Apical sensory neurones mediate developmental retardation induced by conspecific environmental stimuli in freshwater pulmonate snails

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Summary

Freshwater pond snails *Helisoma trivolvis* and *Lymnaea stagnalis* undergo larval development and metamorphosis inside egg capsules. We report that their development is permanently under slight tonic inhibitory influence of the anterior sensory monoaminergic neurones, which are the remnants of the apical sensory organ. Conspecific juvenile snails, when reared under conditions of starvation and crowding, release chemical signals that are detected by these neurones in encapsulated larvae and reversibly suppress larval development, thus providing a link between environmental signals and developmental regulation. Induced retardation starts from the trochophore stage and results in up to twofold prolongation of the larval lifespan. Upon stimulation with the signal, the neurones increase synthesis and release of monoamines [serotonin (5-HT) in *Helisoma* and dopamine in *Lymnaea*] that inhibit larval development acting via ergometrine-sensitive internal receptors. Thus, the novel regulatory mechanism in larval development of molluscs is suggested and compared with the phenomenon of dauer larvae formation in the nematode *Caenorhabditis elegans*.

Key words: Mollusc, Conditioned water, Apical sensory neurones, Larval development, Serotonin, Dopamine, Ergometrine-sensitive receptor

Introduction

The great majority of benthic animals have a complex life cycle with a larval stage prerequisite for juveniles and adults. Duration of larval period varies significantly and in any one species depends upon multiple environmental factors, i.e. temperature, salinity and nutrition regimes (see Miller and Hadfield, 1990; Pechenik et al., 1996; Pechenik et al., 2002a; Pechenik et al., 2003). Far less is known about developmental roles of conspecific signalling. Perhaps the only elaborated model deals with induction of larval settlement and metamorphosis by adults (see Burke, 1984; Burke, 1986; Lambert and Todd, 1994). No data on developmental roles of conspecific signalling at early larval stages exist in literature.

Freshwater pond snails can be a convenient tool with which to address this question. In contrast to marine invertebrates that mainly have free-swimming larvae, they develop inside transparent egg capsules and, hence, are well-suited for in vivo experimental studies of embryonic and larval development (Koss et al., 2003). Thus, in *Helisoma trivolvis* and *Lymnaea stagnalis*, various aspects of neuronal development and larval physiology have been studied (Goldberg and Kater, 1989; Goldberg et al., 1994; Diefenbach et al., 1991; Diefenbach et al., 1995; Croll and Voronezhskaya, 1996; Croll, 2000; Voronezhskaya and Elekes, 2003).

The development of both species is very similar, and provides several advantages. Adult snails lay transparent egg masses, and each egg contains a single embryo. Development of all embryos in each egg mass is highly synchronous. Embryos of both species pass the same larval stages as free-swimming larvae of marine molluscs (blastula-gastrula-trochophore-veliger), undergo metamorphosis inside egg capsules and hatch as miniature juvenile snails. Throughout the intracapsular development, they have a limited number of easily identifiable neurones (reviewed by Croll, 2000). At the trochophore and veliger stages, only one pair of sensory neurones has been described (Diefenbach et al., 1998; Voronezhskaya et al., 1999). These neurones differ in their transmitter content: they express serotonin (5-HT) in *Helisoma* (Goldberg and Kater, 1989; Diefenbach et al., 1991; Diefenbach et al., 1995; Diefenbach et al., 1998; Voronezhskaya and Elekes, 1993; Koss et al., 2003), and dopamine (DA) and FMRFamide-related peptides in *Lymnaea* (Croll and Voronezhskaya, 1996; Voronezhskaya et al., 1999; Voronezhskaya and Elekes, 2003). Until the stage of late veliger these cells are the only monoaminergic neurones in both species.

Important role of monoamines (catecholamines and 5-HT) in development is well known. In larval molluscs, they appear at the pre-nervous stages (Buznikov et al., 2003), and are expressed in the earliest larval neurones including the first nerve centre, the apical sensory organ (see Page and Parries, 2000; Croll at al., 2003). Monoamines are known to mediate

larval ciliary beat frequency (Kuang and Goldberg, 2001), settlement and metamorphosis (Leise at al., 2001; Pechenik et al., 2002b). Thus, it is reasonable to suggest that the first monoaminergic sensory neurones may be also involved in developmental regulation.

The goal of the present study was to test the hypothesis that conspecific signalling regulating development exists at the early larval stages of freshwater pulmonate snails, and the first monoaminergic anterior sensory neurones are involved in mediating this signals. We report below that juveniles of *Helisoma* and *Lymnaea* reared under conditions of starvation and crowding, release water-born cues, which conspecifically retard larval development starting from the trochophore stage. To test whether the anterior monoaminergic neurones mediate transduction of the cues released by juveniles, we pharmacologically emulated changes in their activity. First, endogenous monoamine level was augmented by incubation in its immediate biochemical precursor (Sakharov, 1991; Diefenbach et al., 1995). Conversely, the action of 5-HT and DA was reduced by treating the embryos with commonly used aromatic amino acid decarboxylase inhibitor 3-hydroxybenzylhydrazine (Treseder et al., 2003), specific inhibitor of tryptophan 5-hydroxylase activity L-*p*-chlorophenylalanine (Baker et al., 1993; Diefenbach et al., 1995; Pani and Croll, 1998), methylated analogues of respective monoamines (Sloley and Orikasa, 1988) and receptor antagonists (Goldberg et al., 1994; Pavlova, 2001). Developmental changes were correlated with changes in anti-5-HT immunofluorescence in *Helisoma*, and glyoxylate-induced fluorescence of dopamine in *Lymnaea* within the first anterior neurones. Our data indicate that anterior monoaminergic sensory neurones detect the cues released by juveniles in conditions of food limitation and overcrowding, and increase synthesis of the respective monoamine, which retards larval development. This inhibitory effect is carried out via ergometrine-sensitive receptors. This work has appeared previously in abstract form (Voronezhskaya and Khabarova, 2003).

Materials and methods

Animals and staging

Egg masses of *H. trivolvis* and *L. stagnalis* were collected from inbred colonies at the Institute of Developmental Biology, Moscow. Snails and embryos were maintained at 23-25°C. Snails were raised in glass aquaria (10-40 L) with a 12/12 hours light/dark cycle. Adults and juveniles were fed on lettuce. Freshly laid egg masses were collected daily and transferred to 90 mm Petri dishes (Falcon) containing filtered pond water (FPW). Embryonic development was staged on the basis of a specific set of morphological, morphometric and behavioural features according to Morrill (Morrill, 1982) and Mescheriakov (Mescheriakov, 1990). At the trochophore stage (stage 19; 20% of embryonic development), embryos were transferred to 35 mm Petri dishes with 2 ml FPW (20-25 eggs per dish) for subsequent drug incubation. In case of *Helisoma*, intact or cut into two-three parts egg masses were used. For *Lymnaea*, egg capsules were isolated from egg masses unless other specified. Isolation of egg capsules purposed to uniform the exchange of substances with the environment, and avoid uncontrolled adsorption and oxidation of the drugs within egg mass jelly.

Conditioned water

For conditioning, 200 juvenile snails (length 1.5-2 mm) deprived of food for 24 hours were rinsed in several changes of FPW, put in a glass jar in 10 ml of FPW, and kept without food for 12 hours. The snails that climbed above the water surface were returned to the bath with a soft brush. Then, conditioned water (CW) was pipetted out, filtered through a 0.2 μm Millipore filter and used immediately. Juveniles were transferred for the next 12 hours to 1 l glass tanks and fed with lettuce. Then, the cycle repeated (not more then five times for each group of juveniles). Water was also conditioned by juveniles continuously fed on lettuce or fish chew (i.e. without preliminary food deprivation). During conditioning, mortality did not differ from controls (2-3%). To test the stability of CW, it was heated to 80°C for 10 minutes, boiled for 10 minutes, and frozen for up to one month at –20°C. Developing eggs were also reared in FPW boiled for 20 minutes to reduce oxygen content. Whenever water was heated or frozen, it was adjusted to 23°C before larvae were added.

Drug incubations

The following drugs were used (all from Sigma-RBI, USA, unless other specified): serotonin (5-hydroxytryptamine creatine sulphate, 5-HT); dopamine (DA); 5-hydroxy-L-tryptophan (5-HTP, metabolic precursor of 5-HT); L-3,4-dihydroxyphenylalanine (L-DOPA, metabolic precursor of dopamine); α-methyl-tryptophan and α-methyl-DOPA (α-m-T and α-m-DOPA, methylated analogues of the precursors); 3-hydroxybenzilhydrazine (NSD-1015); L-*p*-chlorophenylalanine (PCPA); ergometrine maleate (EM, antagonist of dopamine receptors, Serva, USA); sulpiride; spiperone hydrochloride (antagonists of DA and 5-HT receptors); mianserin hydrochloride; cyproheptadine hydrochloride; ritanserin; and ketanserin tartrate (antagonists of 5-HT receptors). NSD-1015 and PCPA were applied 4 hours prior to the first application of CW or monoamine precursors. Receptor antagonists were applied simultaneously. All drug solutions were prepared immediately before use. Sulpiride was first prepared as 1 mM solution in 40% ethanol. Ascorbic acid (50 μM) was added to all solutions to avoid oxidative breakdown. Equal amounts of ascorbic acid and ethanol (in case of experiments with sulpiride) were added to controls, and were shown to have no effect on the development. Embryos were kept in darkness and solutions were changed daily.

Cell visualization

Immunochemical procedures have been described in detail elsewhere (Voronezhskaya et al., 1999). In brief, embryos were fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 4 hours at 10°C, and washed in 0.1 M PB. For double immunolabelling, the specimens were incubated in a mixture of anti-5-HT (DiaSorin, USA, #20080, polyclonal, rabbit, diluted 1:3000) and anti-acetylated α-tubulin antibody (Sigma, T-6793, monoclonal, mouse, diluted 1:1500) in PB with 10% normal goat serum, 0.25% bovine serum albumin, 1% Triton X-100 (TX) and 0.03% sodium azide for 72 hours at 10°C. The specimens were washed in PB three times for 20 minutes, incubated in a mixture of goat-anti-rabbit Alexa 488-conjugated IgG and goat-anti-mouse Alexa 546-conjugated IgG (Molecular Probes, USA), both diluted 1:800 in PB-TX, for 12 hours at 10°C. Alternatively, the specimens were sequentially incubated in anti-TH antibodies (DiaSorin, #22941, monoclonal, mouse, diluted 1:3000) for 72 hours at 10°C, goat-anti-mouse Alexa 488-conjugated IgG (Molecular Probes) for 12 hours at 10°C, anti-acetylated α-tubulin antibody diluted 1:3000 in PB-TX, for 4 hours at 10°C, and goat-anti-mouse Alexa 546-conjugated IgG for 2 hours at 10°C. The specimens were washed in PB, immersed in 50% glycerol in PB and mounted on glass slides in 80% glycerol in PB.

The specimens were examined as wholemounts with an LSM-510 confocal laser-scanning microscope (Carl Zeiss, Germany) with appropriate wavelength-filter configuration settings. For illustrations, series of optical sections were projected into one image with greater focal depth using LSM-510 software, and imported into Photoshop 7 (Adobe, USA) where only brightness and contrast were adjusted if necessary. The number and step size of optical sections are given for each image in the legends.

The specificity of the ABs used in our experiments has been shown for various molluscan larvae (see Croll, 2000). Controls included replacement of the primary ABs with non-immune serum. No specific staining was observed in control preparations. Reverse of the colours of the secondary ABs (anti-rabbit Alexa 546 IgG and anti-mouse Alexa 488 IgG) gave identical results.

For glyoxylic acid reaction (De la Torre, 1980; Voronezhskaya et al., 1999), embryos were removed from egg capsules and immersed in a freshly prepared, buffered glyoxylic acid-sucrose solution (500 mM sodium glyoxylate, 150 mM sucrose, 50 mM Tris buffer, pH 7.4) on glass slides at 4°C. After 60 minutes of incubation, the solution was removed, and the embryos were air-dried at room temperature for 30 minutes. Preparations were then heated to 60°C for 30 minutes, embedded in paraffin oil, and examined and imaged by using Jenaval (Zeiss) microscope equipped for ultraviolet epifluorescence (D filter block) and a CCD camera.

Measurements and statistics

The embryos were imaged daily at approximately the same time (from 9 to 12 AM) with a CCD camera attached to a stereomicroscope MBS-10 (L-ZOS, Russia), and their maximal length was measured with Photoshop 7. To measure brightness of fluorescence after glyoxylic acid reaction, the cells were imaged with constant exposure time, and averaged brightness of cell bodies was measured with Photoshop 7 using the Histogram Tool. Measurements of confocal images were carried out using LSM-510 software. Statistical analysis and graph plotting were carried out using Statistica 6 (StatSoft, USA) and Grapher 3 (Golden Software, USA). Results were expressed as means±standard deviation (s.d.). The significance of differences among groups was evaluated using Student's *t*-test. Differences were considered significant at $P<0.05$.

Results

Normal development

Embryonic and larval development of *Lymnaea* and *Helisoma* has already been described in detail (Raven, 1966; Morrill, 1982; Mescheriakov, 1990; Diefenbach et al., 1991; Marois and Croll, 1991; Voronezhskaya et al., 1999). In both species, the cleavage results in formation of a ciliated trochophore larva (stage 19; 20% of development), which rotates in the egg fluid. By the stage 22 (33-35% of development), the trochophore develops into a veliger and the rotary movement is supplemented by a forward swimming movement. By the stage 27 (82%), when the foot is functionally developed, swimming movements cease and the embryo attaches to the inner surface of the egg capsule and starts to creep along it. This corresponds to the settlement and metamorphosis of free-swimming larvae. In 2-3 days, the juvenile snail rasps a hole through the egg capsule and hatches. Each developmental stage is determined by a specific set of characters: the degree of shell, tentacles and foot development, position of the heart, eye pigmentation, foot and tentacles pigmentation, frequency of rotation, buccal mass activity, pneumostome opening/close ratio and gliding locomotion (Morrill, 1982; Mescheriakov, 1990; Diefenbach et al., 1991; Voronezhskaya et al., 1999). The increase in length of embryos precisely corresponds to successive developmental stages (Fig. 1) therefore below we express staging as length of embryos.

Embryos from each single egg mass develop synchronously though the timing between different egg masses may insignificantly vary. At the mean temperature 24±1°C, the duration of development from the trochophore (stage 19) until the adult-like form (stage 27) was 6.6±0.9 days for *Helisoma* (n=990) and 6.8±1.1 days for *Lymnaea* (n=1324). No differences from controls were observed during larval development in boiled FPW (n=193).

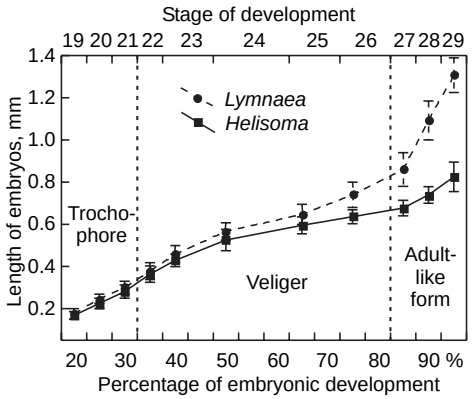

Fig. 1. Normal larval development of *Helisoma trivolvis* (n=215) and *Lymnaea stagnalis* (n=340) from the trochophore stage till the adult-like form showing interrelations between timing of embryogenesis in percents (lower *x*-axis), developmental stages after Mescheriakov (Mescheriakov, 1990) (upper *x*-axis), and the length of embryos (*y*-axis).

Effect of conditioned water

Incubation of embryos of *Helisoma* (n=122) and *Lymnaea* (n=180) in water conditioned by conspecific juveniles (CW) induced developmental retardation, which was expressed as elongation of each developmental stage (Fig. 2A,B). This retardation manifested itself starting from the trochophore stage 19 (20% of embryonic development), and followed the appearance of the first anterior sensory neurones. As a consequence of this retardation, at the time when control animals completed metamorphosis and became miniature adult-like snails (stage 28, Fig. 2C,E), the treated animals were at the veliger stage (stage 23) well before metamorphosis (Fig. 2D,F). The overall effect of CW resulted in up to twofold prolongation of the larval lifespan (see Fig. 7). No differences in the action of CW were detected after incubation of intact or cut egg masses (n=201 and 215 embryos, respectively) of *Helisoma*, and intact egg cocoons or isolated egg capsules (n=284 and 189 embryos, respectively) of *Lymnaea* (data not shown).

The effect of CW was reversible, and the tempo of development was restored when CW was replaced with FPW (Fig. 2A,B). Freezing to –20°C for up to one month did not change the activity of CW (n=71), though heating to 80°C for 10 minutes (n=74) completely abolished it (data not shown). Water similarly conditioned by fed juveniles (n=148) showed no effect on development (Fig. 2A,B and Fig. 7). Water after long term pre-incubation of juvenile snails (100 snails per 1 l for 1 month, fed on lettuce; n=160) was also ineffective.

The role of monoamines in developmental retardation

To test the hypothesis that the retarding effect of CW is mediated by the pair of monoaminergic anterior sensory neurones, we pharmacologically emulated changes in their

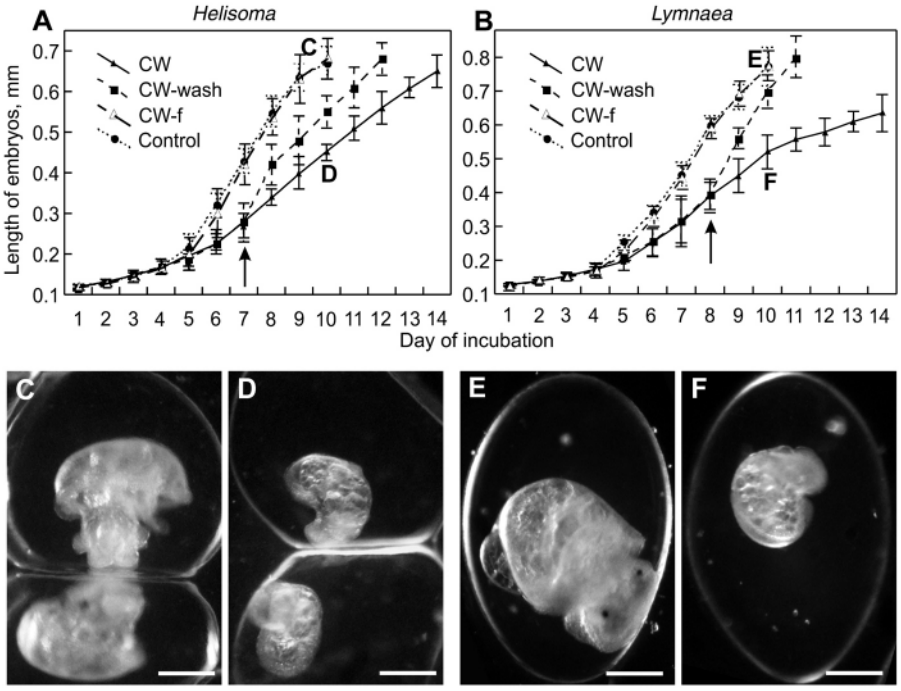

Fig. 2. Effects of conditioned water (CW) on larval development of *Helisoma* (A,C,D) and *Lymnaea* (B,E,F). (A,B) Incubation in CW induced developmental retardation in both *Helisoma* and *Lymnaea*. The effect disappeared after washout (CW-wash; the beginning of washout is indicated by arrows). Water conditioned by juveniles raised with plentiful food (CW-f) was ineffective. At the time when control animals developed into postmetamorphic adult like forms (C,E), the animals in CW were still at the veliger stage (D,F). Scale bars: 100 μm.

activity. As the developmental effect of CW manifested itself starting from the stage 19 (20% of embryonic development) only, all subsequent drug applications started from this stage. Incubation in solutions of monoamine precursors inhibited larval development in a species-specific manner. Thus, in *Helisoma*, the precursor of 5-HT, 5-HTP (1 mM) retarded the development similar to CW (*n*=96), while the precursor of DA, L-DOPA, was ineffective (*n*=205; tested up to 10 mM) (Fig. 3A). By contrast, in *Lymnaea*, the retardation was induced by 1 mM L-DOPA (*n*=128), while 5-HTP (*n*=217; tested up to 10 mM) insignificantly slowed the development only at the time when embryos reached metamorphic stages 25-27 (65-85%) (Fig. 3B). The effects of monoamine precursors were reversible and ceased after washout (*n*=102) (Fig. 3C,D). The overall effect of respective monoamine precursors was similar to that of CW (Fig. 7).

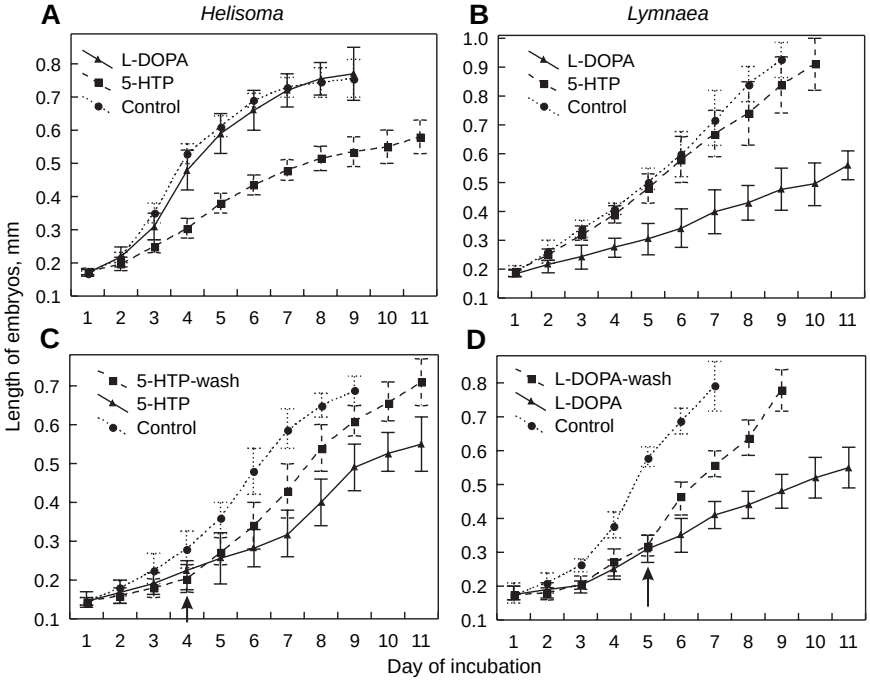

Fig. 3. Effects of monoamine precursors on larval development. (A) In *Helisoma*, 5-HTP (1 mM) retarded the development similar to CW, while L-DOPA (10 mM) was ineffective. (B) In *Lymnaea*, L-DOPA (1 mM) retarded the development, while 5-HTP (10 mM) insignificantly slowed the development at the late stages only. (C,D) In both species, the effect of the precursors disappeared after washout (the beginning of washout is indicated by arrows).

The inhibitor of monoamine synthesis, NSD-1015 in concentration of 10-15 μM did not affect the development (*n*=79) but significantly attenuated the effects of CW in *Helisoma* (*n*=112) and *Lymnaea* (*n*=94), as well as 5-HTP (*n*=120) in *Helisoma*, and L-DOPA (*n*=107) in *Lymnaea* (Figs 4, 7). The selective inhibitor of 5-HT synthesis, PCPA in 5 μM concentration accelerated normal development of *Helisoma* (*n*=83) and suppressed the inhibitory effect of CW (*n*=98) (Fig. 5A,B; Fig. 7). In *Lymnaea*, PCPA affected neither normal development nor the CW induced retardation (Fig. 7).

The antagonist of monoamine receptors, ergometrine, at a concentration of up to 50 μM, did not affect normal development of *Helisoma* (*n*=196; Fig. 7) but attenuated the effects of CW (*n*=138) and 5-HTP (*n*=128) (Fig. 5C,D). In *Lymnaea*, 10 μM ergometrine accelerated normal development (*n*=92) and attenuated the

inhibitory effects of CW (*n*=85) and L-DOPA (*n*=94) (Fig. 6). The rescue action of ergometrine was concentration dependent at 0.1-10 μM (*n*=150) (Fig. 6D). Sulpiride, spiperone, mianserine, cyproheptadine, ritanserin and ketanserin were ineffective at concentrations up to 50 μM (data not shown; 200-250 embryos of each species were tested for each drug).

For both species, incubation in 1-5 mM α-m-T or α-m-DOPA had no significant effect on their development (Fig. 7). Neither DA nor 5-HT (1 μM-1 mM) had any significant effect on development (Fig. 7), while behavioural effects of the neurotransmitters were present. Thus, 5-HT increased the rate of embryonic rotation, and DA increased the buccal mass activity at post-metamorphic stages (data not shown; 200-250 embryos of each species were tested for each drug). The overall effect of the drugs on the larval lifespan is summarized in Fig. 7.

Cellular correlates of developmental retardation

In both *Helisoma* and *Lymnaea*, at the stages 19-24 (20-55% of development), the only cells containing biogenic monoamines and thus being candidates for mediating the described developmental effects were the anterior pairs of neurones (Fig. 8) (see also Voronezhskaya et al., 1999; Koss et al., 2003). In *Helisoma*, these cells were 5-HT immunopositive (Fig. 8A). In *Lymnaea*, the cells were mainly producing dopamine and thus best visualized by glyoxylic acid histochemical reaction (Fig. 8B) or using anti-tyrosine hydroxylase antibodies (Fig. 8C), although they also demonstrated weak anti-5-HT immunoreactivity (Fig. 8D). In both species, at the trochophore stage 19 (20% of embryonic development), the cells looked similar. They were symmetrically located dorsolateral to the mouth opening (Fig. 8A,B). Each neurone had a short thick apical fibre that penetrated the epithelium and bore a tuft of short non-motile cilia, a long basal fibre and two to five short thin fibres emanating from the soma and terminating without ramifications in surrounding tissues (Fig. 8D,E). The basal fibre ramified extensively and formed varicose fibre network underneath the apical ciliary plate in *Lymnaea* (Fig. 8C) and underneath the ciliated areas on the foot in *Helisoma* (Fig. 8A,F).

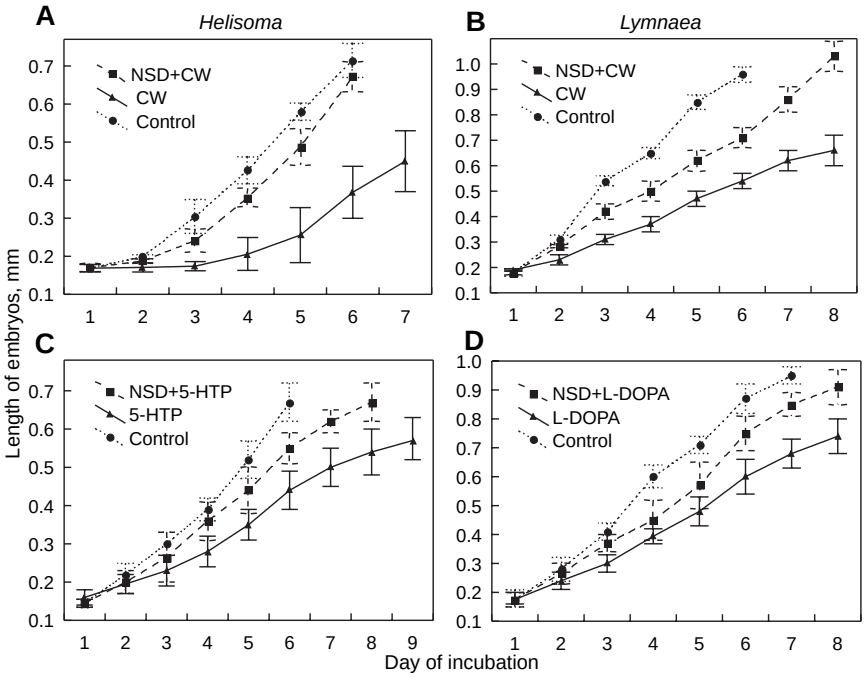

Fig. 4. Decarboxylase inhibitor NSD-1015 (10 μM) significantly attenuated the retarding effect of CW in both *Helisoma* (A) and *Lymnaea* (B). When added together with 1 mM 5-HTP in *Helisoma* (C) or 1 mM L-DOPA in *Lymnaea* (D), NSD-1015 also attenuated their effects.

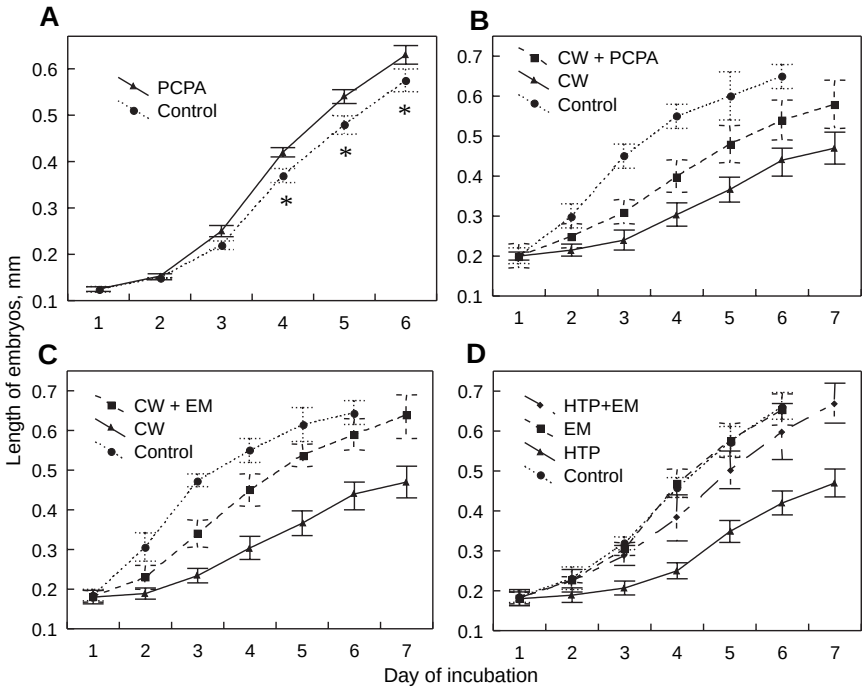

Fig. 5. In *Helisoma*, 5-HT synthesis inhibitor PCPA (5 μM) accelerated larval development (**P*<0.05) (A) and reduced the retarding effect of CW (B). The antagonist of monoamine receptors, ergometrine (EM, 10 μM) attenuated the effect of CW (C) and 1 mM 5-HTP (D).

Incubation of *Helisoma* embryos in the solution of 5-HTP in the concentration that induced developmental retardation (1 mM), resulted in the increase of anti-5-HT immunofluorescence in the anterior neurones (*n*=22). Bathing in 5 mM α-m-T decreased the level of immunofluorescence (*n*=30). In *Lymnaea*, incubation in both CW (*n*=24) and 1 mM L-DOPA (*n*=25) similarly increased the brightness of blue-green glyoxylic acid induced

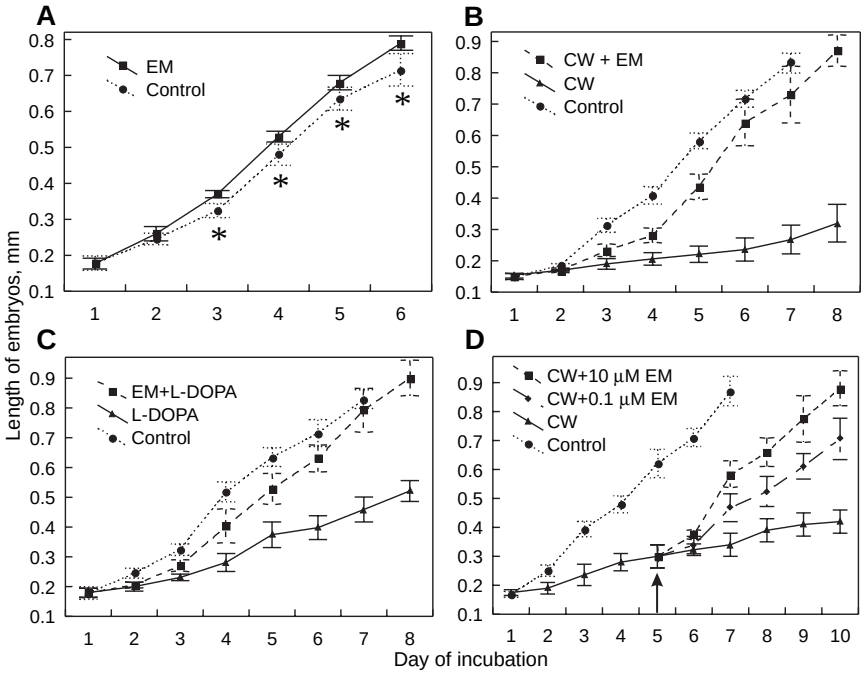

Fig. 6. In *Lymnaea*, ergometrine (10 μM) accelerated normal development (*P<0.05) (A), and attenuated the retarding effect of CW (B) and L-DOPA (C). The rescue effect of ergometrine was concentration dependent (D). Arrow shows the start of ergometrine administration.

fluorescence of catecholamines in the anterior cells, whereas 5 mM α-m-DOPA (n=32) suppressed it (Fig. 9).

Bathing in 5 μM PCPA resulted in suppression of anti-5-HT immunofluorescence in the anterior neurones of *Helisoma* to 72±10% (n=24) of the control (n=20) (Fig. 10). No changes were observed in *Lymnaea* anterior neurones after PCPA treatment (n=51; data not shown).

Discussion

Our main finding is the existence of natural cues, which are released by juveniles of *Helisoma* and *Lymnaea* under conditions of starvation and crowding, are detected by anterior monoaminergic sensory neurones (ASNs) in embryos, and reversibly retard embryonic development. The nature of the released factor(s) is yet unclear. Lack of differences in its action on isolated egg capsules and intact cocoons shows that it easily penetrates through their jelly mass. Heating is known to eliminate volatile organic substances, lipids and large proteins, thus suggesting this cast as potential candidates for molecules inducing developmental retardation. Some of the retarding effects of CW could be in theory due to accumulation of metabolites after conditioning and hypoxia, as anterior monoaminergic cells mediate behavioural responses to hypoxia (Kuang et al., 2002), and hypoxia is known to slow down larval development (Marois and Croll, 1991; Strathmann and Strathmann, 1995). However, in our experiments, neither boiled water nor water conditioned by fed juveniles affected the development, thus making such possibility unlikely.

The involvement of the anterior monoaminergic cells is clear by several reasons. First, at the tested embryonic stages (trochophore and early veliger) these neurones were the only monoaminergic cells present and the only sensory cells described (Goldberg and Kater, 1989; Voronezhskaya et al., 1999; Koss et al., 2003). Second, the inhibitory effect of CW expressed only after the appearance of these cells in

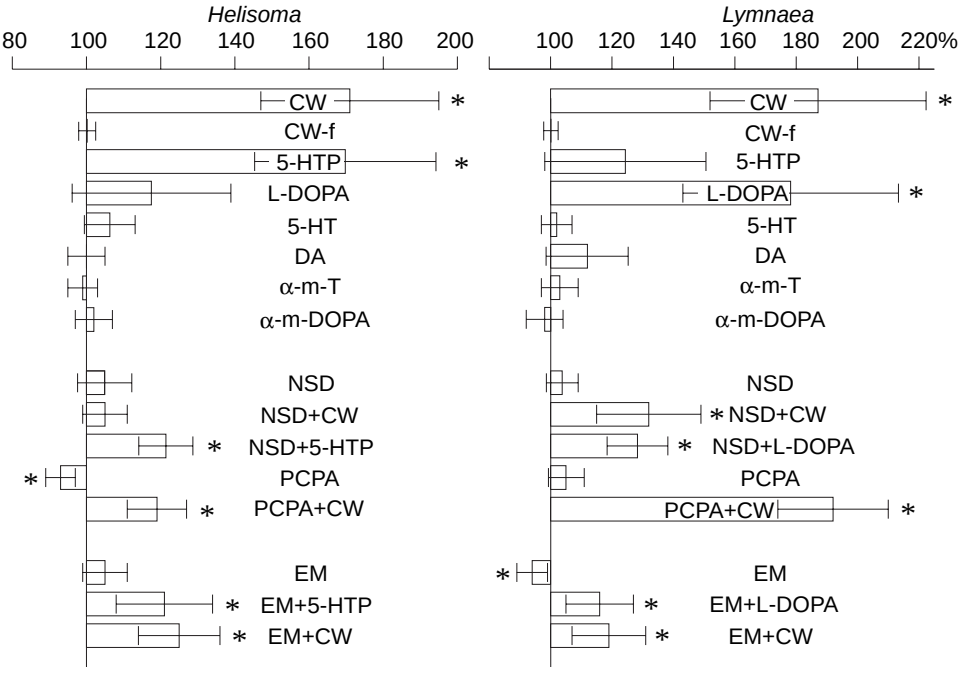

Fig. 7. Effect of the tested drugs on the duration of larval development in *Helisoma* (A) and *Lymnaea* (B) measured from stage 19 till stage 27, and normalized by controls (*significantly different from control; P<0.05). CW, conditioned water; CW-f, water conditioned by fed juveniles; 5-HTP, 5-hydroxy-L-tryptophan; L-DOPA, L-3,4-dihydroxyphenylalanine; 5-HT, 5-hydroxytryptamine creatine sulphate; DA, dopamine; α-m-T, α-methyl-tryptophan; α-m-DOPA, α-methyl-DOPA; NSD, 3-hydroxybenzilhydrazine; PCPA, L-*p*-chlorophenylalanine; EM, ergometrine maleate.

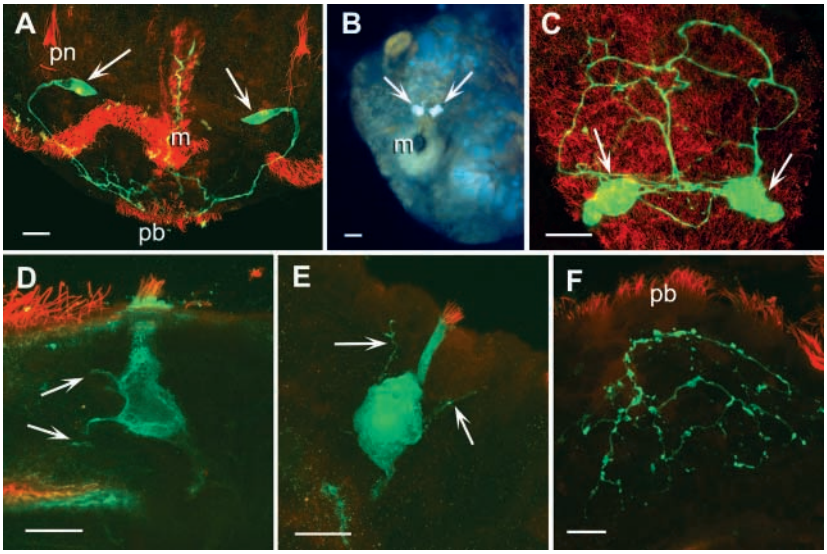

Fig. 8. The first monoaminergic neurones in trochophores of *Helisoma* and *Lymnaea* at stage 19 (20%). (A,C-F) Anti-tubulin Immunostaining (red). (A) *Helisoma*; anti-5-HT immunolabelling (green). Anterior sensory neurones (arrows) are located on both sides of the mouth (m), and project to the pedal ciliary band (pb); pn, cilia in protonephridia (LSM projection, 48×0.6 μm). (B) *Lymnaea*; glyoxylic acid-induced fluorescence of catecholamines; conventional epifluorescence. The pair of the first anterior cells (arrows) is located above the mouth (m). (C) *Lymnaea*; anti-tyrosine hydroxylase immunolabelling (green). Anterior neurones (arrows) make a varicose network underneath the apical ciliary plate (LSM projection, 26×1 μm). (D) *Lymnaea*; anti-5-HT immunolabelling (green). High power image of the anterior neurone with a short thick ciliated apical fibre, thin basal fibre and two short lateral fibres (arrows) emanating from the soma (LSM projection, 54×0.35 μm). (E) *Helisoma*; anti-5-HT immunolabelling (green). The anterior neurone also has a thick ciliated apical fibre, a thin basal fibre and two short lateral fibres (arrows) (LSM projection, 43×0.3 μm). (F) *Helisoma*; anti-5-HT immunolabelling (green). Varicose network made by the fibres of the anterior neurones underneath the pedal ciliary band (pb) (LSM projection, 26×0.4 μm). Scale bars: 20 μm in A,B; 10 μm in C-F.

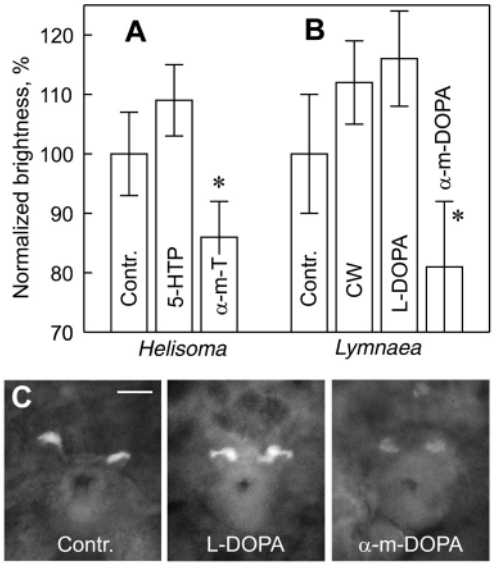

Fig. 9. (A) Changes of average anti-5-HT immunofluorescence in the anterior neurones of *Helisoma*, after 6 hours of incubation with 5-HTP and α-m-T; normalized to controls. (B) Changes of average glyoxylic acid-induced fluorescence of catecholamines in the anterior neurones of *Lymnaea* after 6 hours of incubation with CW, L-DOPA and α-m-DOPA normalized to controls. *Significantly different from control, $P<0.05$. (C) Representative micrographs of glyoxylic acid-induced fluorescence in the anterior neurones of *Lymnaea*, showing increase after incubation with L-DOPA, and reduction after incubation with α-m-DOPA. Scale bar: 20 μm.

development. Third, the effect was emulated by biochemical precursors of monoamines (5-HTP and L-DOPA) corresponding to the transmitter content of the anterior cells (5-HT in *Helisoma* and DA in *Lymnaea*). Fourth, the effects of both CW and the respective precursor were equally attenuated by the monoamine synthesis inhibitors NSD-1015 and PCPA, and the receptor antagonist ergometrine.

In pre-metamorphic veligers (stage 24; 60%), more monoaminergic cells are added in both species, e.g. two pairs in pedal ganglia and sensory cells in the foot (Marois and Croll, 1992; Voronezhskaya et al., 1999). Nonetheless, we are confident that the developmental retardation described above was mediated by ASNs only. First, it already became profound at the trochophore stage 20 (25%), long before the cells in the foot appeared. Second, the transmitter contents of the cells in the foot and pedal ganglia are the same in *Helisoma* and *Lymnaea*, whereas the inhibitory effect of CW was mimicked by different chemicals that corresponded to the transmitter content of ASNs in each species.

Apical cells are activated rather than inhibited in response to the environmental stimuli

Applications of biochemical precursors are widely used to increase synthesis and release of monoamines in respective neurones (e.g. McCaman et al., 1984; Sakharov, 1991; Diefenbach et al., 1995; Fickbohm and Katz, 2000; Pires et al., 2000a), which is generally thought to be a result of neuronal activation. Methylated analogues and synthesis inhibitors are known to significantly reduce concentrations of respective monoamines as demonstrated by HPLC (e.g. Hunter et al., 1993; Diefenbach et al., 1995; Linard et al., 1996; Pani and Croll, 1998; Pires et al., 2000b). In addition, visible changes in immunofluorescence of 5-HT in identified neurones have been shown to correspond to more than twofold changes in its content measured by HPLC (Diefenbach et al., 1995; Croll et al., 1997), and to change the potency of its synaptic and modulatory actions (Fickbohm and Katz, 2000). In our experiments, incubation in the respective precursor resulted in both increase of histo- or immunofluorescence within the ASNs and developmental retardation similar to that induced by CW. By contrast, methylated analogues or synthesis inhibitors did not affect and, sometimes, even facilitated larval development. Together these facts indicate that ASNs are more probably activated rather then inhibited by the factor present in CW.

Attenuation of the retarding effect of monoamine precursors by the synthesis inhibitor, NSD-1015, shows that conversion of the precursors into the respective monoamines is required. Thus, the active substance in this process is the respective

monoamine but not the precursor itself. Nonetheless, applications of 5-HT and DA did not affect the development, though exogenous 5-HT is known to induce metamorphosis acting via internal receptors in the marine gastropod *Ilyanassa obsoleta* (Couper and Leise, 1996). One of the possible explanations is that penetration of monoamines is much easier in marine larvae than in fresh-water ones. Indeed, in the marine polychaete *Phyllodoce maculata*, developmental effects of 5-HT and 5-HTP are similar (E.E.V. and L.P.N., unpublished). In fresh-water animals however, at the tested larval stages, exogenous monoamines can act only on the surface receptors inducing the increase in embryo rotation speed, but do not penetrate into the embryo and reach the internal targets responsible for developmental retardation. However, the precursors can be taken up and can induce both the increase of monoamine synthesis in selective sets of neurones and the directional release of monoamines. This issue is unclear and needs further examination.

Monoamines act through the ergometrine-sensitive receptor

None of the tested specific DA and 5-HT receptor antagonists rescued retardation effect induced by CW and monoamine precursors. Only ergometrine, the derivate of ergot alkaloids, averted the developmental retardation in both *Helisoma* and *Lymnaea*. This and relative substances have been used earlier as dopamine receptor antagonists in molluscs (Juel, 1983; Sawada and Maeno, 1987; Sakharov and Salanki, 1982; Pavlova, 2001). Besides that, some of the 5-HT_1 and 5-HT_5 receptors in vertebrates exhibit high affinity to ergot derivates (Gerhardt and Heerikhuizen, 1997). Thus, the receptor mediating the process of developmental retardation seems to be an internal monoamine ergometrine-sensitive receptor similar to that described earlier in the CNS of *Aplysia* (Shozushima, 1984; Shozushima et al., 1987), and its pharmacological profile is different from the receptors, which are known to be involved in embryonic behaviour of developing gastropods (Goldberg et al., 1994).

Normal development is under slight tonic inhibition by the activity of ASNs

Acceleration of larval development in *Helisoma* by PCPA, and *Lymnaea* by ergometrine was slight but statistically significant (Fig. 7). This may indicate that in normal conditions monoamines are constantly released from the ASNs and tonically inhibit the development. The degree of inhibition depends on the activity of this neurones subjected by environmental stimuli. Spontaneous release of transmitter from growth cones has been shown for embryonic neurones in culture (Young and Poo, 1983). Diefenbach and co-authors (1995) have demonstrated intensive neurite outgrowth of the anterior serotonergic neurones in *Helisoma* trochophores, which was promoted by PCPA and inhibited by 5-HTP, and suggested that the neurones use their own transmitter in an autoregulatory fashion to regulate neurite formation during embryonic development. Perhaps neurite growth autoregulation is one of the mechanisms involved in the developmental effects of monoamines described above. Further experiments are required to verify this supposition.

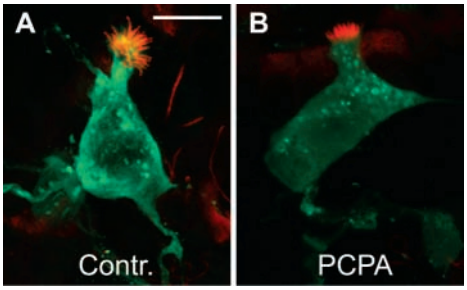

Fig. 10. Representative fluorescence micrographs of PCPA induced reduction of anti-5-HT immunofluorescence (green) in the anterior neurones of *Helisoma*; cilia are labelled in red (LSM projections; 20×0.5 μm). (A) Control. (B) After 3 days of incubation with 5 μM PCPA. Scale bar: 10 μm.

Anterior sensory cells in trochophore animals

Two anterior monoaminergic sensory cells, which develop before the onset of the CNS formation, express 5-HT, innervate locomotory cilia, and degenerate after metamorphosis, have been described in various pulmonate embryos (Goldberg and Kater, 1989; Voronezhskaya and Elekes, 1993; Diefenbach et al., 1998; Voronezhskaya et al., 1999; Kuang and Goldberg, 2001; Koss et al., 2003; Voronezhskaya and Elekes, 2003), and are generally believed to be the remnants of the apical sensory organ (ASO), the structure characteristic for trochophore animals (Nielsen, 2001). The basic plan of the ASO is highly conserved regardless of the planctotrophic or intracapsular developmental mode (Kempf et al., 1997; Marois and Carew, 1997; Dickinson et al., 1999; Page and Parries, 2000; Nielsen, 2001; Schaefer and Ruthensteiner, 2001). It is known to take part in the induction of larval settlement and metamorphosis (Chia and Rice, 1978; Baxter and Morse, 1992; Hadfield et al., 2000; Leise at al., 2001). However, the ASO fully develops much earlier (from several days to several weeks) than the larva becomes competent for metamorphosis (Page, 2002a), and in some species partly degenerates before metamorphic competence (Page, 2002b; Wanninger and Haszprunar, 2003). The essential neurotransmitter of the apical neurones, 5-HT, was able to induce metamorphosis in one species only (Couper and Leise, 1996). These observations contradict speculations about a general role of the ASO for the metamorphic event.

Our data suggest that the function of the apical sensory neurones in early development of pulmonates is to control premetamorphic larval development and inhibit it in response to negative environmental stimuli. Based on structural uniformity of the ASO in diverse larvae, one may speculate that the developmental mechanism we describe is uniform throughout molluscs. Perhaps, after larvae reach metamorphic competence, the functions of the ASO change. The supposition that the ASO plays different roles in pre-metamorphic and competent larvae resolves the contradictions mentioned above.

Comparison with *Caenorhabditis elegans*

Inhibition of larval development by chemosensory neurones was first described in the nematode *C. elegans* (Bargman and Horvitz, 1991; Thomas, 1993). Under conditions of crowding and starvation, a specific pheromone was released by young animals, and detected by chemosensory neurones of larvae. In the presence of the pheromone, after the second larval molt they differentiated into a so called dauer larva, an alternative

larval form, which did not feed, did not develop further and was resistant to harsh environmental conditions. The response to the pheromone was mediated by identified neurones, some of them being serotonergic (Schackwitz et al., 1996; Sze et al., 2000).

The authors suggested that similar mechanisms may be used by other organisms to evaluate environmental signals and regulate their development (Bargman and Horvitz, 1991). The development of *Helisoma* and *Lymnaea* is the second example of such regulation, when conspecific chemical signals informing about food shortage and overpopulation, are detected by sensory cells and retard the development. However, several basic differences in the mechanisms of the inhibition must be mentioned. Sensory neurones were inhibited by the pheromone in *C. elegans*, and excited in *Helisoma* and *Lymnaea*. In *C. elegans*, inhibition or absence of these neurones, as well as lack of 5-HT, resulted in the dauer larva formation, whereas their activation and 5-HT stimulated normal larval development or dauer recovery (Daniels et al., 2000; Sze et al., 2000). By contrast, in *Helisoma* and *Lymnaea*, both activation of chemosensory neurones and increased synthesis of the respective monoamine inhibited the development, while their inactivation facilitated it. In *C. elegans*, the action of chemosensory neurones was indirect. They targeted the nervous system and the nervous system in turn regulated dauer formation by a different mechanism (Inoue and Thomas, 2000). In *Helisoma* and *Lymnaea*, however, no central nervous system is yet developed until the stage 25 (late veliger), thus the action of monoamines released by the anterior sensory neurones is either direct or mediated by early transient peripheral peptidergic neurones.

Conclusions

Our results describe a novel regulatory mechanism in larval development of pulmonate snails. We show that the development is permanently under slight tonic inhibitory influence of the anterior sensory monoaminergic neurones, which are the remnants of the apical sensory organ. Monoamines released by these neurones bind to the internal ergometrine-sensitive receptor, and induce developmental retardation. In case of untoward environmental conditions, larval anterior cells are activated by yet unknown conspecific chemical signals emitted by juvenile snails. The cells enhance secretion and release of the monoamine, which, in turn, enhances the developmental retardation.

In addition to demonstrating a novel role for the apical sensory neurones in the development, our study suggests new avenues for future investigations of a link between environmental signals and developmental regulation. Larval pulmonates provide a unique model in which the environmental stimulus, the sensory cells, the acting chemicals and the resulting developmental effects are all known and can be experimentally manipulated. Thus, this model allows the study of downstream mechanisms underlying developmental regulation.

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