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Dopamine and serotonin modulate the onset of metamorphosis in the ascidian *Phallusia mammillata*

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Abstract

Neurotransmitters play an important role in larval metamorphosis in different groups of marine invertebrates. In this work, the role of dopamine and serotonin during metamorphosis of the ascidian *Phallusia mammillata* larvae was examined. By immunofluorescence experiments, dopamine was localized in some neurons of the central nervous system and in the adhesive papillae of the larvae. Dopamine and serotonin signaling was inhibited by means of antagonists of these neurotransmitters receptors (*R*(+)-SCH-23390, a D_1 antagonist; clozapine, a D_4 antagonist; WAY-100635, a 5-HT_{1A} antagonist) and by sequestering the neurotransmitters with specific antibodies. Moreover, dopamine synthesis was inhibited by exposing 2-cell embryos to α-methyl-L-tyrosine. Dopamine depletion, obtained by these different approaches, caused early metamorphosis, while serotonin depletion delayed the onset of metamorphosis. The opposite effects were obtained using agonists of the neurotransmitters: lisuride, a D_2 agonist, inhibited metamorphosis, while DOI hydrochloride and 8-OH-DPAT HBr, two serotonin agonists, promoted it. So, it is possible to suppose that dopamine signaling delayed metamorphosis while serotonin signaling triggers it. We propose a mechanism by which these neurotransmitters may modulate the timing of metamorphosis in larvae.

Keywords: Neurotransmitter; Urochordata; Ascidian metamorphosis; Larval development; Nervous system

Introduction

Ascidians are marine invertebrates which possess a swimming tadpole larva and a benthic adult. After hatching, the larva swims for a short period and then attaches to a substratum by means of adhesive papillae (three mucus secreting organs at the end of the trunk). Depending on the species, ascidian larvae settle within minutes, hours or days after hatching and then start to metamorphose (reviewed in Satoh, 1994). Metamorphosis can occur only after the larva develops the ability to respond to external inducing signals; the responsive state is termed competence. During metamorphosis, the larva undergoes a series of drastic morphogenetic events starting with release of adhesives or papillary eversion, tail resorption, phagocytosis of the transitory larval organs (TLO), rearrangement of larval–juvenile organs (LJO) and leading to the differentiation of prospective juvenile organs (PJO) (Cloney, 1982). Many environmental factors can influence the settlement of the larva, such as light intensity fluctuations, presence of conspecific or of prey species, bacterial cues or mineral composition of the substrate (Groppelli et al., 2003; Rodriguez et al., 1993; Svane and Dolmer, 1995). Moreover, several artificial inducers, which can shorten the average time between hatching and the onset of metamorphosis have been identified. These include larval-tissue extracts, vital dyes, conditioned sea water, dimethylsulphoxide, dicapryloylglycerol, NH_4^+, copper, iron, zinc salt and K^+ (Berking and Herrmann, 1990; Cloney, 1978, 1982; Degnan et al., 1997; Grave, 1944; Grave and Nicoll, 1939; Hirai, 1961; Lynch, 1961).

However, nothing is known about how these agents trigger metamorphosis. This complex process seems to be regulated both by exogenous signals, interacting with larval receptors, and intrinsic signaling factors, controlling the acquisition of competence and coordinating morphogenetic changes in the larva (Morse, 1990).

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It has been demonstrated that larvae need novel transcription in order to acquire competence. In the ascidian *Herdmania curvata*, a protein called Hemps (EGF-like factor) is strongly involved in acquisition of larval competence and in coordination of metamorphic events (Arnold et al., 1997; Eri et al., 1999). Also in *Boltenia villosa* larvae, specific transcripts are found during the acquisition of metamorphic competence; among these, there is a transcriptional factor, *cornichon*, which is thought to be involved in EGF signaling (Davidson and Swalla, 2001). Nakayama et al. (2001) isolated two genes expressed during *Ciona intestinalis* metamorphosis: *Ci-meta1*, encoding a polypeptide with EGF-like repeats, and *Ci-meta2*, encoding a secreted protein probably involved in epithelial cells rearrangement during metamorphosis.

Some studies suggest that the larval nervous system is implicated in the initiation of the metamorphosis (Rodriguez et al., 1993). Nervous system, neuroid conduction and diffusion of one or more factors are probably involved in controlling this process in ascidians (Satoh, 1994). Neurotransmitters are endogenous substances that are released from neurons and can act at the level of postsynaptic receptors in a broader area as neuromodulators or can diffuse in the extracellular space as neurohormones. A number of neurotransmitters have been reported to play a role in regulating the metamorphosis in different marine invertebrates: choline and succinylcholine chloride (polychaetes, Pawlik, 1990); DOPA and other cathecolamines (gastropods: Pires et al., 2000); serotonin (hydrozoan, McCauley, 1997); DOPA and serotonin (barnacles: Yamamoto et al., 1999). In ascidians, neuroactive compounds, such as acetylcholine, L-tyroxine and cathecolamines, were reported to induce metamorphosis (Coniglio et al., 1998; Patricolo et al., 2001; Kimura et al., 2003), while NO was found to inhibit it (Bishop et al., 2001). But so far, neither the environmental signals that trigger metamorphosis nor the genetic cascades activated in response to these signals are known.

In a previous work on *Phallusia mammillata* larvae, we detected the presence of serotonin (5-HT) in primary neurons of the papillae in cells surrounding the ocellus and in primary neurons of the tail (Pennati et al., 2001). In this study, we report the localization of another neurotransmitter, dopamine (DA), in *P. mammillata* larvae. Moreover, the effect of DA, 5-HT and agonists/antagonists of both serotonin and dopamine receptors on metamorphosis was examined and the involvement of both neurotransmitters in modulating the onset of metamorphosis is discussed.

Materials and methods

Animals

Adults of *P. mammillata* were collected in the gulf of Lerici, La Spezia, Italy and reared in an aquarium at 16°C with 12 h light cycle. Animals were anesthetized with MS222 (3-aminobenzoic-acid-ethyl-ester methane sulfonate salt, Sigma, Italy), 1 g/l, for 1 h at least and then dissected to remove male and female gametes from the gonoducts. Gametes were used for in vitro fertilization. Fertilized eggs were allowed to develop in 9 cm Petri dishes in Millipore-filtered seawater (MFSW) at 18°C. In order to avoid bacterial infection, 50 mg/l streptomycin (Sigma, Italy) were added to MFSW. One or two hours after hatching, larvae were collected for treatments.

Immunohistochemistry

Specimens were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.2) (PBS) at room temperature for 2 h. All steps were performed with gentle rocking. After rinsing with 0.1 M PBS (phosphate buffer saline), the samples were stored in methanol at −20°C. After rehydration, specimens were permeabilized with 0.1% Tween-20, 0.25% Triton X-100 in PBS for 20 min, washed three times in PBS, 10 min each and incubated for 30 min in 50% PBS/50% normal goat serum, previously disactivated at 55°C for 30 min. Then, the samples were treated for 48 h at 4°C with polyclonal antibody AB122S rabbit anti-dopamine antibody (Chemicon) diluted 1:500. After several washes in PBS, the samples were incubated in 1% bovine serum albumine (BSA) in PBS for 1 h at room temperature and then incubated at 4°C overnight in PBS, in which FITC-conjugated goat anti-rabbit IgG, diluted 1:100, was added. Next, the specimens were washed three times in PBS, 20 min each, and mounted in 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma, Italy) on microscope slides.

As negative controls, larvae and juveniles were processed without incubation in primary antibody: such specimens exhibited no detectable fluorescence in the nervous system.

Samples were examined using a confocal laser scanning microscope Leica TCS-NT (Leica Microsystems, Heidelberg, Germany), equipped with laser argon/krypton, 75 mW multiline. Series of “optical sections” attained by scanning whole-mount specimens were projected into one image with greater focal depth. The number and step size of “optical sections” are given for each image in figure captions. FITC fluorescence was observed using a 488 nm excitation filter and a 530/30 band pass filter.

Histology

PFA fixed larvae were sectioned for detailed observation of the sensory vesicle. After staining in 1% carmin red for 3 h, specimens were embedded in Technovit 7100 plastic (Heraeus Kulzer GmbH, Werheim, Germany) and sectioned at 5 μm. Sections were counterstained with 0.5% methylene blue in water for few minutes and mounted in Entellan (Merck, Italy).

Assays for larval metamorphosis

Assays for larval metamorphosis with agonists and antagonists of neurotransmitters were performed using 4-well plates (NUNC, Denmark). Three replicates of each experiment were performed on different culture batches on different dates. In each replicate, 2 h post hatching larvae (hph) were collected from a single batch and evenly divided among the treatments. Each treatment for each batch was replicated five times. For each treatment, 10 ± 2 larvae were carefully pipetted in wells containing 1 ml of MFSW in which the drug to be tested had been diluted. Treatments with α-methyl-L-tyrosine, a dopamine synthesis inhibitor, were performed on 2-cell stage embryos reared in 5 cm Petri dishes, containing 5 ml of the drug diluted in MFSW. Each experiment was performed three times with two replicates, using 30 ± 2 embryos for each replicate. A 10 mM stock solution was prepared for each drug and then diluted to the different concentrations: 1, 10 and 100 μM. Combined treatments were performed with 1 μM and 10 μM lisuride and 1 μM and 10 μM WAY-100635 maleate respectively. Chemical agents tested were dissolved mainly in distilled water, whereas those which were insoluble in water were dissolved in DMSO. Control tests were performed in MFSW alone and in MFSW plus DMSO diluted to the same concentration used in treatments. The plates were kept at 20°C throughout the experiments. Larvae were observed by a stereo-microscope equipped with optic fibers 1 h after treatment and then every 2 h, for at least 9 h, to estimate proportion of tail resorption (number of larvae with tail resorption/number of total larvae). Percentage of larvae attached to the dish was scored. Particular care was taken to observe each plate under the microscope the same number of times and for no longer than 5 min in order to avoid long exposures of the larvae to intense light, which could influence the metamorphosis.

Dopamine, L-DOPA, *S*−(−)−Lisuride, *R*(+)-SCH-23390, clozapine, α-methyl-L-tyrosine, 5-HT (5-hydroxytryptamine), serotonin creatinine sulfate complex (3-[2-Aminoethyl]-5-hydroxyindole creatinine sulfate complex), (±)-8-Hydroxy-2-(di-*n*-propyl-amino)tetralin hydrobromide (8-OH-DPAT HBr), *R*(−)-DOI hydrochloride, fluoxetine, WAY-100635 maleate were purchased from SIGMA, Italy.

Assays with anti-dopamine and anti-serotonin antibodies

Newly hatched larvae were cultured in 4-well plates (NUNC, Denmark) containing 300 μl of MFSW in which polyclonal AB122S rabbit anti-dopamine antibody (Chemicon) or the polyclonal human anti-5-HT antibody (Medac, Hamburg, Germany) were added. Three different dilutions of the antibody were tested: 1:50, 1:100 and 1:200. Control larvae were cultured in MFSW. For each experiment, 10 ± 2 larvae for each treatment in 5 replica were employed. The plates were kept at 20°C throughout the experiments. Rate of metamorphosis was scored as above. To test the penetration of the primary antibodies, treated larvae were fixed as previously described and processed for immunolocalization with the appropriate TRITC-conjugated secondary antibody.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the significance of differences in the metamorphosis rate. Tukey's post hoc test (significant at $P<0.05$) was used to identify specific effects. Prior to perform analysis of variance, normal frequency distribution of data and homogeneity of variance were tested. No significant deviation from the parametric assumption was found (normality, Kolmogorov–Smirnov test, all $P > 0.4$; homogeneity of variance, Levene's test, all $P > 0.06$).

In some cases (see Results), significant differences were found among controls of different batches, then in each experiment, rate of metamorphosis of treated larvae was compared to that of the same batch controls.

Results

Normal development of P. mammillata

Larvae of *P. mammillata* hatched 16 h after fertilization at 20°C and continued swimming for several hours. Newly hatched larvae had a round-shaped trunk with short conical papillae (Fig. 1A). Three hours after hatching, the larvae showed an elongated trunk and elevated adhesive papillae (Fig. 1B). Under laboratory conditions, larvae never started metamorphosis before 3 h after hatching. By this time, some larvae became attached to the Petri dishes using the adhesive papillae (about 80%) or to the superficial film of the water and started to metamorphose. The first evidence of metamorphosis was tail resorption (Fig. 1C).

Metamorphosis rate in control larvae is shown in Fig. 2. Values are means of the metamorphosis rate of all the experiments: between 7 hph and 9 hph, about 50% of the larvae started metamorphosis (7 hph: 39.8% ± 13.9; 9 hph: 55.2% ± 22.8). Differences in the metamorphosis rate among batches were not significant at 5 hph and 7 hph (ANOVA 5 hph: $F_{4,20} = 1.662$, $P = 0.198$; 7 hph: $F_{4,20} = 2.697$, $P = 0.060$). Nine hph differences were significant (ANOVA 9 hph: $F_{4,20} = 4.493$, $P = 0.009$). The observed variability in the metamorphosis rate was probably due to the natural variability among individuals, may be related also to the sampling season. Within 24 hph, 82.1% ± 7.6 of larvae had started metamorphosis; the other larvae continued swimming for 1–2 days and then they settled or died (Fig. 2). After completed tail resorption and adhesive papillae retraction, outgrowing ampullae became evident (Figs. 1D, E).

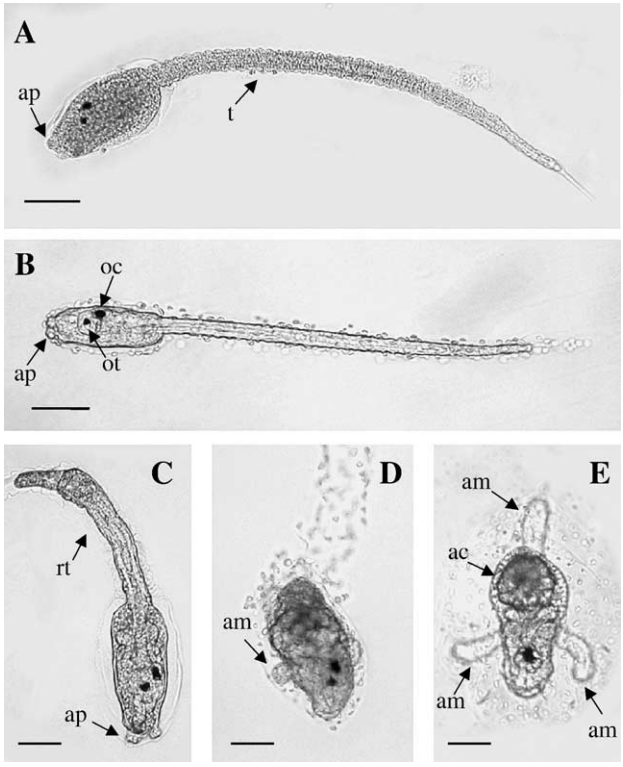


Fig. 1. Normal development of *P. mammillata* larvae. (A) Just hatched larva with a typical round-shaped trunk and conical short adhesive papillae (ap); the tail (t) is not completely stretched yet. (B) Three-hour-old larva with elongated adhesive papillae and a more extended trunk. It is possible to distinguish the ocellus (oc) and the otolith (ot). (C) Settled larva with partially retracted tail (rt). (D) Completed tail resorption: adhesive papillae are retracted, while ampullae (am), secondary organs of attachment, are outgrowing. (E) At this stage, the ampullae (am) are fully developed, and the axial complex (ac) is formed. Scale bar: 100 μm (A, B); 60 μm (C, D, E). ac: axial complex; am: ampullae; ap: adhesive papillae; oc: ocellus; ot: otolith; rt: retracted tail; t: tail.

Dopamine localization

In 1 hph larvae, dopamine-like immunoreactivity was localized in fibers extending along the neural tube in the tail and in numerous fibers in the visceral ganglion (Figs. 3A, B). Diffuse immunostaining was found in the adhesive papillae, the most anterior organs of the larva, which probably have a chemosensory function (Fig. 3B) (Groppelli et al., 2003; Takamura, 1998).

Dopamine-like immunoreactivity was also found in the bodies of three cells localized in the posterior left side of the sensory vesicle (Figs. 3C, D). Two fibers extended from these cells. Comparing 3-D reconstructions of 4 larvae obtained by confocal microscopy, it was possible to describe the pathways of these fibers: one projected in the anterior direction, running along the left wall of the sensory vesicle; the other one ran along the floor of the vesicle and contacted the stalk of the otolith (Figs. 3D, E). Another positive fiber ran in the right wall of the sensory vesicle, but it was not directly connected with the three dopaminergic neurons (Figs. 3D, E). In the same position, some cells with protrusions in the sensory vesicle were evident in histological sections (Figs. 3F, G): these cells may correspond to those described by Nicol and Meinertzhagen (1991), as presumed hydrostatic pressure receptors. They appeared as small protrusions, which evaginated from the left posterior wall into the vesicle cavity.

Effects of dopaminergic and serotonergic drugs on the onset of metamorphosis

In order to test the role of some neurotransmitters during metamorphosis, 2 h post hatching (hph) larvae were treated with different doses of dopamine and of 5-HT and with agonists and antagonists of dopaminergic and serotonergic receptors. Just hatched larvae did not show any response to the inducing drugs. Treatments (10 μM, 100 μM) with dopamine and with L-DOPA, a precursor of dopamine synthesis, had no effects on metamorphosis (data not shown), as reported by other authors (Degnan et al., 1997).

Exposure of 2-cell stage embryos to the action of α-methyl-L-tyrosine, an inhibitor of dopamine synthesis, caused a significant increase of metamorphosis rate at 5 hph. At 7 hph, more than 90% of treated larvae had started metamorphosis (Fig. 4).

Exposure of larvae to serotonin or to serotonin creatinine sulfate complex (10 μM, 100 μM) caused no effects on metamorphosis (data not shown).

Fluoxetine is a 5-HT reuptake inhibitor which increases extracellular levels of endogenous serotonin. Larvae exposed to 10 μM fluoxetine exhibited a higher rate of metamorphosis at 7 hph, 9 hph and at 11 hph (Table 2, Fig. 5). Treatments with 1 μM fluoxetine were ineffective.

The effect of agonists and antagonists of the neurotransmitter receptors was tested to understand better the role of dopamine and serotonin during metamorphosis. The substances were: lisuride, a D_2 agonist (Ferrari et al., 1992); *R*(+)-SCH-23390, a D_1 selective antagonist (Iorio et al., 1983); clozapine, a D_4 antagonist (Van Tol et al., 1991); 8-OH-DPAT HBr, a selective 5-HT_{1A} agonist; DOI, a 5-HT_2/

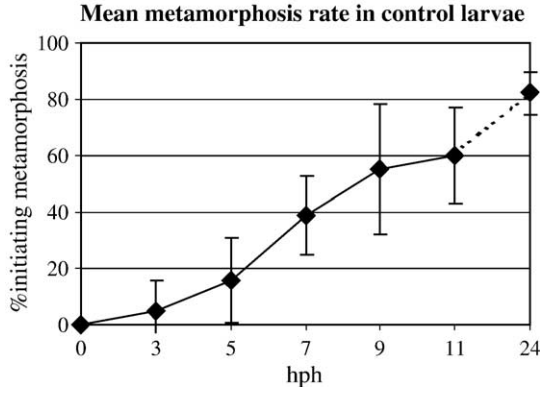


Fig. 2. Metamorphosis in control larvae. Mean rate of individuals initiating metamorphosis observed at different times after hatching. Data are means ± SD of the controls of all the different batches used in this work.

5-HT_{1C} agonist (Johnson et al., 1990); WAY-100635, a 5-HT_{1A} selective antagonist (Foster et al., 1995) (Tables 1, 2). All tested substances had toxic effects at the 100 μM dose: the larvae stopped swimming and died or started tail resorption but failed to complete metamorphosis. In larvae treated with lower concentrations, metamorphosis proceeded successfully. Proportion between number of metamorphosing larvae attached to the Petri dishes vs. number of larvae metamorphosing to the superficial film of the water was the same observed in controls.

Treatment of 2 hph larvae with 10 μM lisuride, a D_2 agonist, strongly delayed the onset of metamorphosis. The difference between treated and control larvae was significant 7 hph, 9 hph and 11 hph. The effect of lisuride was dose-dependent. Larvae exposed to the solvent alone (DMSO) metamorphosed at the same rate as controls (Table 1, Fig.

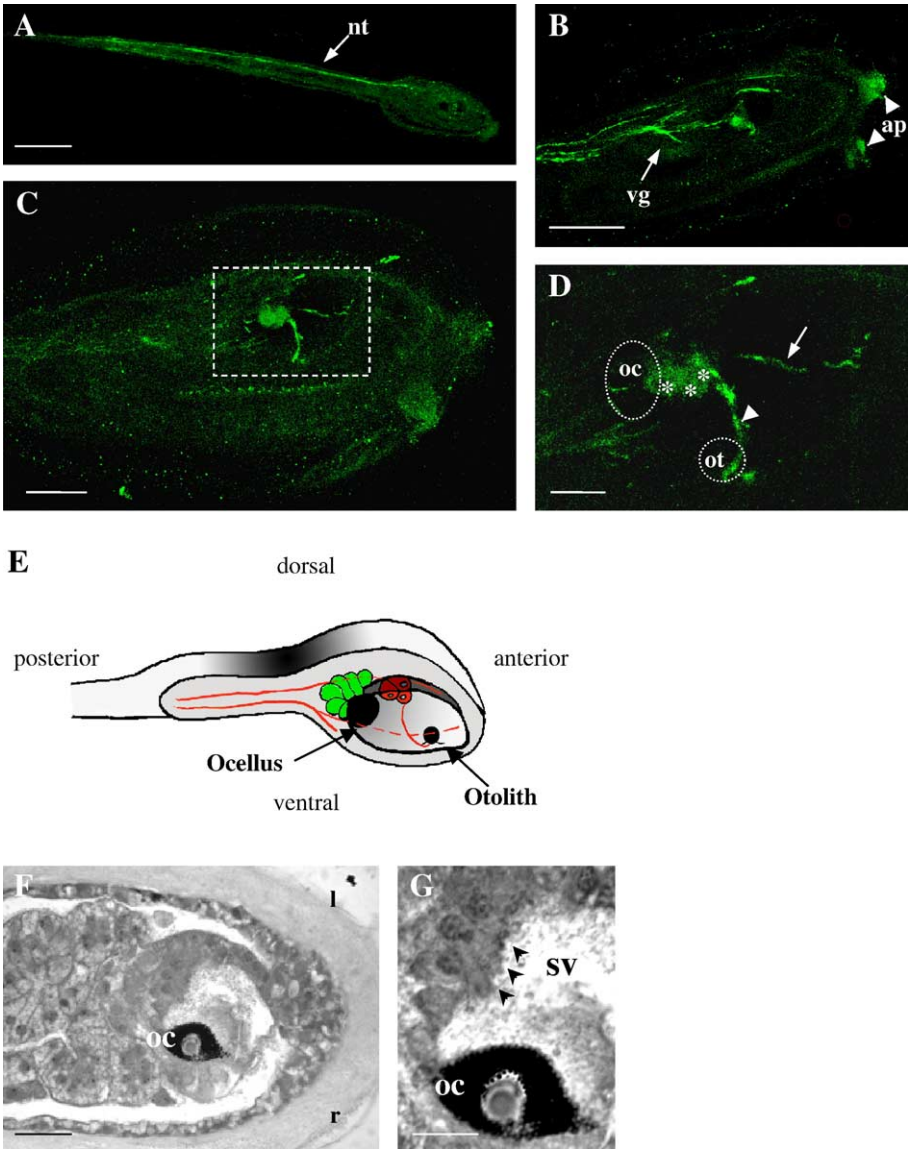
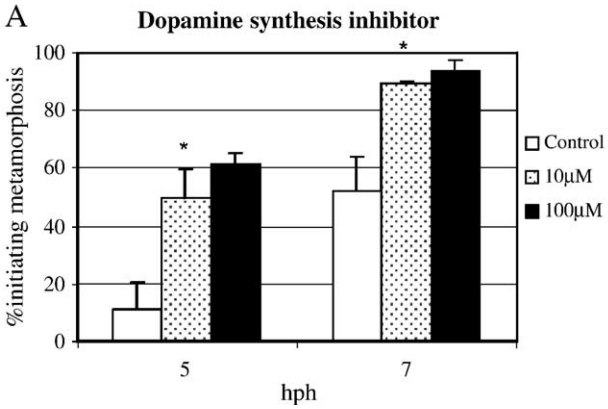


Fig. 3. Dopamine localization in *P. mammillata* larvae. (A) Dopamine (DA) localization in 1 h post hatching larvae. Dopamine immunoreactivity is present along the whole dorsal neural tube (nt) (arrow) (optical sections: 30, step size: 2.72 μm). (B) Immunopositive staining is particularly evident in the adhesive papillae (ap) (arrowheads) and in fibers in the central nervous system. Some fibers arise from the visceral ganglion (vg) (arrow) and are directed to the tail (optical sections: 50; step size: 1.63 μm). (C) Localization in the sensory vesicle indicated by the dotted square (optical sections: 50; step size: 1.63 μm). (D) Greater magnification of the area indicated by the dotted square in panel (C). Dopamine is present in 3 cells (asterisks) on the posterior left side of the sensory vesicle and in fibers connecting them to the otolith (ot), running in the sensory vesicle floor (arrowhead); another dopamine positive fiber projects in anterior direction, running in the right wall of the sensory vesicle (arrow). Dotted circles indicate the position of ocellus (oc) and otolith. (E) Schematic drawing of the sensory vesicle of *P. mammillata* larva after right wall ablation. Dopaminergic fibers and cells are in red (see text for details). The group of green cells surrounding the ocellus corresponds to serotonin immunopositive cells (Pennati et al., 2001). Relationships between dopamine and serotonin immunopositive cells and fibers are deduced from confocal microscopy images. (F) Frontal section of the sensory vesicle: the ocellus (oc) is on the right side of the vesicle (l: left, r: right). (G) Higher magnification of panel (F): arrowheads mark the presumptive dopamine positive cells which protrude from the left posterior wall into the cavity of the sensory vesicle (sv). Scale bar: (A) 100 μm, (B) 50 μm, (C) 25 μm, (D, G) 10 μm, (F) 20 μm.



B

	Merthyl-L-tyrosine Dopamine synthesis inhibitor	
hph	**5**	**7**
Controls	11.1 ± 9.6	52.1± 12.2
10µM	49.5 ± 10.1	89.2 ± 1.1
100µM	61.3 ± 4.2	94.0 ± 3.4
ANOVA test $F_{2,3}$ =	19.494	19.633
***P* =**	0.019	0.019
Post hoc tests		
Co *vs.* 10µM	0.04	0.03
Co *vs.* 100µM	0.019	0.021

Fig. 4. Effect of 2-cell embryos treatments with α-methyl-L-tyrosine, a dopamine synthesis inhibitor, on metamorphosis. (A) Larvae hatched in 10 µM and 100 µM of α-methyl-L-tyrosine solution showed a higher rate of metamorphosis than in controls. Data presented are means ± SD (statistical significance: $*P \leq 0.05$). (B) Mean percentage of larvae initiating metamorphosis ± SD corresponding to the graph, ANOVA test and post hoc tests values.

6). Features of treated larvae were the same as in control larvae.

Treatments with 10 µM *R*(+)-SCH-23390, a D_1 selective antagonist, significantly promoted the onset of metamorphosis. Percentage of treated larvae initiating metamorphosis was significantly different from that of control larvae both 9 hph and 11 hph. Treatments with 1 µM *R*(+)-SCH-23390 had no effects (Table 1). Similar results were obtained exposing the larvae to the action of 10 µM clozapine, a D_4 antagonist. Seven hours post hatching, more than 90% of treated larvae had started metamorphosis (Table 1, Fig. 6). Events related to tail resorption in *R*(+)-SCH-23390 and in clozapine-treated larvae were the same as those observed in control larvae.

Treatments with 10 µM DOI, a 5-HT_2/5-HT_{1C} agonist, promoted metamorphosis. At 7 hph, 9 hph and 11 hph, the percentage of larvae which had started tail resorption was significantly higher than percentage of control larvae. Treatments with the lower dose of DOI, 1 µM, had no effects (Table 2, Fig. 6). On the opposite, WAY-100635, a potent and selective 5-HT_{1A} antagonist, delayed the onset of metamorphosis: 7 hph, 9 hph and 11 hph percentage of 10 µM treated larvae was significantly lower than in controls. The lower dose of WAY-100635 tested had no significant effects (Table 2, Fig. 6).

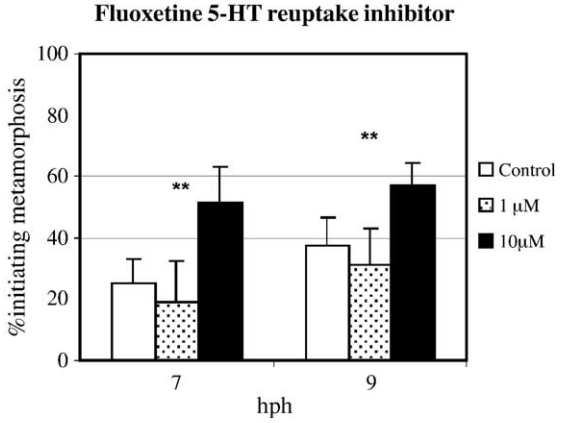


Fig. 5. Effect of fluoxetine, a 5-HT reuptake inhibitor, on metamorphosis. Fluoxetine increases extracellular levels of 5-HT promoting metamorphosis at the concentration of 10 µM. Data presented are means ± SD (statistical significance: $**P \leq 0.01$). Data reported in Table 2.

These results showed that metamorphosis was promoted by inhibiting dopamine synthesis or by exposing the larvae to dopamine antagonists or to serotonin agonists. On the contrary, metamorphosis was delayed by dopamine agonists and by serotonin antagonists.

Combined treatment with lisuride, a D_2 agonist, and WAY-100635, a 5-HT_{1A} antagonist, delayed the onset of metamorphosis to a similar extent to that obtained by treatments with the single substances. This demonstrated that the action of these drugs was not additive and that a complete block of metamorphosis could not be achieved (Fig. 7).

Effects of antibody incubation on the onset of metamorphosis

In order to confirm the role of dopamine and serotonin during the early metamorphosis of *P. mammillata*, 1 hph larvae were treated with antibodies which were effective in immunolocalizing these substances (dopamine: first part of this paper; serotonin: Pennati et al., 2001). We expected that these antibodies would bind and sequester dopamine and serotonin molecules diffused in the extracellular space. Thus, these treatments would lower the effective concentration of the free molecules playing neuromodulator or neurohormonal roles and would not affect the fraction of molecules at the synaptic level. Other authors (Eri et al., 1999) demonstrated that antibodies can efficiently diffuse through the tunic and epidermis of living larvae of the ascidian *H. curvata* and bind to specific proteins. To confirm this, larvae treated with the two primary antibodies were fixed and processed for immunolocalization using the

Table 1
Effect of dopaminergic substances on metamorphosis

Dopaminergic substances	Lisuride			*R*(+)-SCH-38393 hydrochloride			Clozapine		
	D_2 agonist			D_1 antagonist			$D_4 > D_3/D_2$ antagonist		
hph	7	9	11	7	9	11	7	9	11
Controls	44.1 ± 15.0	68.2 ± 21.8	75.5 ± 19.2	22.5 ± 10.7	33.6 ± 22.9	52.7 ± 17.2	44.1 ± 15.0	68.2 ± 21.8	75.5 ± 19.2
DMSO	37.9 ± 3.0	81.5 ± 8.2	92.4 ± 7.7	–	–	–	37.9 ± 3.0	81.5 ± 8.2	92.4 ± 7.7
1 μM	11.7 ± 9.1	25.9 ± 12.4	36.8 ± 10.5	20.4 ± 17.5	31.5 ± 24.5	36.3 ± 26.2	31.8 ± 26.5	70.1 ± 17.4	73.7 ± 13.6
10 μM	13.9 ± 8.6	15.9 ± 11.1	15.9 ± 11.1	41.4 ± 35.4	95.3 ± 7.0	100 ± 0	92.4 ± 7.3	94.0 ± 8.9	94.0 ± 8.9
ANOVA test F^* =	13.992	24.863	37.040	1.197	16.779	17.813	15.486	3.074	3.106
P =	0.0001	0.000003	0.0000002	0.336	0.0003	0.0003	0.0001	0.058	0.056
Post hoc tests									
Co vs. DMSO	ns	ns	ns	–	–	–	ns	ns	ns
Co vs.1 μM	0.001	0.001	0.001	ns	ns	ns	ns	ns	ns
Co vs. 10 μM	0.0005	0.0002	0.00001	ns	0.001	0.004	0.001	ns	ns

Lisuride and clozapine $F_{3,16}$; *R*(+)-SCH-38393 hydrochloride $F_{2,12}$.
ns: not significant.

secondary antibodies. Fig. 8 shows that anti-5-HT antibody administered to living larvae efficiently penetrated into the adhesive papillae, into the pharynx and in some sensory neurons of the tail.

Larvae of *P. mammillata* treated with anti-dopamine antibody, diluted 1:200, underwent a rapid metamorphosis compared to larvae maintained in MFSW only (Fig 9). Treatment of larvae with anti-5-HT antibody, diluted 1:100, delayed the onset of metamorphosis (Fig. 9). Gross morphological analysis of juveniles developed from individuals treated with the antibodies indicated that these antibodies did not affect normal metamorphosis (data not shown).

Discussion

It has been suggested that neurotransmitter signaling is an integral step in the signal pathway for metamorphosis of the ascidian *C. intestinalis* (Kimura et al., 2003). We investigated the role of two neurotransmitters, dopamine and serotonin, in regulating the onset of metamorphosis of

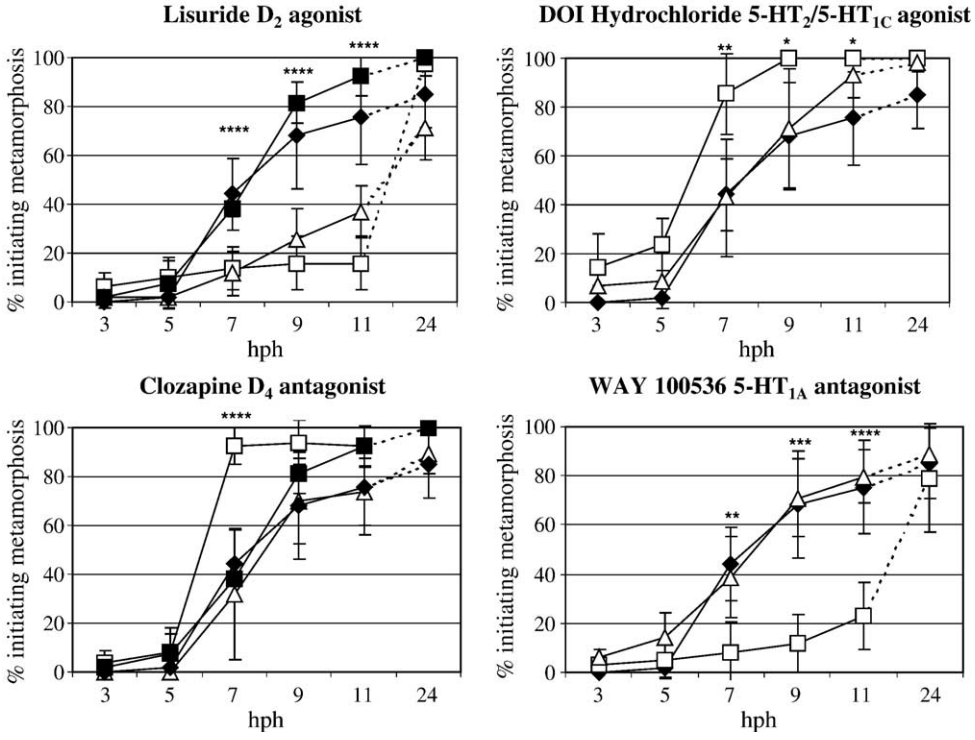


Fig. 6. Effect of dopamine and serotonin receptor agonists and antagonists on metamorphosis. Lisuride, D_2 agonist, delayed tail resorption in a dose-dependent way. Clozapine, D_4 antagonist, promoted tail resorption. DOI hydrochloride, 5-HT_2/5-HT_{1C} agonist, promoted tail retraction, while WAY-100635 maleate, 5-HT_{1A} antagonist, delayed it. Data presented are means ± SD (♦ control larvae, ■ DMSO, □ 10 μM treated larvae, △ 1 μM treated larvae (statistical significance: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$). Data reported in Tables 1, 2.

Table 2
Effect of serotonergic substances on metamorphosis

Serotonergic substances	8-OH-DPAT			*R*-(−)-DOI hydrochloride			WAY-100635 maleate			Fluoxetine		
	5-HT_{1A} agonist			5-HT_2/5-HT_{1C} agonist			5-HT_{1A} antagonist			5-HT reuptake inhibitor		
hph	7	9	11	7	9	11	7	9	11	7	9	11
Controls	22.5 ± 10.7	33.6 ± 22.9	52.7 ± 17.2	44.1 ± 15.0	68.2 ± 21.8	75.5 ± 19.2	44.1 ± 15.0	68.2 ± 21.8	75.5 ± 19.2	25.3 ± 8.0	37.3 ± 9.1	51.6 ± 6.4
1 μM	14.0 ± 19.5	19.6 ± 21.3	31.1 ± 26.2	43.1 ± 24.0	71.4 ± 24.6	93.3 ± 9.6	38.9 ± 16.3	70.1 ± 15.8	79.7 ± 10.8	19.0 ± 13.8	31.1 ± 11.9	50.2 ± 6.4
10 μM	42.5 ± 29.4	84.9 ± 14.6	94.4 ± 8.2	85.5 ± 16.5	100 ± 0	100 ± 0	8.3 ± 12.3	11.6 ± 11.7	23.0 ± 13.7	51.4 ± 12.0	56.9 ± 7.5	91.0 ± 13.1
ANOVA test												
$F_{2,12}$ =	2.355	14.951	14.793	8.167	4.272	5.227	8.721	19.485	22.268	11.203	9.665	31.766
P =	0.137	0.001	0.001	0.006	0.040	0.023	0.005	0.0002	0.0001	0.002	0.003	0.00002
Post hoc tests												
Co vs. 1 μM	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Co vs. 10 μM	ns	0.004	0.011	0.012	0.051	0.022	0.006	0.001	0.003	0.01	0.019	0.0001

ns: not significant.

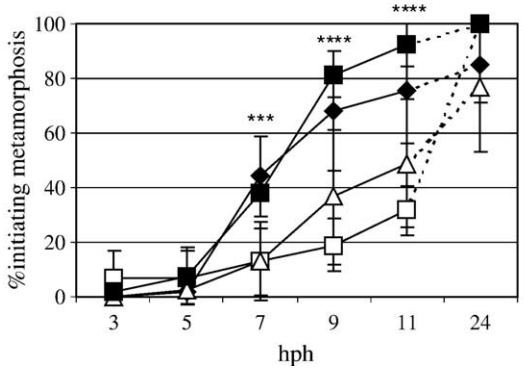
P. mammillata larvae. Our results showed that inhibition of dopamine signaling promoted metamorphosis, while inhibition of serotonin signaling delayed the onset of this process. Dopamine depletion was obtained by three methods: inhibiting dopamine synthesis, treating the larvae with dopamine receptor antagonists and sequestering the substance by a specific antibody. All these approaches gave similar results, promoting metamorphosis in a high percentage of treated larvae. Instead, the dopamine agonist had an inhibitory effect on metamorphosis. These results suggest that dopamine signaling may promote the swimming behavior of the larvae, delaying the onset of metamorphosis.

Serotonergic signaling seems to play an opposite role: serotonin depletion, by means of antagonists and by antibody incubation, delayed tail resorption and the onset of metamorphosis. Instead, serotonin agonists had a stimulating effect on metamorphosis. Therefore, it is likely that 5-HT plays a role in the mechanism triggering metamorphosis in *P. mammillata* larvae. Moreover, since two serotonergic substances which had strong effects, WAY-100635 and 8-OH-DPAT HBr, are both selective for 5-HT_{1A} receptor, probably the serotonin receptor controlling metamorphosis in ascidians belongs to this subtype.

Then, dopamine and serotonin showed to have an opposite effect on metamorphosis. This is consistent with the results obtained by Yamamoto et al. (1999) in cypris larvae of the barnacle *Balanus amphitrite.* These authors found that dopamine agonists inhibited larval attachment in a dose-dependent way, while serotonin promoted attachment and metamorphosis, although the receptor type could not be specified.

Kimura et al. (2003) reported that, in larvae of *Ciona savignyi*, metamorphosis is triggered by interactions between noradrenaline or adrenaline and β_1 adrenergic receptors. The same authors reported that treatment of *C. savignyi* larvae with other neurotransmitters such as serotonin, dopamine, DOPA, thyroxine, acetylcholine, octopamine, GABA and glutamate did not promote tail resorption. These findings are not in contrast with our results. We also did not observe any effect after treatments with the neurotransmitters, dopamine and serotonin, perhaps because catecholamines and serotonin are easily oxidized in sea water (Pires and Hadfield, 1991). Moreover, noradrenaline and adrenaline are dopamine metabolites, and it could be that an increase of these substances concentration coincides with a lowering of the dopamine endogenous level, which is compatible with the mechanism of metamorphosis triggering proposed in this paper. Anyway, it is possible that, even among ascidians, the mechanism of metamorphosis is regulated by different neurotransmitters, and that the same molecule has an inducing activity in one species and an inhibitory activity in a different one. Among the molluscs, for instance, elevated levels of endogenous serotonin activate metamorphosis in the prosobranch gastropod *Ilyanassa obsoleta* (Couper and Leise, 1996), while in the oyster of the genus *Crassostrea* (Bonar et al., 1990) and

A



B

	Lisuride D2 agonist - WAY-100635 maleate 5-HT$_{1A}$ antagonist		
hph	**7**	**9**	**11**
Controls	44.1±15.0	68.2±21.8	75.5±19.2
DMSO	37.9±3.0*	81.5±8.2*	92.4±7.7*
1µM	13.1 ± 14.5	36.7 ± 24.7	48.9 ± 23.5
10µM	12.9 ± 12.3	19.0 ± 9.5	31.7 ± 9.0
ANOVA test $F_{3,16}$ =	8.960	13.213	13.780
***P* =**	0.001	0.0001	0.0001
Post hoc tests			
Co *vs.* DMSO	ns	ns	ns
Co *vs.* 1µM	0.005	0.054	ns
Co *vs.* 10µM	0.005	0.002	0.003

Fig. 7. (A) Effect on metamorphosis of the combined action of lisuride, a D_2 agonist, and WAY-100635 maleate, 5-HT_{1A} antagonist. Data presented are means ± SD (♦ control larvae, ■ DMSO, □ 10 µM treated larvae, △ 1 µM treated larvae. Statistical significance: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$). (B) Mean percentage of larvae initiating metamorphosis ± SD corresponding to the graphic, ANOVA test and post hoc tests values.

in the nudibranch *Phestilla sibogae* (Pires et al., 1997), metamorphosis is regulated by catecholamines, such as dopamine and norepinephrine.

Further evidence of the role of neurotransmitters comes from the localization of dopamine in larvae of *P. mammillata*. By means of confocal laser scanning microscopy (CLSM), it was possible to detect dopamine in the cell bodies of three cells of the sensory vesicle of *P. mammillata* larvae. These cells may correspond to the presumed hydrostatic pressure reception organ, described by Nicol and Meinertzhagen (1991). A dopamine-like immunoreactive fiber connects these cells with the stalk of the otolith, the gravity sensing organ. Thus, it is possible to assume that the synthesis of dopamine may be related to stimuli coming from this sensory organ. The localization of dopamine is consistent with the localization of catecholamines described by Kimura et al. (2003), using glyoxylate-induced fluorescence. These authors reported that the existence of catecholamines was always detected around the brain vesicle of *C. savignyi* larvae, even if the technique did not allow a more precise localization.

In *P. mammillata* larvae, another neuroamine, 5-HT, was localized in cells surrounding the ocellus, the photoreceptor organ (Pennati et al., 2001), thus serotonin synthesis may be activated in response to light stimuli. Serotonin is present also in primary neurons of the adhesive papillae. These organs, situated at the anterior tip of the trunk, enable the larva to attach to the substrate by secreting mucus. The structure of adhesive papillae was described in many species (Gianguzza and Dolcemascolo, 1994; Gianguzza et al., 1999; Takamura, 1998; Torrence and Cloney, 1983). In *P. mammillata* larva, each papilla contains two primary sensory neurons bearing a single cilium at the cell apex and an axonal prolongation (Groppelli et al., 2001; Pennati et al., 2003). During the exploratory period, the adhesive papillae play a role in substrate choice and in the settlement of the larva, thus they were supposed to have a chemosensory function (Hirai, 1964; Kimura et al., 2003).

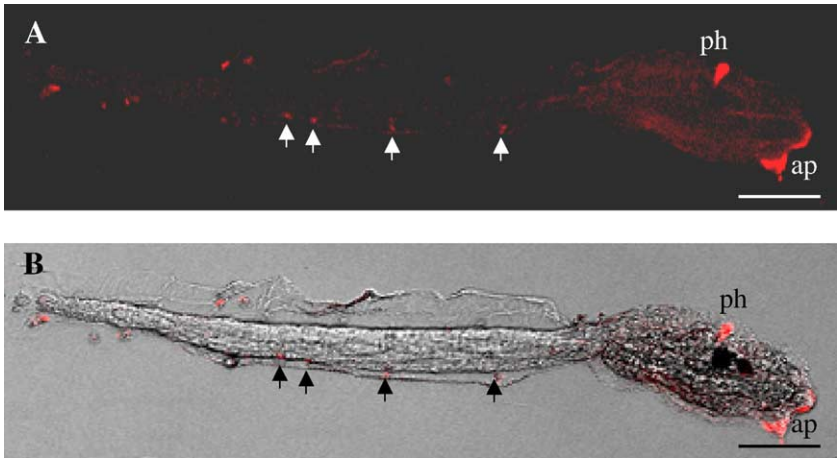
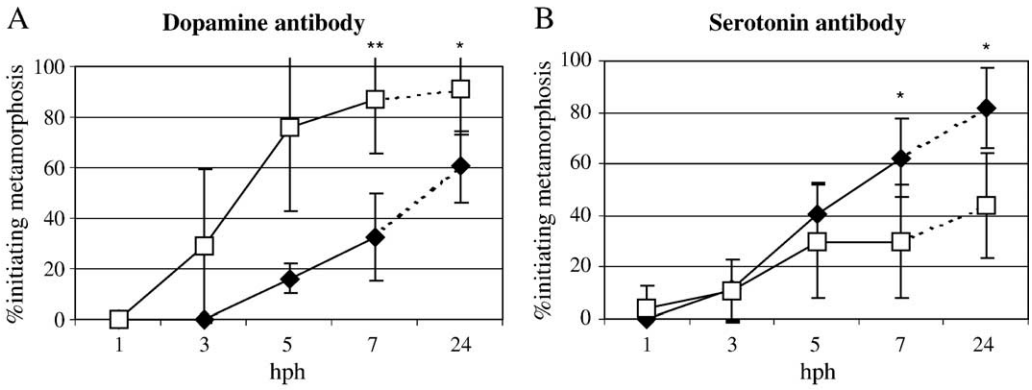


Fig. 8. Immunofluorescence localization of anti-serotonin antibody in larvae after in vivo treatment with the same antibody. The antibody penetrated into the adhesive papillae (ap) and into the pharynx (ph). A faint fluorescent signal is present also in some primary sensory neurons (arrows) of the tail. (A) Confocal microscope image obtained merging 10 optical sections (step size: 1.17 µm). (B) Superimposition of fluorescence image and light transmission image. Scale bar = 100 µm.



C

	Dopamine antibody 1:200		Serotonin antibody 1:100	
hph	7	24	7	24
Controls	32.3 ± 17.1	60.5 ± 14.2	62.4 ± 15.0	81.6 ± 15.7
Ab	86.9 ± 21.6	91.1 ± 17.8	30.0 ± 21.7	43.9 ± 20.4
ANOVA test $F_{1,8}$ =	17.073	7.854	7.566	10.655
P =	0.003	0.023	0.025	0.011

Fig. 9. Effect on metamorphosis of treatments with anti-dopamine and anti-serotonin antibodies. (A) Anti-dopamine antibody, diluted 1:200, promoted metamorphosis. (B) Anti-serotonin antibody, diluted 1:100, delayed it. Data presented are means ± SD. (♦ control larvae, □ antibody treated larvae. Statistical significance: *$P \leq 0.05$; **$P \leq 0.01$). (C) Mean percentage of larvae initiating metamorphosis ± SD corresponding to the graph, ANOVA test and post hoc tests values.

It is possible to propose the following mechanism to explain how dopamine and serotonin can modulate metamorphosis acting both in the central and in the peripheral nervous system of the larva. In young larvae, dopamine may promote the swimming activity in response to gravity: in fact, ascidian larvae are negatively geotactic as soon as they hatch and they swim upward towards the light (Svane and Young, 1989). Then, the light may stimulate the synthesis of serotonin. High levels of 5-HT in the central nervous system may cause the switch from negative geotaxis-positive phototaxis, typical of newly hatched larvae, to positive geotaxis-negative phototaxis, typical of pre-settlement ones. Next, when the larva comes in contact with the environmental cue, by the papillary primary sensory neurons, serotonin may be released by these neurons and may act as neuromodulator to stimulate the signal transduction pathway leading to tail resorption.

In the proposed mechanism, dopamine could contribute to the modulation of the onset of metamorphosis, controlling the duration of the early swimming phase of the larva. In contrast, serotonin is supposed to act in two different stages of larval life and through different compartments of the nervous system: it could control negative phototactic-positive geotactic swimming behavior during the dispersal phase acting on the central nervous system, and it could promote metamorphosis upon contact with the external cue, acting in the peripheral nervous system in pre-settlement phase.

Our results indicated that dopamine and serotonin are involved in ascidian metamorphosis: their combined action may modulate the beginning of this process. Moreover, serotonin may act as an intermediary between the inducing external stimuli and the internal signaling pathway which leads to the morphogenetic events of metamorphosis.

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