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RESEARCH ARTICLE

Identification and evolutionary implications of neurotransmitter–ciliary interactions underlying the behavioral response to hypoxia in *Lymnaea stagnalis* embryos

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SUMMARY

Acceleration of embryonic rotation is a common response to hypoxia among pond snails. It was first characterized in *Helisoma trivolvis* embryos, which have a pair of sensorimotor neurons that detect hypoxia and release serotonin onto postsynaptic ciliary cells. The objective of the present study was to determine how the hypoxia response is mediated in *Lymnaea stagnalis*, which differ from *H. trivolvis* by having both serotonergic and dopaminergic neurons, and morphologically distinct ciliated structures at comparative stages of embryonic development. Time-lapse video recordings of the rotational behavior in *L. stagnalis* revealed similar rotational features to those previously observed in *H. trivolvis*, including rotational surges and rotational responses to hypoxia. Serotonin and dopamine increased the rate of rotation with similar potency. In contrast, serotonin was more potent than dopamine in stimulating the ciliary beat frequency of isolated pedal cilia. Isolated apical plate cilia displayed an irregular pattern of ciliary beating that precluded the measurement of ciliary beat frequency. A qualitative assessment of ciliary beating revealed that both serotonin and dopamine were able to stimulate apical plate cilia. The ciliary responses to dopamine were reversible in both pedal and apical plate cilia, whereas the responses to serotonin were only reversible at concentrations below 100 μmol l^{-1}. Mianserin, a serotonin receptor antagonist, and SKF83566, a dopamine receptor antagonist, effectively blocked the rotational responses to serotonin and dopamine, respectively. The rotational response to hypoxia was only partially blocked by mianserin, but was fully blocked by SKF83566. These data suggest that, despite the ability of serotonin to stimulate ciliary beating in *L. stagnalis* embryos, the rotational response to hypoxia is primarily mediated by the transient apical catecholaminergic neurons that innervate the ciliated apical plate.

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Key words: dopamine, gastropod, hypoxia, serotonin.

INTRODUCTION

Many aquatic animals undergo embryonic development inside egg capsules that are deposited in aggregates called egg masses [for examples of different types, see Salthe and others (Salthe, 1963; Hurst, 1967; Strathmann and Chaffee, 1984)]. Encapsulated embryos benefit from an abundance of nutrients in the surrounding capsular fluid, as well as protection from bacterial invasion (Benkendorff et al., 2005). Encapsulation also carries several disadvantages, including susceptibility to predation and the presence of diffusion barriers. Unstirred boundary layers associated with the embryonic surface, egg capsule membrane and outer egg mass membrane can severely impede the inward and outward diffusion of O_2, CO_2 and metabolites. Reduced O_2 levels (hypoxia) have been observed near the center of egg masses or within egg capsules of many gastropod, amphibian and fish species (Burggren, 1985; Seymour and Roberts, 1991; Pinder and Friet, 1994; Booth, 1995; Kuang et al., 2002), and have been implicated in slowed development and decreased survival (Chaffee and Strathmann, 1984; Giorgi and Congleton, 1984; Seymour and Roberts, 1991; Mills and Barnhart, 1999; Seymour et al., 2000; Shartau et al., 2010a). Intracapsular hypoxia can be generated intrinsically through the embryo's metabolism or extrinsically from environmental fluctuations in O_2 levels. Many aquatic environments undergo seasonal, diurnal and even hourly fluctuations in O_2 content through complex interactions between factors such as photosynthesis, water surface gas exchange rate, temperature and metabolic O_2 demand (Cole, 1994; Shartau et al., 2010b). Encapsulated organisms are especially vulnerable to the adverse effects of hypoxia because of their inability to relocate through locomotion.

Encapsulated species have evolved various strategies that enhance O_2 diffusion and maintain an adequate O_2 supply during hypoxia. First, many encapsulated embryos display movements propelled by cilia that help mix the intracapsular fluid (Burggren, 1985; Diefenbach et al., 1991; Voronezhskaya et al., 1999), reduce unstirred boundary layers and promote diffusion (Burggren, 1985; Hunter and Vogel, 1986; Goldberg et al., 2008). Second, the O_2 conductance of capsular membranes can be regulated to facilitate O_2 diffusion, as occurs in various amphibians (Seymour and Bradford, 1987; Mills et al., 2001). Third, some amphibian egg masses contain photosynthetic symbiotic algae that provide a localized O_2 supply for the encapsulated embryos (Pinder and Friet, 1994).

Freshwater pond snails have emerged as excellent model systems for studying adaptations associated with embryonic encapsulation. They originated from more ancient marine gastropod lineages that typically display planktotrophic larval development, whereby veligers disperse long distances in the vast marine environment (Klussmann-Kolb et al., 2008). The conversion to encapsulated direct development by freshwater pond snails required adaptations that likely involved the redeployment of existing cellular machinery into new roles. For instance, the embryonic neuron C1 (ENC1) of the pulmonate *Helisoma trivolvis* (also known as *Planorbella trivolvis*; family Planorbidae) (Brown, 1991) is thought to be derived from the para-ampullary apical ganglion neurons described in marine gastropods (Kempf et al., 1997; Diefenbach et al., 1998). ENC1 mediates detection of hypoxia and egg capsule ventilation in *H. trivolvis* embryos (Goldberg et al., 2008), whereas the para-ampullary neurons participate in substrate detection and larval settlement in planktotrophic gastropod veligers (Hadfield et al., 2000).

ENC1s are sensorimotor neurons that release serotonin onto postsynaptic ciliary cells in response to hypoxia, resulting in faster ciliary beating and embryonic rotation (Goldberg et al., 1994; Kuang and Goldberg, 2001; Koss et al., 2003). Hypoxia elicits sustained, dose-dependent and reversible accelerations of embryonic rotation in control embryos, whereas this response is absent in embryos that have undergone bilateral laser ablation of their ENC1s (Kuang et al., 2002). ENC1s directly respond to hypoxia with membrane depolarization and generation of action potentials (Goldberg et al., 2008). The 'stir-bar hypothesis' proposes that the rotational behavior is a ventilation response that facilitates O_2 diffusion to the embryo by reducing unstirred boundary layers (Goldberg et al., 2008).

Similar rotational responses to hypoxia as those seen in *H. trivolvis* have recently been demonstrated in two other families of pond snails, the Physidae and Lymnaeidae (Goldberg et al., 2008; Byrne et al., 2009). The widespread occurrence of this behavior suggests that it is an important adaptation to encapsulation. Although little is known about physid embryos, numerous studies on embryos of the lymnaeid species *Lymnaea stagnalis* have revealed a variety of morphological and physiological differences between *L. stagnalis* and *H. trivolvis*. For example, only *L. stagnalis* has a ciliated apical plate region, whereas both species have pedal and dorsolateral (prototrochal) ciliary bands (Voronezhskaya et al., 1999; Koss et al., 2003). Furthermore, the transient apical catecholaminergic (TAC) neurons of *L. stagnalis*, which are considered to be homologous to the ENC1 neurons of *H. trivolvis*, contain dopamine rather than serotonin and innervate the apical plate cilia rather than the other ciliary bands (Diefenbach et al., 1998; Voronezhskaya et al., 1999; Koss et al., 2003). Finally, application of either dopamine or serotonin stimulates embryonic rotation in *L. stagnalis* (Filla et al., 2009), whereas only serotonin does so in *H. trivolvis* (Diefenbach et al., 1991). In light of these differences, determining the physiological mechanisms underlying the rotation response to hypoxia in *L. stagnalis* will help elucidate the evolution of a simple conserved behavior. In this study, the rotation rate of encapsulated embryos and ciliary beat frequency (CBF) of isolated patches of identified cilia were measured in response to treatments with hypoxia, and serotonin and dopamine agonists and antagonists, to determine the relative role of these neurotransmitters in mediating the rotational behavior of *L. stagnalis* embryos.

MATERIALS AND METHODS

Animals

Adult *Lymnaea stagnalis* (Linnaeus 1758) were maintained in flow-through aquaria containing dechlorinated water at ~25°C, or in non-flow-through aquaria containing artificial pond water (APW; 0.025% Instant Ocean, pH 7.2–7.3; Aquarium Systems, Mentor, OH, USA) at 22°C. They were alternatively fed with lettuce and trout pellets (NU-WAY, United Feeds, Okatoks, AB, Canada). Because pond snails preferentially lay eggs on a smooth surface, plastic Petri dishes (150 mm diameter) were placed in the aquaria to facilitate egg laying and egg mass collection. Egg masses were removed from the Petri dishes with a razor blade and transferred into APW for experimental use. Embryos were staged as a percentage of the whole intracapsular development, with E0 corresponding to 0% development (zygote) and E100 corresponding to 100% development (hatching) (Marois and Croll, 1992; Voronezhskaya et al., 1999; Filla et al., 2009). In this study, egg masses containing embryos from stages E35 to E39 were used. The defining characteristics during this period of development include appearance of the pedal-abdominal furrow, minimal elongation along the anterior–posterior axis, a defined shell gland with no shell deposition, and rapid embryo rotation.

Measurement of embryonic rotation

Embryonic rotation was monitored with a CCD video camera (JVC model TK-860U, Wayne, NJ, USA) or a digital video camera (QImaging QICAM Fast, cooled color 12 bit; Surrey, BC, Canada) mounted on a dissecting microscope (Zeiss StemiSR, Zeiss Canada Ltd, Toronto, ON, Canada) or an inverted compound microscope with a 4× objective (Nikon Eclipse 300, Melville, NY, USA). In earlier experiments, the analog signal captured by the CCD camera was recorded by a time-lapse VCR (Panasonic AG-6720, Panasonic Canada, Mississauga, ON, Canada) at 2.5 frames s^{-1} for 10 min. The time-lapse VCR facilitated analysis as it allowed the relatively slow rotational behavior to be examined at 24 times the actual rotational rate when replayed at 60 frames s^{-1}. In later experiments, rotation was recorded for 6 min using the time-lapse function of Northern Eclipse imaging software (Northern Eclipse 6.0, Empix Inc., Mississauga, ON, Canada). The overall rate of rotation was analyzed by counting the total number of rotations during the video, expressed as rotations min^{-1}. Several of the earlier experiments were repeated in the later experiments to confirm that the new method of image capture and playback, as well as the shorter video length, did not have an effect on the results. Counts were accurate to within 0.5 rotations min^{-1}.

The dynamic pattern of rotation was determined by recording 3 min videos at 15 frames min^{-1} using Northern Eclipse imaging software. The degrees of rotation in each 4 s interval was measured using ImageJ software (National Institutes of Health, http://rsbweb.nih.gov/ij/) and an Iconico screen protractor version 4.0 (Iconico, http://www.iconico.com/). Each value of degrees rotated per 4 s interval was then converted into rotations min^{-1}.

Isolation and culture of ciliary tissues

Prior to removing embryos from their egg capsules, egg masses were rinsed in 35% ethanol for 30 s, followed by two 10 min washes in *Lymnaea* saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$ and 5.0 mmol l^{-1} HEPES; pH 7.49). A razor blade was used to split the egg mass into smaller segments on filter paper (Whatman Laboratory Division, Maidstone, Kent, UK) and the egg capsules were separated and placed in a dry 100×15 mm Petri dish (Fisher Scientific Canada, Edmonton, AB, Canada) to allow the capsules to adhere to the plastic. Once adhered, *Lymnaea* saline was added to the dish. Each egg capsule was ruptured with a 30 gauge needle (Becton Dickinson, Franklin Lakes, NY, USA) and the embryo was removed by flushing with *Lymnaea* saline using a Pasteur pipette. These embryos were then collected using Pasteur pipettes and washed in *Lymnaea* saline twice for

10 min each. The washed embryos were transferred to a glass-bottom culture dish containing *Lymnaea* medium [40 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 0.4 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} HEPES, 2.0 mmol l^{-1} galactose, 2.5 mmol l^{-1} sodium pyruvate, 0.014 mmol l^{-1} Phenol Red, 1:200 MEM essential amino acid solution (Invitrogen Canada Ltd, Burlington, ON, Canada), 9:100 MEM non-essential amino acid solution (Invitrogen Canada Ltd), 1:100 MEM vitamin solution (Invitrogen Canada Ltd) and 0.015% glutamine; pH 7.45].

The pedal ciliary cells were identified and isolated under Normarski differential interference contrast (DIC) optics on an inverted compound microscope (Nikon Diaphot-TMD, Chlyoda-ku, Japan) as described previously (Doran et al., 2004). Briefly, a glass micropipette with a tip diameter of 30 to 50 µm was positioned over the pedal cluster of ciliary cells. Negative pressure was applied with a micrometer syringe to introduce the ciliary patch into the micropipette tip, and the ciliary cells were subsequently detached from the rest of the embryo with a 30 gauge needle. The isolated patches were then transferred to poly-L-lysine-coated culture dishes [1 mg ml^{-1} poly-L-lysine; molecular weight 70,000–150,000; 0.15 mol l^{-1} Tris (hydroxymethyl)-aminomethane; pH 8] containing *Lymnaea* medium and were left stationary for approximately 3 h to settle and adhere to the glass-bottomed portion of the dish. Experiments were performed on the same day that ciliary explants were harvested.

A gravity-driven multi-valve perfusion system (Warner Instruments, Hamden, CT, USA) was used to control the flow of solutions into the glass-bottomed dishes containing explants. The system was controlled with a VC-8 valve controller set to external digital mode. After two initial recordings of CBF under static conditions, the recording dish was perfused continuously for the duration of the experiments at a flow rate of 5.2 ml min^{-1}.

Measurement of CBF

The isolated ciliary patches were identified through DIC optics on an inverted compound microscope (Nikon Eclipse 300) with a 100× objective. Two-second movies of ciliary beating were captured using a Retiga 4000R digital camera (QImaging) at 1 min intervals. Each movie was an image sequence of 100 frames captured at a rate above 50 frames s^{-1} with Northern Eclipse software. The image sequences were then converted to a movie using ImageJ, and the movies were analyzed for CBF measurements using autocorrelation software (Particle Analysis; written by C. J. H. Wong, University of Alberta, Edmonton, AB, Canada) (Doran et al., 2004). Each CBF data point was the mean of three runs of the analysis program taken at different ciliary locations, expressed in beats s^{-1}.

Anoxia treatments

In the experiment involving repeated episodes of anoxia (see Fig. 2B), egg masses were stabilized with wax strips in a 2.8 ml sealed perfusion chamber. Normoxic or anoxic APW was perfused by gravity through fluorinated ethylene propylene tubing (Cole Parmer, Montreal, QC, Canada) at a flow rate of approximately 6.5 ml min^{-1}. To ensure water saturation, gases were bubbled vigorously in APW for at least 20 min before and during perfusion.

In all other experiments on embryonic rotation, egg capsules were isolated from egg mass membranes and adhered to the floor of a 25 mm culture plate. Adhesion was achieved by allowing the egg capsules to settle onto the dry surface of the plate for up to 1 min before adding APW. The APW was replaced by 5 ml of drug-containing or anoxic APW. To maintain anoxic levels of oxygen in the APW solution, the culture plate was placed inside a dry 100 mm culture plate, which was then covered with its lid and sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). The lid contained two holes, one for gas injection and the other for exhaust, which allowed the maintenance of an O_2-free environment around the recording culture plate. Anoxia was administered 15 min prior to imaging embryonic rotation.

Pharmacological treatments

All chemicals were purchased from Sigma-Aldrich, including serotonin creatinine sulfate, dopamine HCl, mianserin HCl, SFK83566 HCl (+,–) and domperidone. All solutions were made fresh daily in APW and kept in the dark at 4°C. SKF83566 was dissolved in ethanol and diluted to a final working concentration that contained no more than 0.01% ethanol. Domperidone was dissolved in dimethyl sulfoxide (DMSO) and diluted to a final working concentration that contained no more than 0.1% DMSO. In experiments involving SKF83566 or domperidone, all treatments contained the same level of ethanol or DMSO, respectively.

Embryos were exposed to the neurotransmitters serotonin or dopamine 1 min prior to the start of the 6 min video recording of embryonic rotation. Control experiments indicated that longer incubation periods produced identical results (not shown). Embryos were exposed to the neurotransmitter antagonists mianserin, SKF83566 or domperidone 15 min prior to the start of the 6 min video recording of embryonic rotation.

Immunolocalization of ciliary patches

Stage E39 embryos were isolated from egg masses as described above and suspended in 2× Zamboni's fixative (4% paraformaldehyde, 7.5% picric acid and 0.01 mol l^{-1} phosphate buffer; pH 7.5) at 4°C for 16 h. The embryos were then subjected to a 30 min wash in 0.01 mol l^{-1} Tris buffered saline (TBS) and six 30 min washes in 0.01 mol l^{-1} phosphate buffer (PBS), pH 7.5. Embryos were permeabilized for 15 min in 0.01 mol l^{-1} phosphate buffer containing 0.5% Triton X-100 (TPBS), pH 7.5 and washed twice with PBS. Ciliary bands were immunolabelled by incubating the embryos with a mouse monoclonal anti-acetylated tubulin antibody (Sigma-Aldrich, St Louis, MO, USA) diluted 1:1000 in PBS and 10% goat serum at 4°C for 16 h. Excess antibody was removed by washing the embryos once with PBS, TPBS and PBS for 20 min each. The embryos were then incubated in a secondary goat-anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen Canada Inc.) diluted 1:500 in PBS and 10% goat serum for 1 h. The embryos were subjected to single 20 min washes in PBS, TPBS and PBS, and then suspended in mounting media (10 mmol l^{-1} 1,4-phenylenediamine in glycerol; pH 8) between a glass slide and a #1 cover glass. The edges of the cover glass were sealed with nail polish.

The embryos were examined on a Nikon Eclipse TE300 inverted microscope under epifluorescence excitation through a 40× objective. Images were captured using a Retiga 4000R digital camera controlled by Northern Eclipse acquisition software. Images were acquired at several planes of focus through 20 embryos.

Statistical analysis

The statistical analysis and graphics were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as means ± s.e.m. The anoxia experiments were analyzed using a two-way ANOVA with a Bonferroni *post hoc* test. The pharmacological experiments were analyzed using a one-way ANOVA and Tukey's multiple comparison *post hoc* test. To assess the effect of serotonin or dopamine on CBF, we performed an area under the curve analysis. The sum of the first five CBF

measurements after addition of the drug was normalized to the sum of the five CBF measurements immediately before addition of the drug, and expressed as percentage of pre-treatment CBF.

RESULTS

Acetylated tubulin immunofluorescence revealed that *L. stagnalis* embryos contained the same ciliated structures on the embryonic surface as those previously described in *H. trivolvis* (Kuang and Goldberg, 2001; Doran et al., 2004), including two dorsolateral bands of cilia, a pedal band of cilia, the dorsal stomodeum and scattered single ciliary cells (Fig. 1). *Lymnaea stagnalis* embryos also contained the ciliated apical plate (Fig. 1), which is absent in *H. trivolvis*.

Despite differences in the neuronal complement, neurotransmitter phenotypes and ciliary structures, the rotational behavior of stage E35–39 *L. stagnalis* embryos was generally similar to that of *H. trivolvis* embryos with respect to the rotational components, rotational dynamics and responses to hypoxia. As previously characterized in *H. trivolvis* embryos (Diefenbach et al., 1991), embryonic rotation in *L. stagnalis* had two directional components: upward pitch rotation around the mediolateral axis and yaw rotation around the dorsoventral axis. In contrast to the rightward yaw rotation in *H. trivolvis*, *L. stagnalis* embryos executed their yaw rotation leftward, likely reflecting the adult left-handed (sinistral) and right-handed (dextral) shell coiling of *H. trivolvis* and *L. stagnalis*, respectively (Clarke, 1981; Morrill, 1982). In addition, the relative contribution of these directional components to the overall rotation was more variable in *L. stagnalis*, which produced regular changes in the axis of rotation (supplemental material Movie 1). The dynamic pattern of rotation was also similar to that previously described for *H. trivolvis* (Cole et al., 2002), with periods of slow basal rotation interrupted by transient accelerations termed surges (Fig. 2A). Finally, embryos of both species displayed similar concentration-dependent responses to hypoxia, including an immediate rise in rotation rate to a peak in the first 10–20 min, followed by a return towards baseline levels even under maintained hypoxia (Kuang et al., 2002; Goldberg et al., 2008; Byrne et al., 2009). Repeated 30 min episodes of hypoxia induced similar rotational responses, including a rapid recovery to the baseline rotation rate each time normoxia was restored (Fig. 2B).

Effects of serotonin and dopamine on embryonic rotation and CBF

Serotonin and dopamine are both present in *L. stagnalis* embryos between stages E30 and E39 and are able to augment the rotation rate when applied exogenously (Filla et al., 2009). As a first step in elucidating their relative roles in the hypoxia response, the actions of these neurotransmitters on the rotational behavior of intact embryos and CBF of isolated patches of cilia were compared. Serotonin and dopamine elicited very similar concentration-dependent increases in the rate of embryonic rotation, with half-maximal effective concentration (EC_{50}) values of approximately 100 μmol l^{-1} for both neurotransmitters (Fig. 3). Basal rotation rates were approximately 1.7 rotations min^{-1}, whereas the maximal responses produced by serotonin and dopamine were 4.5±0.4 and 3.8±0.4 rotations min^{-1}, respectively. To determine whether the relatively low sensitivity of embryonic rotation to both neurotransmitters resulted from incomplete diffusion of these compounds through the diffusion barriers of the egg capsule, CBF responses to serotonin and dopamine were measured in isolated ciliary tissues. This provided the additional opportunity to determine whether serotonin and dopamine are selective in activating distinct ciliary structures. Given that serotonergic neurons innervate the ciliated pedal epithelium (Longley, 2008) and dopaminergic TAC neurons innervate the ciliated apical plate cilia (Voronezhskaya et al., 1999), it was predicted that the pedal cilia and apical plate cilia are selectively sensitive to serotonin and dopamine, respectively. Although both neurotransmitters caused a significant increase in pedal CBF, serotonin was more potent (Figs 4, 5). The estimated EC_{50} for serotonin occurred at approximately 5 μmol l^{-1} (Fig. 4A), whereas it was approximately 100 μmol l^{-1} for dopamine (Fig. 4B).

Serotonin and dopamine also produced different response profiles in pedal ciliary cells. CBF measurements taken at 1 min intervals revealed that pedal cilia beat continuously at 14±2 beats s^{-1} under control conditions (Fig. 5). Addition of 10 μmol l^{-1} serotonin caused a rapid and sustained increase in CBF to 27±3 beats s^{-1}, which was fully reversible upon washout (Fig. 5A). Although 100 μmol l^{-1} serotonin did not cause any further increase in the peak amplitude of the CBF response, the reversibility of the response upon washout was significantly reduced at this higher concentration. In contrast to serotonin, the cilioexcitatory effect of dopamine was reversible

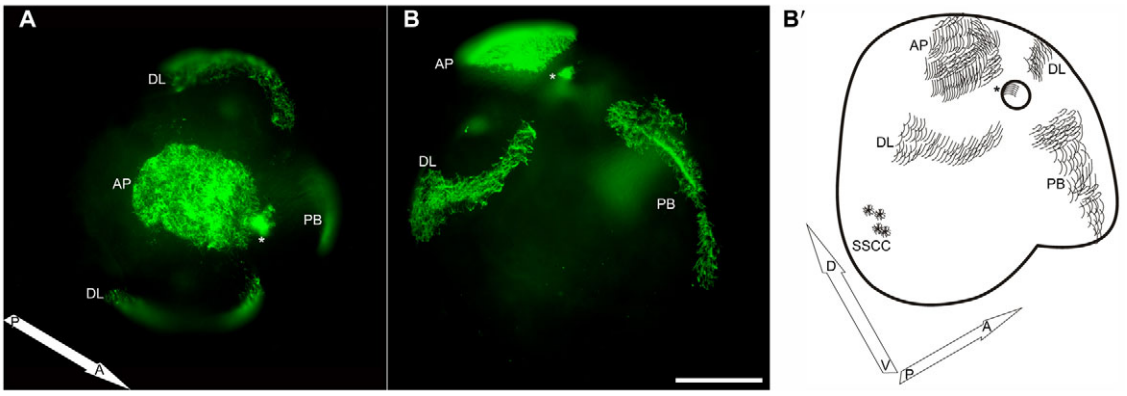

Fig. 1. Superficial ciliary structures in stage E35–39 *Lymnaea stagnalis* embryos revealed by acetylated tubulin immunofluorescence. (A) Dorsal view. The densely ciliated apical plate (AP) occupies a large portion of the dorsal surface. The two dorsolateral bands of cilia (DL), as well as the ciliated dorsal stomodeum (asterisk), are also apparent. Arrow indicates the anterior (A)–posterior (P) orientation. Fluorescence micrograph (B) and schematic (B′) of an embryo from an anterior–lateral view. Five types of ciliary structures were found on the embryonic surface, including the AP, dorsal stomodeum (asterisk), pedal band of cilia (PB), DL and scattered single ciliary cells (SSCC). The focal plane of the micrograph shown in B did not include any SSCC staining. Arrows in B′ indicate the A–P and dorsal (D)–ventral (V) orientations. Scale bar, 100 μm. Only the acetylated tubulin signal on the surface of the embryo was incorporated into the schematic.

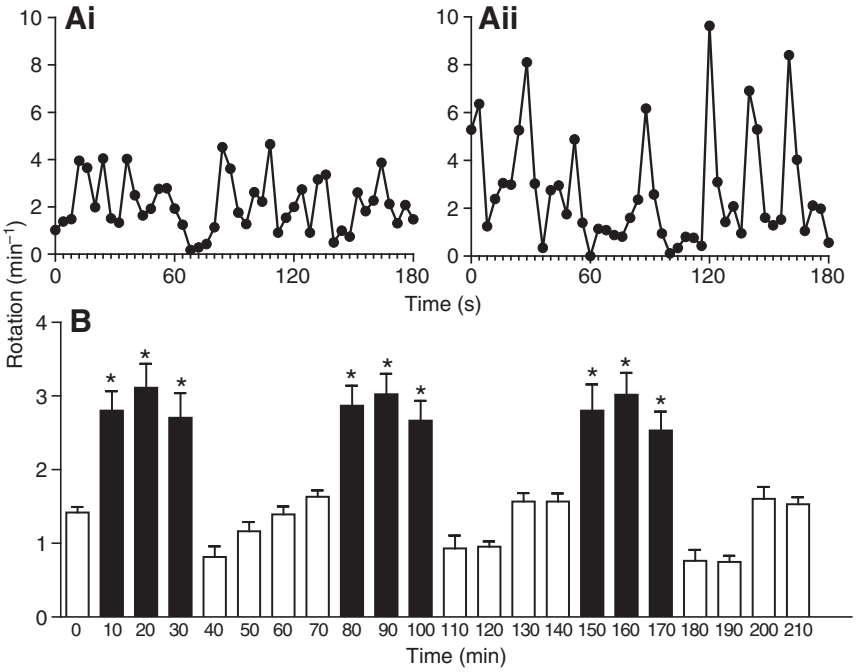

Fig. 2. Fundamental properties of rotational behavior in *L. stagnalis* embryos. (Ai, Aii) Rotational dynamics in two representative embryos. Rotation rate was measured over consecutive 4 s periods for 3 min. The rotation behavior consisted of transient surges in rotation rate, as well as periods of slow rotation. (B) Rotational response to multiple anoxia treatments. Embryos were exposed to three cycles of anoxia (black bars) and normoxia (white bars). The anoxia-induced increase and post-anoxia recovery of rotation rate were similar between each cycle (N=15 embryos, five egg masses; asterisk denotes statistical significance compared with the baseline rotation rate measured at time 0).

upon washout, even at concentrations as high as 1000 μmol l^{-1} (Fig. 5B).

Experiments assessing the ability of serotonin and dopamine to activate apical plate cilia in isolated explants revealed major differences in their activity patterns as compared with pedal cilia. Under control conditions, apical plate cilia beat irregularly, with periods of inactivity interspersed with periods of very rapid beating. As this pattern usually precluded the measurement of CBF using the standard quantitative assay, a qualitative assessment of ciliary beating (QACB) using a four-point scale was developed to provide a semi-quantitative measure of ciliary activity. As described for pedal cilia, ciliary activity was recorded during 2 s videos taken at 1 min intervals. Each recording was then analyzed for a QACB score, whereby: 1=cilia not beating; 2=only a portion of the cilia are beating or beating occurs during a portion of the 2 s period; 3=beating is continuous at a rate below 12 beats s^{-1}; and 4=beating is continuous at or above 12 beats s^{-1}. Under control conditions, cilia typically beat at a level between 1 and 2 on the QACB scale (Fig. 6). As was the case for pedal cilia, both serotonin and dopamine activated apical plate ciliary beating at 100 μM. Furthermore, in all cases the activated cilia met the criterion for a level 4 response on the QACB scale, with CBF values consistently falling in the 12–15 beats s^{-1} range. The apical plate cilia also behaved similarly to the pedal cilia with respect to the reversibility of the responses. The ciliary activity returned towards control levels after washout of 100 μmol l^{-1} dopamine, but remained quite high after washout of 100 μmol l^{-1} serotonin. Given that the apical plate cilia could only be assessed through the semi-quantitative analysis described above, concentration–response relationships for serotonin and dopamine were not determined.

Relative roles of serotonin and dopamine in mediating the hypoxia response

Because both serotonin and dopamine were able to stimulate CBF and embryonic rotation, these neurotransmitters are prime candidates for the mediators of the embryonic rotation response to hypoxia. As an initial step in testing the involvement of serotonin and dopamine, the effectiveness of serotonin and dopamine antagonists was tested. Previous studies on *H. trivolvis* (Goldberg et al., 1994) and *L. stagnalis* (Filla et al., 2009) suggest that mianserin is one of the only effective blockers of the serotonergic response identified

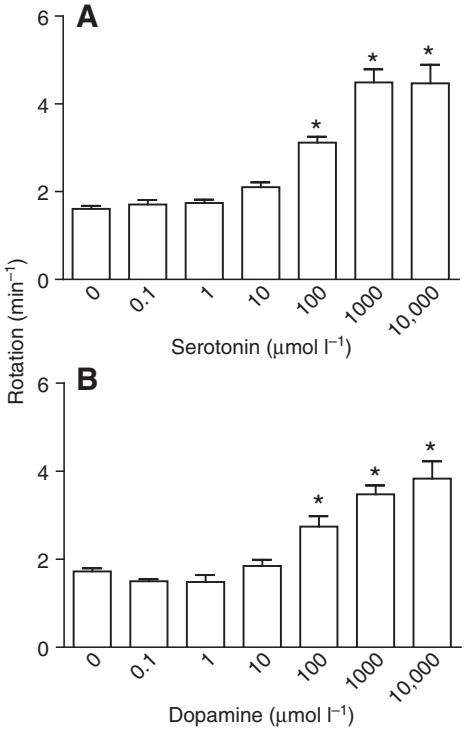

Fig. 3. Concentration-dependent effects of serotonin and dopamine on rotation rate in *L. stagnalis* embryos. Different embryos were used for each treatment. (A) Serotonin. Each bar represents data from at least 11 embryos and five egg masses. (B) Dopamine. Each bar represents data from at least nine embryos and four egg masses. Asterisks in A and B represent statistically significant differences from the control treatment (0 μmol l^{-1}; P<0.001).

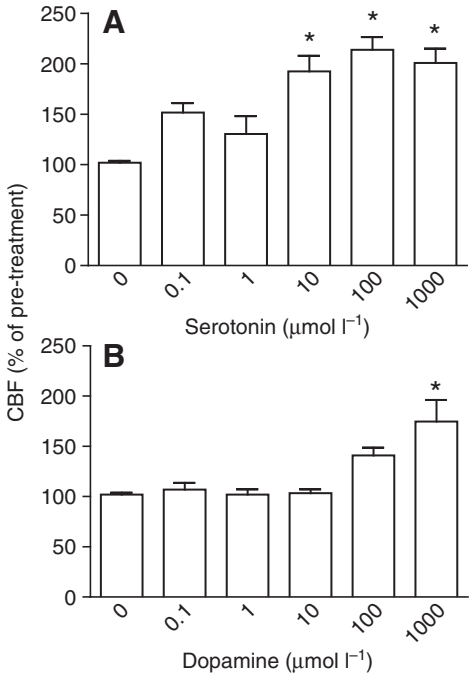

Fig. 4. Concentration-dependent effects of serotonin and dopamine on *L. stagnalis* pedal ciliary explants. The ciliary beat frequency (CBF) during the 5 min exposure to neurotransmitter was calculated by adding the CBF values measured at 1 min intervals, and then normalizing relative to the integrated response obtained in the 5 min prior to the exposure. Each ciliary explant was only exposed to a single application of neurotransmitter. (A) Serotonin (N=5 for each bar). (B) Dopamine (0, 10, 100, 1000 μmol l^{-1}, N=5; 0.1 μmol l^{-1}, N=3; 1 μmol l^{-1}, N=4). Asterisks in A and B represent statistically significant differences from the 0 μmol l^{-1} treatment (P<0.001).

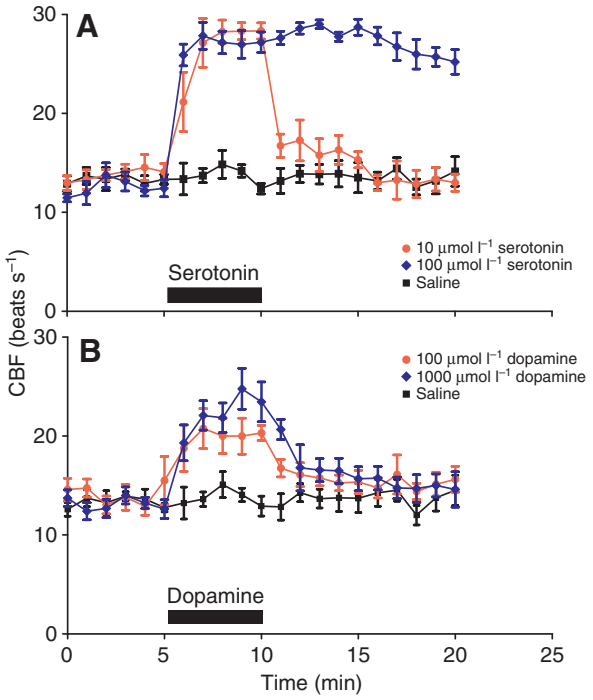

Fig. 5. Time-dependent effects of serotonin and dopamine on *L. stagnalis* pedal ciliary explants. The CBF of pedal cilia was determined at 1 min intervals before, during and following the addition of serotonin (A) and dopamine (B). Bar indicates the duration of exposure to serotonin or dopamine. N=5 for each treatment.

to date. To confirm its antagonist activity in the present study, embryos were exposed to either serotonin (100 μmol l^{-1}), mianserin (100 μmol l^{-1}) or the two combined (Fig. 7). Mianserin on its own had no affect on the rate of embryonic rotation, whereas when presented in combination with serotonin, it abolished the serotonergic response. These data suggest that although serotonin is capable of stimulating embryonic rotation, it is not involved in regulating the rate of rotation under normoxic conditions.

To investigate the dopamine receptor subtype mediating the rotational response to dopamine, the rotational effects of a specific D1 receptor antagonist, SKF83566 (Emaduddin and Takeuchi, 1996), and a specific D2 receptor antagonist, domperidone (Reddymasu et al., 2007), were examined (Fig. 8). SKF83566 (100 μmol l^{-1}) alone caused a significant reduction in embryonic rotation compared with the control rate (P<0.05; Fig. 8A). When applied together with dopamine (both at 100 μmol l^{-1}), SKF83566 completely abolished the dopamine-induced increase in rotation rate (Fig. 8A). In contrast, 100 μmol l^{-1} domperidone had no effect on embryonic rotation when applied alone, and no effect on the dopaminergic response when applied in conjunction with 100 μmol l^{-1} dopamine (Fig. 8B). Together, these results suggest that dopamine stimulates embryonic rotation through activation of a dopamine receptor that is sensitive to D1-type antagonists (see Discussion). Furthermore, the inhibitory effect of SKF83566 on its own suggests that dopamine contributes to the regulation of embryonic rotation under normoxic conditions.

The involvement of serotonergic neurotransmission in hypoxia-stimulated embryonic rotation was tested pharmacologically using the serotonergic antagonist mianserin. Embryonic rotation rate was measured in three groups of *L. stagnalis* embryos at 0, 20 and 60 min. The control group received no anoxia or antagonist treatments and displayed no change in rotation rate over the 1 h experiment, confirming the stability of embryonic rotation rate over this time frame (Fig. 9, white bars). The anoxia group was exposed to anoxia for 15 min prior to the final measurement of rotation rate, which caused a significant elevation of rotation rate (P<0.001; Fig. 9, black bars). The test group was treated with 100 μmol l^{-1} mianserin for 15 min prior to the 20 min measurement, and to anoxia and 100 μmol l^{-1} mianserin for 15 min prior to the 60 min measurement. Mianserin on its own did not affect the baseline rotation rate; however, in combination with the anoxia treatment, mianserin caused a partial reduction of the rotation response to anoxia (P<0.01; Fig. 9, gray bars). These data suggest that serotonin may be partially responsible for mediating the embryonic response to hypoxia.

The same experimental paradigm was used to test the role of dopamine in the hypoxia response (Fig. 10). Because the earlier experiments demonstrated that the dopamine-induced stimulation of embryonic rotation can be selectively blocked by the D1 antagonist SKF83566, this blocker was used in the anoxia experiments. As seen in the previous experiment, the embryonic rotation rate at 60 min in the control group was not different from the 0 and 20 min trials (P>0.05; Fig. 10, white bars), whereas it was significantly elevated in the anoxia group (P<0.001; Fig. 10, black bars). Embryos also displayed the expected decrease in rotation rate in response to 100 μmol l^{-1} SKF83566 on its own (P<0.001; Fig. 10,

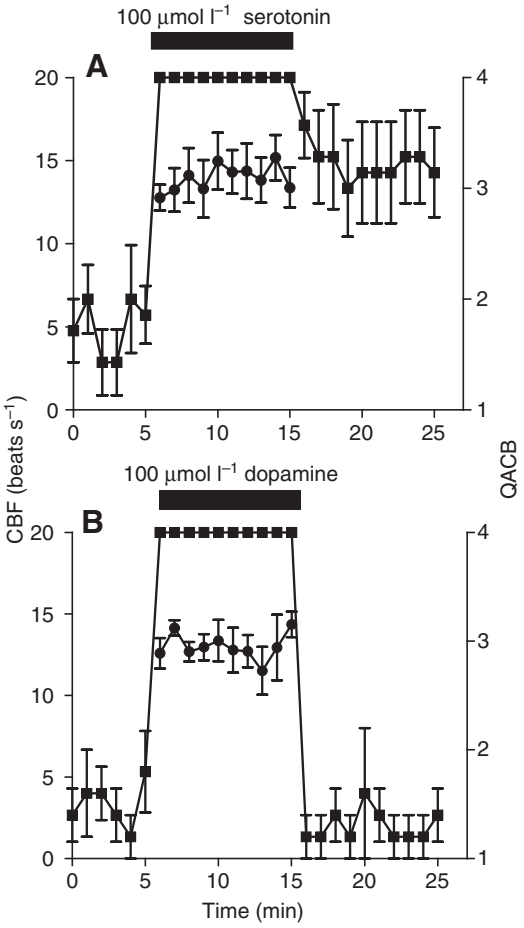

Fig. 6. Effects of serotonin and dopamine on *L. stagnalis* apical plate ciliary explants. The CBF (circles) and qualitative assessment of ciliary beating (QACB; squares) were determined at 1 min intervals before, during and following the addition of serotonin (A) and dopamine (B). The QACB is a four-point scale of ciliary activity (see Results). It was used because apical plate cilia did not beat regularly in the absence of neurotransmitter, preventing accurate CBF measurements. Bar indicates the duration of exposure to serotonin or dopamine. (A) 100 μmol l^{-1} serotonin (N=7); (B) 100 μmol l^{-1} dopamine (N=5).

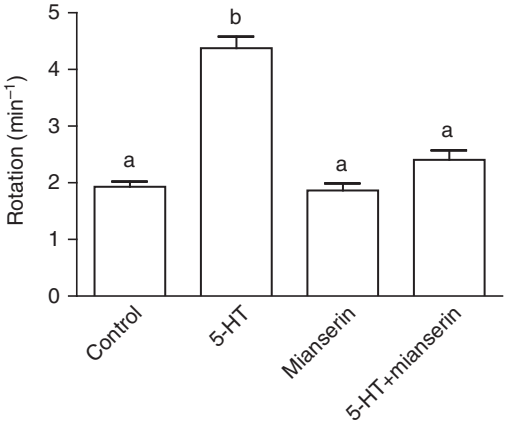

Fig. 7. Antagonistic effect of mianserin on the rotational response to serotonin in *L. stagnalis* embryos. Embryonic rotation rate was measured in embryos treated with artificial pond water (APW; control), 100 μmol l^{-1} serotonin (5-HT), 100 μmol l^{-1} mianserin (mianserin), or 100 μmol l^{-1} serotonin and 100 μmol l^{-1} mianserin together (5-HT+mianserin). Bars with different letter designations had rotation rates that were significantly different (P<0.001). Each bar represents data from at least 10 embryos taken from four egg masses.

gray bars at 20 min). Most importantly, when presented in combination with the anoxia treatment, 100 μmol l^{-1} SKF83566 completely eliminated the anoxia response and lowered the rotation rate below control levels. Collectively, these results suggest that dopaminergic neurotransmission plays the primary role in the signal transduction of the hypoxia response in *L. stagnalis* embryos.

DISCUSSION

This study describes a set of experiments on rotational behavior in embryos of the pond snail *L. stagnalis* as a means for comparison with the well-established *H. trivolvis* model system. As members of the pulmonate families Lymnaeidae and Planorbidae, respectively, these species share a common ancestor that was part of the successful invasion of freshwater environments by the earliest pulmonates (Jorgensen et al., 2004; Klussmann-Kolb et al., 2008). Although the development of a rudimentary lung was a major element of this transition, switching from planktotrophic larval development to lecithotrophic direct development was also crucial for adapting to freshwater environments. In comparison to the relative stability of the marine environment, freshwater environments undergo huge variations in food availability, oxygen concentration, temperature, pH and salinity (Chang and Ouyang, 1988; Cole, 1994; Shartau et al., 2010a). By developing inside egg capsules that are aggregated into egg masses, embryos are ensured an adequate food supply, relatively stable environment and excellent protection from predators and microbial infection (Kamiya et al., 1984; Benkendorff et al., 2001; Benkendorff et al., 2005). However, encapsulated embryos still rely on an adequate supply of environmental oxygen for their survival (Shartau et al., 2010b), and thus display behavioral adaptations that help mitigate the effects of hypoxia, most notably faster rotation. An indicator of the importance of such adaptations is how well they are conserved between different taxa. Phylogenetic studies on gastropods indicate that, within the pulmonates, the planorbids are closely related to the physids and more distantly related to the lymnaeids (Klussmann-Kolb et al., 2008). This degree of phylogenetic distance predicts that various structural and physiological characters in the common ancestor of *H. trivolvis* and *L. stagnalis* will have diverged over evolutionary time. These species are therefore good models to examine how essential adaptations such as rotational responses to hypoxia in encapsulated embryos are mediated in organisms that have distinct structural and physiological machinery.

The present study on *L. stagnalis*, along with earlier studies on both *L. stagnalis* and *H. trivolvis*, have elucidated various similarities and differences in the embryonic rotation behavior between these two model species. The basic behavior itself appears quite similar in both species, with the rotation having two underlying components: periods of slow rotation and transient accelerations called surges (Fig. 2A) (Diefenbach et al., 1991; Cole et al., 2002). Furthermore, the hypoxia-induced stimulation of embryonic rotation is similar in both species, including the time course of the response to hypoxia (Kuang et al., 2002), the post-anoxia recovery of rotation rate (Kuang et al., 2002; Goldberg et al., 2008; Byrne et al., 2009), the oxygen concentration–response relationship (Kuang et al., 2002; Byrne et al., 2009) and the repeatability of the response to multiple episodes of hypoxia (Kuang et al., 2002; Byrne et al., 2009) (Fig. 2B). In

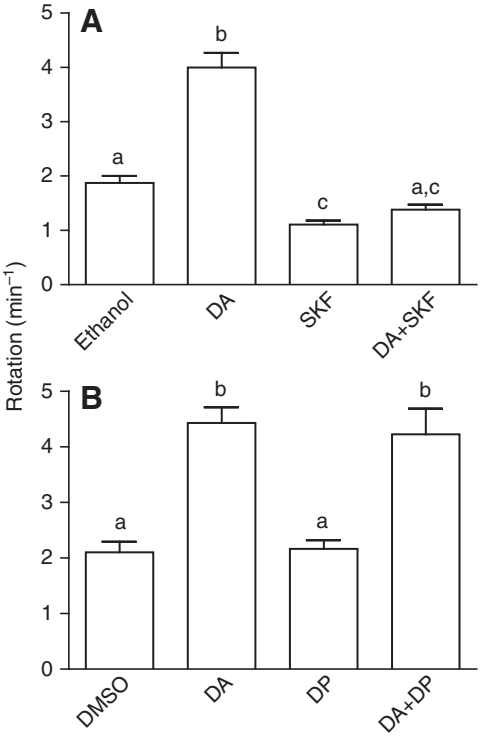

Fig. 8. Antagonistic effect of SKF83566 and domperidone on the rotational response to dopamine in *L. stagnalis* embryos. (A) Embryonic rotation rate was measured in embryos treated with 0.01% ethanol, 100 μmol l^{-1} dopamine (DA), 100 μmol l^{-1} SKF83566 (SKF), or 100 μmol l^{-1} DA and 100 μmol l^{-1} SKF83566 together (DA+SKF). All treatments included the 0.01% ethanol vehicle. Each bar represents data from at least 10 embryos taken from five egg masses. Bars with different letter designations had rotation rates that were significantly different (ethanol *vs* SKF, P<0.05; other differences, P<0.001). (B) Embryonic rotation rate was measured in embryos treated with 0.1% DMSO, 100 μmol l^{-1} DA, 100 μmol l^{-1} domperidone (DP), or 100 μmol l^{-1} DA and 100 μmol l^{-1} DP together (DA+DP). All treatments included the 0.1% DMSO vehicle. Each bar represents data from at least 10 embryos taken from four egg masses, except DA, where data are from five embryos taken from four egg masses. In both A and B, bars with different letter designations had rotation rates that were significantly different (P<0.001).

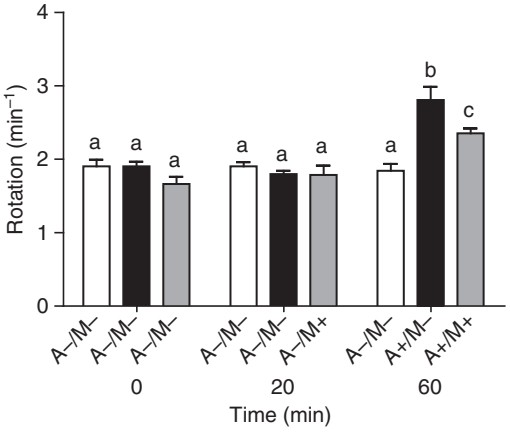

Fig. 9. Effect of mianserin on anoxia-stimulated rotation in *L. stagnalis* embryos. Embryonic rotation rate was measured at time 0, 20 and 60 min in three groups of embryos. The control group (white bars) was not treated with anoxia or mianserin at any time point (A–/M–). The anoxia group (black bars) was exposed to anoxia for 15 min prior to the final measurement of rotation rate (A+/M–). The test group (gray bars) was treated with 100 μM mianserin for 15 min prior to the second measurement of rotation rate (A–/M+), and to anoxia and 100 μmol l^{-1} mianserin for 15 min prior to the final measurement of rotation rate (A+/M+). Bars with different letter designations had rotation rates that were significantly different (P<0.01). Each group contained 14 embryos taken from five egg masses.

contrast, minor behavioral differences between the two species include a consistently faster rotation rate in *L. stagnalis*, as well as greater variation in the basal rate of rotation occurring between surges (Fig. 2A) (Diefenbach et al., 1991; Cole et al., 2002). Overall, the high level of behavioral conservation between the two species suggests that the hypoxia-induced increase in embryonic rotation is an essential adaptation that allows pulmonate embryos to withstand periods of reduced oxygen levels inside the egg capsule (Goldberg et al., 2008).

Given the morphological and physiological changes that have occurred in the Lymnaeidae and Planorbidae families since their divergence, it is quite remarkable that so many aspects of the rotation behavior are similar in the two groups. For example, the embryonic neurons thought to be the primary neural control elements of the behavior have diverged significantly. The ENC1 neurons of *H. trivolvis* are sensory-motor neurons that function both in hypoxia sensation and cilioexcitation (Kuang and Goldberg, 2001; Kuang et al., 2002). These serotonergic neurons have a short apical dendrite tipped with a sensory specialization on the embryonic surface, as well as a primary descending axon that innervates both the dorsolateral and pedal bands of cilia (Diefenbach et al., 1998; Koss et al., 2003). The TAC neurons of *L. stagnalis* are thought to be homologues of ENC1, with similar features including cell body and dendritic morphology, embryonic location, time of occurrence, transient expression of amine neurotransmitter and innervation of ciliary tissues (Voronezhskaya et al., 1999). However, the TAC neurons differ from ENC1 in that they primarily contain the catecholamine neurotransmitter dopamine instead of serotonin, and they innervate the medial-dorsal apical plate region rather than the dorsolateral and pedal bands of cilia. Although this suggests that dopamine may be the primary cilioexcitatory neurotransmitter in *L. stagnalis* embryos, serotonin is also present and capable of stimulating embryo rotation (Filla et al., 2009), and may therefore contribute to the regulation of this behavior. In addition to these neuronal differences, the apical plate region is only ciliated in *L. stagnalis* embryos, and may therefore participate in driving embryo rotation in lymnaeids.

Characterization of pedal and apical plate cilia

Ciliated pedal and apical plate tissues were isolated for *in vitro* analysis of CBF, the first ever examination of specific ciliary subtypes in *L. stagnalis*. Although this was initially done to determine their relative sensitivity to serotonin and dopamine in the absence of the diffusion barriers inherent in the whole embryo experiments, additional unexpected characteristics of the apical plate cilia were identified. Morphologically, the apical plate had a denser distribution of cilia than the other ciliary structures (Fig. 1). Functionally, the apical plate cilia displayed a unique pattern of ciliary beating in comparison to the more conventional constant and synchronous beating of the pedal cilia. Under control conditions, apical plate ciliary beating was temporally and spatially sporadic. Short bouts of ciliary beating occurred randomly in small patches of the isolated tissue, making assessment of CBF nearly impossible. As it is unlikely that the isolated ciliary tissue receives any neural

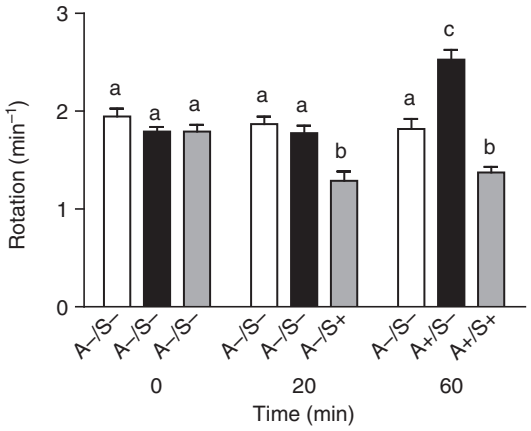

Fig. 10. Effect of SKF83566 on anoxia-stimulated rotation in *L. stagnalis* embryos. Embryonic rotation rate was measured at time 0, 20 and 60 min in three groups of embryos. The control group (white bars) was not treated with anoxia or SKF83566 at any time point (A–/S–). The anoxia group (black bars) was exposed to anoxia for 15 min prior to the final measurement of rotation rate (A+/S–). The test group (gray bars) was treated with 100 μmol l^{-1} SKF83566 for 15 min prior to the second measurement of rotation rate (A–/S+), and to anoxia and 100 μmol l^{-1} SKF83566 for 15 min prior to the final measurement of rotation rate (A+/S+). Bars with different letter designations had rotation rates that were significantly different (P<0.001). Each group contained 12–13 embryos taken from six egg masses.

input, this pattern appears to be an inherent property of this ciliary subtype. Moreover, observation of apical plate cilia in intact embryos revealed a similar sporadic beating pattern (S.P.M. and J.I.G., unpublished observations), suggesting that it is not an artifact of tissue isolation. Although in most systems ciliary beating is either arrested or more uniformly active, the sporadic beating pattern in unstimulated apical plate cilia is reminiscent of *Chlamydomonas reinhardtii* cilia. Each of these unicellular green algae contains two cilia that display unique, independent beat responses to light stimuli (Josef et al., 2005). Whether the intracellular mechanisms giving rise to the independent activities of neighboring cilia are similar in these two very different model systems remains to be determined.

Unlike the apical plate cilia, the pedal cilia displayed continuous beating under control conditions throughout the field of cilia. This was the same beat pattern reported for pedal and dorsolateral cilia of *H. trivolvis* (Doran et al., 2004). In contrast, pedal cilia isolated from *L. stagnalis* consistently beat at approximately 12–14 beats s^{-1} under control conditions, which is approximately 4–6 beats s^{-1} faster than the baseline rates observed in the various studies on isolated pedal cilia of *H. trivolvis* (Christopher et al., 1999; Doran et al., 2004; Doran and Goldberg, 2006). This difference is likely one of the factors causing *L. stagnalis* embryos to rotate faster than *H. trivolvis* embryos under control conditions (Diefenbach et al., 1991; Shartau et al., 2010b). Additional factors, including ciliary biomechanics, the additional apical plate cilia and capsule fluid viscosity, may also contribute to the faster rotation displayed by *L. stagnalis*.

A previous study established that both serotonin and dopamine can stimulate embryonic rotation when applied to whole embryos (Filla et al., 2009). The present study aimed to determine the relative roles of these neurotransmitters and the different ciliary structures in mediating whole-embryonic rotational responses, in part by examining isolated pedal and apical plate ciliary tissues. Earlier experiments comparing the effects of serotonin on embryonic rotation and ciliary activity in *H. trivolvis* showed that encapsulated embryos were less sensitive than isolated ciliary cells, likely as a result of the additional diffusion barriers associated with encapsulation (Goldberg et al., 1994). Although we expected to confirm that embryonic rotation was less sensitive than isolated tissues to neurotransmitters in the present study, the difficulty in generating concentration–response curves for apical plate cilia prevented a satisfactory test of this hypothesis.

The experiments testing the selectivity and sensitivity of isolated ciliary cells to dopamine and serotonin also produced some unexpected findings. We hypothesized that apical plate cilia would be selectively sensitive to dopamine because they are innervated by the dopaminergic TAC neurons (Voronezhskaya et al., 1999). Similarly, we hypothesized that pedal cilia would be selective for serotonin because of their extensive innervation by serotonergic fibers later in embryonic development (Longley, 2008). However, neither the apical plate nor pedal cilia were selective, as both neurotransmitters effectively stimulated CBF in both types of cilia (Figs 4, 5). There are various possibilities regarding the functional significance of this lack of selectivity. First, serotonin and dopamine receptors may be ectopically expressed and not functional in apical plate and pedal cilia, respectively. Second, there may be only a single receptor type that has cross-reactivity to both neurotransmitters. The very high concentration of dopamine required to stimulate pedal cilia may reflect such cross-reactivity, with dopamine acting upon pedal serotonin receptors. Third, ciliary activation may occur through a combination of synaptic and paracrine signaling, such that both types of cilia are normally exposed to both serotonin and dopamine. Although more experiments are required to distinguish between these possibilities, the idea that paracrine signaling contributes to the regulation of embryonic rotation is also favored. At the early embryonic stages when the rotational behavior occurs, embryos have a geometrically simple, non-compartmentalized structure that would facilitate the diffusion of released neurotransmitters to non-synaptic active sites (Morrill, 1982).

The sensitivity of isolated ciliary tissues to serotonin and dopamine was best assessed for pedal cilia because their activation by neurotransmitters involved an increase in CBF from basal values, allowing for easy construction of concentration–response relationships. Paradoxically, serotonin had greater potency than dopamine in stimulating pedal cilia (Fig. 4), but similar potency to dopamine in stimulating embryonic rotation (Fig. 3). Furthermore, dopamine produced statistically significant rotational responses at lower concentrations compared with its effects on pedal CBF. Together, these differential sensitivities strongly suggest that the pedal cilia do not play the primary role in driving embryonic rotation. Therefore, the apical plate cilia are likely the primary drivers of rotation. Consistent with this interpretation is the innervation of the pedal and apical plate cilia at the time when embryonic rotation occurs. The dopaminergic TAC neurons selectively innervate the apical plate (Voronezhskaya et al., 1999), whereas the initial innervation of the pedal cilia by serotonergic neurons occurs at later stages, suggesting that these cilia are primarily involved in mediating the latent crawling behavior (Longley, 2008).

Interestingly, the reversibility of ciliary activation upon washout of the neurotransmitter was different for serotonin and dopamine, with stronger recovery occurring after washout of high concentrations of dopamine compared with serotonin (Fig. 5). This difference in response characteristics suggests that there are indeed distinct receptors that mediate the serotonergic and dopaminergic responses, contrary to one of the possibilities raised above. In *H. trivolvis* embryos, cilia contain at least two different serotonin

receptors, 5-HT_{1Hel} and 5-HT_{7Hel}, and it has been proposed that one of these is involved in prolongation of the response (Mapara et al., 2008). The results of the present study suggest that *L. stagnalis* also has a serotonin receptor that mediates the prolonged response. A potential candidate would be the 5-HT_{1Lym} serotonin receptor, which is a closely related homolog of 5-HT_{1Hel} (Sugamori et al., 1993; Mapara et al., 2008). It will be interesting to determine whether this receptor is expressed in ciliary tissues of *L. stagnalis* embryos and functions to produce sustained responses.

Pharmacological analysis of the hypoxia response

As an initial step in testing the role of serotonin and dopamine in mediating the hypoxia response, three receptor antagonists were tested for their ability to block the rotational responses to serotonin and dopamine. The aim of these experiments was to identify effective blockers, rather than establish the receptor subtypes involved in the responses. Molecular cloning studies of invertebrate receptors have established that members of specific receptor families as determined by sequence homology do not reliably recapitulate the pharmacological profile of agonist and antagonist activity determined for the homologous vertebrate receptors (Tierney, 2001; Mustard et al., 2005). For example, if a dopamine D1 receptor blocker is effective in blocking the dopamine-induced rotation response in *L. stagnalis*, it does not definitively mean that the response is mediated by a member of the DOP1 receptor family (dopamine D1), as opposed to one of the two other known families of invertebrate dopamine receptors (Mustard et al., 2005). Nevertheless, if it effectively blocks the dopaminergic response, it can be used to determine if the hypoxia-induced increase in rotation rate action is mediated by dopamine.

The dopamine D1 antagonist SKF83566 fully blocked the rotational response to dopamine, and inhibited the basal rate of rotation when applied on its own (Fig. 8A). In contrast, the dopamine D2 antagonist domperidone was ineffective (Fig. 8B), consistent with other studies on gastropods in which responses were typically blocked by only one of these antagonist types (Mukai et al., 2004; Dobson et al., 2006). The ability of SKF83566 to lower the rate of rotation indicates that the cilia driving embryonic rotation receive tonic dopaminergic stimulation. Perhaps the prominent surges revealed in the analysis of rotational dynamics (Fig. 2A) are dopamine-induced events associated with bursts of activity in the TAC neurons. These data are consistent with the TAC neurons of *L. stagnalis* functioning very similarly to their ENC1 homologs in *H. trivolvis* (Kuang and Goldberg, 2001), despite having a different primary neurotransmitter.

The serotonin receptor antagonist mianserin, although completely effective in blocking the rotational response to serotonin, had no effect on the basal rate of rotation when applied on its own (Fig. 7). Therefore, despite the fact that pedal cilia are more sensitive to serotonin, dopamine appears to be the primary signal controlling embryonic rotation under control conditions. However, serotonin may help mediate the hypoxia response, which was partially blocked by mianserin (Fig. 9). Whether this occurs through the activation of additional serotonergic neurons by hypoxia or through the release of serotonin as a secondary transmitter in the TAC neurons is unknown. The former possibility seems unlikely because the earliest neurons displaying serotonin-like immunoreactivity first appear at stage E37–38 (Marois and Croll, 1992), whereas hypoxia responses occur as early as stage E30 (Byrne et al., 2009). More likely is the latter possibility, which is supported by an earlier study that indicated that the TAC neurons contain not only tyrosine hydroxylase, but also FMRFmide and serotonin in lesser amounts (Voronezhskaya et al., 1999). In this scenario, hypoxia-induced activation of the TAC neuron would cause it to co-release dopamine and serotonin, whereas it releases only dopamine as its primary neurotransmitter during its basal activity state under normal levels of oxygen. Further experiments are required to test the validity of this hypothesis.

As mentioned above, it is necessary to be cautious when employing a pharmacological approach in a functional study on invertebrate systems. Drugs are typically developed for well-characterized vertebrate systems, and despite this, most drugs show a lack of complete specificity even in vertebrates. Mianserin, for example, has antagonist activity on vertebrate adrenergic and histamine receptors in addition to its well-established role in blocking 5-HT_2 serotonin receptors (Peroutka and Snyder, 1981; Pinder, 1985). Furthermore, it is also an effective antagonist of octopamine responses in invertebrates (Roeder, 1994). It is not surprising, therefore, that we observed some cross-reactivity by the drugs employed in the present study (data not shown). Nevertheless, with the ability of mianserin to fully block the serotonergic response, only partially block the hypoxia response and have no effect on basal rotation, these data strongly discount serotonin as the primary regulator of rotational behavior in *L. stagnalis* embryos. Although the evidence strongly favors dopamine in this role, its final confirmation awaits the molecular identification and experimental knockdown of the specific neurotransmitter receptors involved in regulating the rotation behavior.

In conclusion, the analysis of embryonic rotation complemented by experiments on isolated patches of identified ciliary subtypes strongly suggests that the TAC–apical plate cilia neural circuit controls the hypoxia-induced rotational response, as well as the rotational behavior under normoxic conditions. Although the pedal cilia are active and capable of responding to both serotonin and dopamine in *L. stagnalis* embryos, these cilia are not the primary drivers of embryonic rotation. In future studies, *in situ* analysis of apical plate and pedal cilia could reveal whether the pedal cilia display surges in activity in parallel with those displayed by the apical plate cilia (Kuang and Goldberg, 2001). This would determine whether dopamine released from the TAC neurons also causes paracrine activation of the pedal cilia as a secondary pathway driving the embryonic rotation behavior. In the more derived planorbid *H. trivolvis* (Klussmann-Kolb et al., 2008), embryonic rotation is regulated through direct innervation of the pedal cilia by the TAC homolog ENC1 (Kuang and Goldberg, 2001; Kuang et al., 2002; Koss et al., 2003). Thus, switching the TAC neurotransmitter phenotype from dopamine to serotonin, loss of cilia from the apical plate region and re-routing the neurite projections from the apical plate cilia to the pedal cilia appear to be evolutionary events that afforded planorbids greater efficiency in producing an essential behavior. These steps eliminated the need for embryos to develop and power the apical plate cilia, given that the pedal cilia were already present and functional in anticipation of their role in mediating the crawling behavior at later stages. Because the family Physidae are thought to share a more recent common ancestor with the Planorbidae, with the Lymnaeidae more distantly related (Klussmann-Kolb et al., 2008), it will be interesting to determine whether this proposed evolutionary pattern also occurs in *Physa gyrina*, whose encapsulated embryos display similar rotational responses to hypoxia as seen in *H. triviolvis* and *L. stagnalis*.

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REFERENCES

Benkendorff, K., Davis, A. R. and Bremner, J. B. (2001). Chemical defense in the egg masses of benthic invertebrates: an assessment of antibacterial activity in 39 mollusks and 4 polychaetes. *J. Invertebr. Pathol.* **78**, 109-118.

Benkendorff, K., Davis, A. R., Rogers, C. N. and Bremnerc, J. B. (2005). Free fatty acids and sterols in the benthic spawn of aquatic molluscs, and their associated antimicrobial properties. *J. Exp. Mar. Biol. Ecol.* **316**, 29-44.

Booth, D. T. (1995). Oxygen availability and embryonic development in sand snail (*Polinices sordidus*) egg masses. *J. Exp. Biol.* **198**, 241-247.

Brown, K. M. (1991). Mollusca: Gastropoda. In *Ecology and Classification of North American Freshwater Invertebrates* (ed. J. H. Thorpe and A. P. Covich), pp. 285-314. San Diego, CA: Academic Press.

Burggren, W. (1985). Gas exchange, metabolism and ventilation in gelatinous frog egg masses. *Physiol. Zool.* **58**, 503-514.

Byrne, R. A., Rundle, S. D., Smirthwaite, J. J. and Spicer, J. I. (2009). Embryonic rotational behaviour in the pond snail *Lymnaea stagnalis*: influences of environmental oxygen and development stage. *Zoology* **112**, 471-477.

Chaffee, C. and Strathmann, R. R. (1984). Constraints on egg masses. I. Retarded development within thick egg masses. *J. Exp. Mar. Biol. Ecol.* **84**, 73-83.

Chang, Y. B. and Ouyang, H. (1988). Dynamics of dissolved oxygen and vertical circulation in fish ponds. *Aquaculture* **74**, 263-276.

Christopher, K. J., Young, K. G., Chang, J. P. and Goldberg, J. I. (1999). Involvement of protein kinase C in 5-HT-stimulated ciliary activity in *Helisoma trivolvis* embryos. *J. Physiol.* **515**, 511-522.

Clarke, A. H. (1981). *The Freshwater Molluscs of Canada*. Ottawa: National Museum of Natural Sciences, National Museums of Canada.

Cole, A. G., Mashkournia, A., Parries, S. C. and Goldberg, J. I. (2002). Regulation of early embryonic behavior by nitric oxide in the pond snail *Helisoma trivolvis*. *J. Exp. Biol.* **205**, 3143-3152.

Cole, G. A. (1994). *Textbook of Limnology*. Long Grove, IL: Waveland Press Inc.

Diefenbach, T. J., Koehncke, N. K. and Goldberg, J. I. (1991). Characterization and development of rotational behavior in *Helisoma* embryos: role of endogenous serotonin. *J. Neurobiol.* **22**, 922-934.

Diefenbach, T. J., Koss, R. and Goldberg, J. I. (1998). Early development of an identified serotonergic neuron in *Helisoma trivolvis* embryos: serotonin expression, de-expression, and uptake. *J. Neurobiol.* **34**, 361-376.

Dobson, K. S., Dmetrichuk, J. M. and Spencer, G. E. (2006). Different receptors mediate the electrophysiological and growth cone responses of an identified neuron to applied dopamine. *Neuroscience* **141**, 1801-1810.

Doran, S. A. and Goldberg, J. I. (2006). Roles of Ca^{2+} and protein kinase C in the excitatory response to serotonin in embryonic molluscan ciliary cells. *Can. J. Physiol. Pharmacol.* **84**, 635-646.

Doran, S. A., Koss, R., Tran, C. H., Christopher, K. J., Gallin, W. J. and Goldberg, J. I. (2004). Effect of serotonin on ciliary beating and intracellular calcium concentration in identified populations of embryonic ciliary cells. *J. Exp. Biol.* **207**, 1415-1429.

Emaduddin, M. and Takeuchi, H. (1996). Lineweaver-Burk analysis for the blocking effects of mammalian dopamine receptor antagonists on dopamine-induced currents in *Achatina* giant neurones. *Gen. Pharmacol.* **27**, 1209-1213.

Filla, A., Hiripi, L. and Elekes, K. (2009). Role of aminergic (serotonin and dopamine) systems in the embryogenesis and different embryonic behaviors of the pond snail, *Lymnaea stagnalis*. *Comp. Biochem. Physiol.* **149C**, 73-82.

Giorgi, A. E. and Congleton, J. L. (1984). Effects of current velocity on development and survival of ling cod, *Ophiodon elongata*, embryos. *Environ. Biol. Fish* **10**, 15-27.

Goldberg, J. I., Koehncke, N. K., Christopher, K. J., Neumann, C. and Diefenbach, T. J. (1994). Pharmacological characterization of a serotonin receptor involved in an early embryonic behavior of *Helisoma trivolvis*. *J. Neurobiol.* **25**, 1545-1557.

Goldberg, J. I., Doran, S. A., Shartau, R. B., Pon, J. R., Ali, D. W., Tam, R. and Kuang, S. (2008). Integrative biology of an embryonic respiratory behaviour in pond snails: the 'embryo stir-bar hypothesis'. *J. Exp. Biol.* **211**, 1729-1736.

Hadfield, M. G., Meleshkevitch, E. A. and Boudko, D. Y. (2000). The apical sensory organ of a gastropod veliger is a receptor for settlement cues. *Biol. Bull.* **198**, 67-76.

Hunter, T. and Vogel, S. (1986). Spinning embryos enhance diffusion through gelatinous egg masses. *J. Exp. Mar. Biol. Ecol.* **96**, 303-308.

Hurst, A. (1967). The egg masses and veligers of thirty Northeast Pacific opithobranchs. *Veliger* **9**, 255-288.

Jorgensen, A., Kristensen, T. K. and Stothard, J. R. (2004). An investigation of the "Ancyloplanorbidae" (Gastropoda, Pulmonata, Hygrophila): preliminary evidence from DNA sequence data. *Mol. Phylogenet. Evol.* **32**, 778-787.

Josef, K., Saranak, J. and Foster, K. W. (2005). Ciliary behavior of a negatively phototactic *Chlamydomonas reinhardtii*. *Cell Motil. Cytoskeleton* **61**, 97-111.

Kamiya, H., Muramoto, K. and Ogata, K. (1984). Antibacterial activity in the egg mass of a sea hare. *Experientia* **40**, 947-949.

Kempf, S. C., Page, L. and Pires, A. (1997). Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. *J. Comp. Neurol.* **386**, 507-528.

Klussmann-Kolb, A., Dinapoli, A., Kuhn, K., Streit, B. and Albrecht, C. (2008). From sea to land and beyond – new insights into the evolution of euthyneuran Gastropoda (Mollusca). *BMC Evol. Biol.* **8**, 57.

Koss, R., Diefenbach, T. J., Kuang, S., Doran, S. A. and Goldberg, J. I. (2003). Coordinated development of identified serotonergic neurons and their target ciliary cells in *Helisoma trivolvis* embryos. *J. Comp. Neurol.* **457**, 313-325.

Kuang, S. and Goldberg, J. I. (2001). Laser ablation reveals regulation of ciliary activity by serotonergic neurons in molluscan embryos. *J. Neurobiol.* **47**, 1-15.

Kuang, S., Doran, S. A., Wilson, R. J., Goss, G. G. and Goldberg, J. I. (2002). Serotonergic sensory-motor neurons mediate a behavioral response to hypoxia in pond snail embryos. *J. Neurobiol.* **52**, 73-83.

Longley, R. D. (2008). Development of the 5-HT-like immunoreactive pedal plexus in the pond snail *Lymnaea stagnalis appressa*. *Biol. Bull.* **215**, 280-294.

Mapara, S., Parries, S., Quarrington, C., Ahn, K. C., Gallin, W. J. and Goldberg, J. I. (2008). Identification, molecular structure and expression of two cloned serotonin receptors from the pond snail, *Helisoma trivolvis*. *J. Exp. Biol.* **211**, 900-910.

Marois, R. and Croll, R. P. (1992). Development of serotonin-like immunoreactivity in the embryonic nervous system of the snail *Lymnaea stagnalis*. *J. Comp. Neurol.* **322**, 255-265.

Mills, N. E. and Barnhart, M. C. (1999). Effects of hypoxia on embryonic development in two *Ambystoma* and two *Rana* species. *Physiol Biochem Zool* **72**, 179-188.

Mills, N. E., Barnhart, M. C. and Semlitsch, R. D. (2001). Effects of hypoxia on egg capsule conductance in *Ambystoma* (Class Amphibia, Order Caudata). *J. Exp. Biol.* **204**, 3747-3753.

Morrill, J. G. (1982). Development of the pulmonate gastropod, *Lymnea*. In *Developmental Biology of Freshwater Invertebrates* (ed. F. W. Harrison and R. R. Cowden), pp. 399-483. New York: Alan R. Liss Inc.

Mukai, S. T., Kiehn, L. and Saleuddin, A. S. M. (2004). Dopamine stimulates snail albumen gland glycoprotein secretion through activation of a D1-like receptor. *J. Exp. Biol.* **207**, 2505-2518.

Mustard, J. A., Beggs, K. T. and Mercer, A. R. (2005). Molecular biology of the invertebrate dopamine receptors. *Arch. Insect Biochem. Physiol.* **59**, 103-117.

Peroutka, S. J. and Snyder, S. H. (1981). [^{3}H]Mianserin: differential labeling of serotonin and histamine receptors in rat brain. *J. Pharmacol. Exp. Ther.* **216**, 142-148.

Pinder, A. W. and Friet, S. C. (1994). Oxygen transport in egg masses of the amphibians *Rana sylvatica* and *Ambystoma maculatum*: convection, diffusion and oxygen production by algae. *J. Exp. Biol.* **197**, 17-30.

Pinder, R. M. (1985). Adrenoreceptor interactions of the enantiomers and metabolites of mianserin: are they responsible for the antidepressant effect? *Acta Psychiatr. Scand. Suppl.* **320**, 1-9.

Reddymasu, S. C., Soykan, I. and McCallum, R. W. (2007). Domperidone: review of pharmacology and clinical applications in gastroenterology. *Am. J. Gastroenterol.* **102**, 2036-2045.

Roeder, T. (1994). Biogenic amines and their receptors in insects. *Comp. Biochem. Physiol.* **107C**, 1-12.

Salthe, S. N. (1963). The egg capsules in the Amphibia. *J. Morph.* **113**, 161-171.

Seymour, R. S. and Bradford, D. F. (1987). Gas exchange through the jelly capsule of the terrestrial eggs of the frog, *Pseudophryne bibroni*. *J. Comp. Physiol. B* **157**, 477-481.

Seymour, R. S. and Roberts, J. D. (1991). Embryonic respiration and oxygen distribution in foamy and nonfoamy egg masses of the frog *Limnodynastes tasmaniensis*. *Physiol. Zool.* **64**, 1322-1340.

Seymour, R. S., Roberts, J. D., Mitchell, N. J. and Blaylock, A. J. (2000). Influence of environmental oxygen on development and hatching of aquatic eggs of the australian frog, *Crinia georgiana*. *Physiol. Biochem. Zool.* **73**, 501-507.

Shartau, R. B., Harris, S., Boychuk, E. C. and Goldberg, J. I. (2010a). Rotational behaviour of encapsulated pond snail embryos in diverse natural environments. *J. Exp. Biol.* **213**, 2086-2093.

Shartau, R. B., Tam, R., Patrick, S. and Goldberg, J. I. (2010b). Serotonin prolongs survival of encapsulated pond snail embryos exposed to long-term anoxia. *J. Exp. Biol.* **213**, 1529-1535.

Strathmann, R. R. and Chaffee, C. (1984). Constraints of egg masses. II. Effect of spacing, size, and number of eggs on ventilation of masses of embryo in jelly, adherent groups, or thin-walled capsules. *J. Exp. Mar. Biol. Ecol.* **84**, 85-93.

Sugamori, K. S., Sunahara, R. K., Guan, H.-C., Bulloch, A. G. M., Tensen, C. P., Seeman, P., Niznik, H. B. and Van Tol, H. H. M. (1993). Serotonin receptor cDNA cloned from *Lymnaea stagnalis*. *Proc. Natl. Acad. Sci. USA* **90**, 11-15.

Tierney, A. J. (2001). Structure and function of invertebrate 5-HT receptors: a review. *Comp. Biochem. Physiol.* **128A**, 791-804.

Voronezhskaya, E. E., Hiripi, L., Elekes, K. and Croll, R. P. (1999). Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis*: I. Embryonic development of dopamine-containing neurons and dopamine-dependent behaviors. *J. Comp. Neurol.* **404**, 285-296.