective were ligands characterized as 5-HT_1-antagonists in mammals. The whole range of comparative ligand activity is as follows:

> methiothepine = 5-nonyl-oxytryptamine > inmecarb > cyproheptadine > (S)-WAY-100635 = SB 242084 = clozapin > MDL 72832 = BW 723C86 > GR 55562 > NAN-190

It needs to be noted that not only serotonin antagonists were able to block or inhibit first cleavage division but also some agonists such as BW 723C86. At the same time $5HT_3$-antagonists tropisetron and granisetron had no embryostatic effects.

A slightly different range of ligand activity was obtained in the parallel experiments with *A. lixula* embryos (Table 3):

> (S)-WAY-100635 > 5-nonyl-oxytryptamine >> inmecarb > cyproheptadine = mianserin >> SB 242084 = clozapin = MDL 72832 = BW 723C86 = 8-OH-DPAT > GR 55562 = NAN-190 >>> methiothepine (ineffective)

The activity of $5HT_1$-receptor ligands was more pronounced compared with other types of ligands in this sea urchin species. In contrast with *P. lividus*, early *A. lixula* embryos were sensitive to mianserin and 8-OH-DPAT but not to methiothepine. *A. lixula* embryos also have far higher sensitivity to two substances as compared with *P. lividus* – 4 μM versus 50 μM for (S)-WAY-100635 and 7.5 μM versus 20 μM for 5-nonyl-oxytryptamine, correspondingly.

The specificity of the serotonergic mechanism of ligand action was investigated using the addition of transmitters or their conjugates with arachidonic

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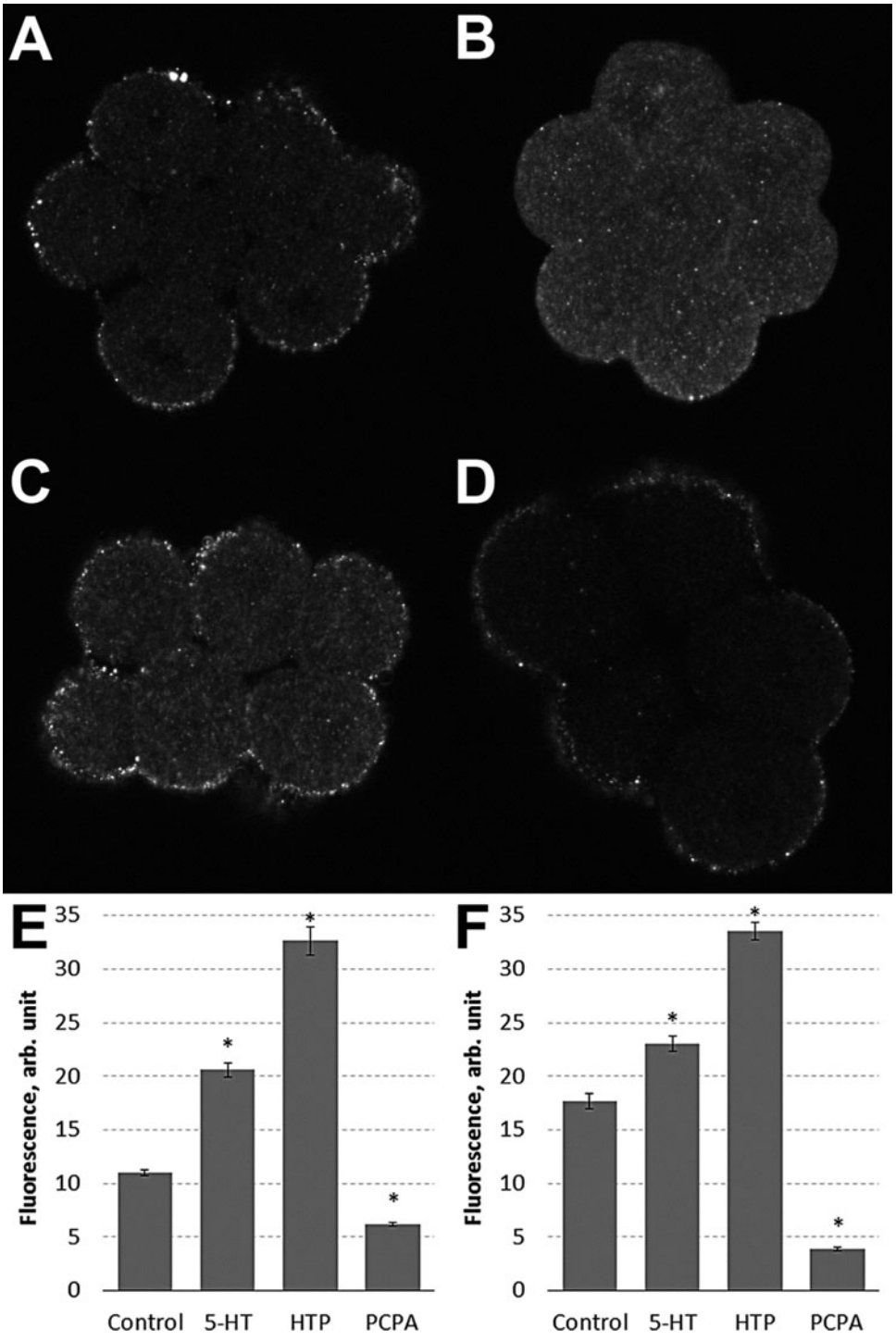

Figure 3 Immunohistochemical detection of serotonin in *P. lividus* blastula stage embryos in the control (*A*) and after 3 h incubation in the 5-HT (*B*), HTP (*C*) and PCPA (*D*). Changes of serotonin immunoreactivity intensity after 3 h incubation of cleavage embryos (*E*) and blastula stage embryos (*F*) with 5-HT, HTP and PCPA. Bars show standard error. Significance by Mann–Whitney *U*-test: $^{*}P < 0.005$.

acid together with antagonists. Serotonin has a relatively weak protective action against the effects of serotonin receptor antagonists in *P. lividus* embryos (Table 4). At the same time, arachidonoyl transmitter derivatives, firstly serotonin (AA-5-HT), had a far more pronounced protective effect. AA-5-HT effectively protected the development against inmecarb, (S)-WAY-100635, cyproheptadine and

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Table 3 Minimal concentrations of serotonergic ligands blocking cleavage divisions of *P. lividus* and *A. lixula* embryos

Ligand	Specificity	*P. lividus* block. conc. (μM)	*A. lixula* block. conc. (μM)
5-Nonyl-oxytryptamine	5-HT_{1B} selective agonist	20	7.5
Methiothepin	Methiothepin mesylate salt is a 5-HT_1, 5-HT_2, 5-HT_6, 5-HT_7 serotonin receptor antagonist	20	No effect
Inmecarb	Preparation widely used in earlier studies (Buznikov, 1989). Characterized as 'serotonin antagonist'	25	25
Cyproheptadine	5-HT_2/5-HT_{1C} serotonin receptor antagonist; H1 histamine receptor antagonist	30	30
(S)-WAY-100635	Highly selective 5-HT_{1A} serotonin receptor antagonist	50	4
MDL 72832	A potent ligand at 5-HT_{1A} receptors, with mixed agonist and antagonist properties	>50	50
SB 242084	5-HT_{2C} receptor antagonist that displays 158- and 100-fold selectivity over 5-HT_{2A} and 5-HT_{2B} receptors, respectively	50	50
BW 723C86	Selective 5-HT_{2B} receptor agonist	> 50	50
GR 55562	5-HT_{1B}/5-HT_{1D} serotonin receptor antagonist	60	60
NAN-190	Potent 5-HT_{1A} serotonin receptor antagonist	100	60
8-OH-DPAT	Selective 5-HT_1 agonist with high affinity for subtype 5-HT_{1A} receptor	No effect	50
Cinanserin	5-HT_2 antagonist	No effect	No effect
Ketanserin	Selective 5-HT2 serotonin receptor antagonist	No effect	No effect
Mianserin	Antagonist/inverse agonist at 5-HT_2 serotonin receptors; has moderate affinity for 5-HT_6, also blocks the H_1 histamine receptor and the α_2 adrenoceptor	No effect	30
Tropisetron	Selective serotonin 5-HT_3 receptor antagonist	No effect	No effect
Granisetron	5-HT_3 receptor antagonist	No effect	No effect
Clozapine	Selective antagonist for D_4-dopamine receptor. Antagonist at 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_6, and 5-HT_7 serotonin receptors	50	50
Eticlopride	Potent and selective dopamine D_2/D_3 receptor antagonist	30	
Galoperidol	D_2, D_3, and D_4 dopamine receptor antagonist	20	
Spiperone	Selective D_2 dopamine receptor antagonist; α_{1B}-adrenoceptor antagonist; mixed 5-HT_{2A}/5-HT_1 serotonin receptor	50	

Note: No effect: has no cytostatic effect at concentrations below 100 μM.

methiothepin. Arachidonoyl-5-methoxy-tryptamine (AA-5-MOT) and arachidonoyl-dopamine (AA-DA) had less pronounced protective effects. Arachidonic acid itself never showed a protective action against serotonin antagonists. Together with arachidonic transmitter analogues, the activator of adenylyl cyclase forskolin (100 μM) and dibutyryl-cAMP (100 μM) also had a protective effect against cytostatic effect of methiothepin. Eticlopride and haloperidol, dopamine antagonists, effectively blocked the development of *P. lividus* embryos at concentrations of 50 and 20 μM, correspondingly. None of the substances used had a protective effect against eticlopride, whereas AA-DA effectively protected from the haloperidol effect.

Discussion

The results of the present work allow some preliminary conclusions concerning the structure of the embryonic transmitter system. Serotonin is present in all species of early embryos studied (Buznikov, 1989). In particular in sea urchins it is distributed over the cytoplasm (Buznikov *et al.*, 1979; Markova *et al.*, 1985; Buznikov *et al.*, 2003; Amireault & Dubé, 2005). The present immunohistochemical research showed the uniform localization of serotonin.

Immunohistochemical results of incubation of sea urchin embryos with serotonin precursor HTP and with tryptophan hydroxylase inhibitor PCPA (Fig. 3*C*, *D*) have shown continuous activity of both synthetic

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Table 4 Effects of various protectors against cytostatic action of transmitter ligands in *P. lividus* embryos

Ligand (conc. μM)	Protector (100 μM)	Protective effect (%)
Methiothepin (25)	5-HT	15
	AA-5-HT	39
	AA-DA	17
	Forskolin	10
	dBcAMP	11
5-Nonyl-oxytryptamine	5-HT	2
	AA-5-MOT	5
	AA-DA	23
Inmecarb	5-HT	10
	AA-5-HT	51
	AA-5-MOT	12
	AA-DA	15
Cyproheptadine	5-HT	8
	AA-5-HT	32
	AA-5-MOT	15
	AA-DA	18
(S)-WAY-100635	5-HT	0
	AA-5-HT	35
	AA-5-MOT	30
	AA-DA	20
NAN-190 (100)	5-HT	10
	AA-5-HT	1
Galoperidol (20)	5-HT	0
	DA	3
	AA-DA	4
	AA-Adr	36
	AANor	31
	Forskolin	3
	dBcAMP	2
Clozapin	5-HT	0
	AA-5-HT	0
	AA-5-MOT	0
	AA-DA	15
Eticlopride (50)	AA-DA	1
	AAAdr	1
	AANor	0
	Forskolin	0
	dBcAMP	0
Spiperone (50)	5HT	8
	AA-DA	13
	AAAdr	10
	AANor	23
	Forskolin	13
	dBcAMP	24

Note: Effects are presented as the increase in a number of cleaving embryos after addition of protectors (%). Concentration of protectors in all experiments was 100 μM.

enzymes – tryptophan hydroxylase and aromatic amino acid decarboxylase – during whole early development. Sea urchins are not exclusive in this respect as the activity of a serotonin synthesis system at the early stages of the development was shown in a number of species (Buznikov *et al.*, 2003). In some cases this system was detected in yolk granules, however serotonin is always detected in the cytoplasm only (Buznikov, 1989).

Membrane and vesicular monoamine transporters are important components of monoaminergic systems. The present work has shown that several genes

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homologous to monoamine transporters are expressed in the early stages of embryonic development. Analysis of protein amino acid sequences encoded by these genes proved the presence of conservative residues in *SERT-like* and *NET-like2*. Both genes are expressed in the early *P. lividus* developmental stages, and a *SERT-like* homologue has interesting expression dynamics: it expresses most pronouncedly in the early developmental stages and then practically disappears at the gastrula stage which coincides with the dynamics of serotonin itself in sea urchin embryos (Renaud *et al.*, 1983). Together with immunohistochemical data on the increase of intracellular serotonin levels after incubation with this transmitter this probably shows the functional role of serotonin transporters at the early stages of the development – during cleavage divisions and blastulation. These results also support earlier data on the activity of serotonin membrane transport in early sea urchin embryos (Buznikov, 1984) and other animals (Amireault & Dubé, 2005). The gene from *S. purpuratus* genome homologous to *VMAT* is annotated, whose amino acid sequence contains conservative residues that are characteristic for the vesicular monoamine transporter. PCR analysis has shown that the expression of this gene is absent in the early stages of development. Vesicular monoamine transporters are needed for the accumulation of monoaminergic transmitters, including serotonin, into vesicles for the realization of their intercellular signalling function in adult organisms. This mechanism is probably absent in early sea urchin embryos. It was earlier demonstrated that reserpine and other inhibitors of vesicular monoamine transport do not influence the level of monoamines inside cells at the pre-gastrulation stages, proving the absence of a system of vesicular transport in early embryos (Markova *et al.*, 1985). Nevertheless, it is known that early sea urchin embryos transport the serotonin to the outer medium (Buznikov, 1967; Renaud *et al.*, 1983), and serotonin can be accumulated at the interblastomere spaces of early embryos (Markova *et al.*, 1985). Mechanisms of such serotonin excretion remain unclear when taking into account the absence of vesicular transport. This is probably achieved through any membrane transporters able to undertake reverse activity (Richerson & Wu, 2003). The function of transmitter transport in early embryos may be coupled to some factors. First, in connection with the weakness of the enzymatic system of transmitter degradation, efflux of transmitter to the outer medium can be the main way of its inactivation in intracellular regulatory processes (Buznikov, 1989). Moreover, transmitters transported to the external medium can take part in blastomere interaction (Shmukler, 1993), and reuptake of serotonin into the blastomeres thus limits this process.

A key link of transmitter signalling is a receptor, and it is therefore important to know which receptors mediate transmitter effects in early embryonic development. Generally the results of embryophysiological experiments show significant differences between neurotransmitter receptors of sea urchins and mammalian ones, in which the ligands used were characterized. In particular, some serotonin agonists in mammals (5-nonyl-oxytryptamine, BW 723C86, 8-OH-DPAT) may act as blockers of the development in sea urchin embryos. Besides, there are interspecies differences in the sensitivity of *A. lixula* and *P. lividus* embryos that are proved by variations of the ranges of ligand activities of these two species. This is probably the reason for the ineffectiveness of $5HT_{1A}$-receptor ligands, embryostatic in species used in the present work, in cleaving embryos of *L. variegatus* (Buznikov *et al.*, 2005).

Nevertheless, it is clear that the cytostatic effects of transmitter ligands are mediated by metabotropic receptors. This opinion is supported in particular by a lack of embryostatic effects of channel receptor antagonists, as in previous study (Buznikov *et al.*, 2005). Ligands, characterized as antagonists of $5HT_1$-receptors, were most effective that generally corresponds to previous results although obtained in an unusual experimental design – with the administration of ligands after the first cleavage division not after fertilization (Buznikov *et al.*, 2005). However, taking into account our data on the expression of transmitter receptors during the early development of sea urchins, the data of the later publication have to be taken with special caution, because pharmacological characteristics obtained in mammals were applied there to sea urchins directly. At least partially, ligand effects are mediated by the adenylyl cyclase signal pathway, a finding that is supported by the protective action of forskolin and dibutyryl-cAMP and corresponds to earlier data obtained in another sea urchin species (Shmukler *et al.*, 1986; Rostomyan *et al.*, 1985). Similar data were obtained earlier (Carginale *et al.*, 1992) for dopaminergic ligands, including, however, metergoline, which is now recognized as a 5-HT_1-, 5-HT_2- and 5-HT_7-antagonist. Thus, transmitter receptors taking part in serotonin signal function in sea urchin embryos are probably GPCR.

Our molecular genetic study of receptors homologous to transmitter GPCR, detected five genes that are expressed in early *P. lividus* development, probably playing a role in the serotonergic regulation of the cell cycle during cleavage. However, the amino acid sequence of *D2-like* only has an aspartate residue in the third transmembrane domain that is conservative among the metabotropic receptors of serotonin and other monoamines (van Rhee & Jacobson, 1996). The fact that the receptors studied are annotated as HTR-

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like but do not have this highly conservative amino acid residue means that prediction of GPCRs types using GNOMON methods may be imprecise (Nagy *et al.*, 2008). Taking into account pharmacological data obtained from *P. lividus* embryos, the *D2-like* receptor is the most probable link of pre-nervous monoamine functions in sea urchins. Further functional studies of this receptor may allow characterization of its real type and pharmacological properties also as elicit its role in the regulation of early sea urchin development.

Study of the specificity of early sea urchin embryo transmitter receptors using arachidonoyl derivatives of the transmitters brought some contradictions. Protective action against the cytostatic effects of methiothepin, inmecarb, cyproheptadine, and (S)-WAY-100635 had not only AA-5-HT but also AA-5-MOT and AA-DA. Furthermore, serotonin and AA-5-HT had no protective action against the cytostatic effect of clozapine which is active on the serotonin and dopamine receptors of mammals, whereas AA-DA had some protective action. At the same time, the cytostatic effect of spiperone, which is an antagonist of serotonin and dopamine receptors, was decreased by the addition of both serotonin and AA-DA. This again stresses a possible similarity of embryonic dopamine and serotonin receptors. These results suggest that either dopamine receptors are functionally active in early *P. lividus* development together with serotonin ones, or that embryonic receptors have combined sensitivity to both of these transmitters. Simultaneous expression of the receptors of different transmitters coupled to the same system of second messengers is characteristic for early development of some other animals (Dubé & Amireault, 2007; Nikishin *et al.*, 2012a). Moreover, various types of GPCRs may interact and thus change their functional properties (Kamal & Jockers, 2011). Such mechanisms might be present in the transmitter regulation of early sea urchin development.

Finally, one peculiarity of the embryonic transmitter system that needs to be noted that is indirectly supported by our experiments with arachidonoyl transmitter derivatives, having high lipophility and easily penetrating the embryonic cells is the intracellular localization of the receptors (Landau *et al.*, 1977; Buznikov & Shmukler, 1978; Buznikov, 1989). This suggestion is supported by the significantly more pronounced protective action of arachidonoyl derivatives of the transmitters that easily penetrate the cell as compared with serotonin and dopamine *per se*, that cannot enter the cell without the participation of specific membrane transporters. It needs to be noted that in the analogous experiments of Buznikov and co-authors (2005) lipophilic AA-5-HT and hydrophilic 5HTQ, which practically do not penetrate the cell of sea urchin embryos, show their protective effects in principally different ways. The specificity of protective action of the conjugates of transmitters with fatty acids is supported by the absence of any protective action of arachidonic acid.

Thus, previous understandings of the transmitter regulation of sea urchins' early development have been confirmed by the present work in its main features. However, this is in need of some updates both concerning the specificity of pharmacological tools used and in the solution of the problem of key links in the process – embryonic receptors, which occur in a more complex way in sea urchins than in amphibians and mammals (Nikishin *et al.*, 2012a; Veselá *et al.*, 2003). The study of transmitter receptors in early sea invertebrates needs to be continued.

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