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Serotonin prolongs survival of encapsulated pond snail embryos exposed to long-term anoxia

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SUMMARY

Embryos of the pond snail, *Helisoma trivolvis*, develop bilateral serotonergic neurons that innervate ciliary bands and stimulate cilia-driven rotation. This behaviour is postulated to increase oxygen availability during hypoxia by mixing the capsular fluid. We hypothesised that the stimulation of ciliary-driven rotation by serotonin (5-HT) enhances the survival of embryos during prolonged hypoxia. Embryo rotation and survival were monitored in different levels of oxygen for 24–48 h while in the presence or absence of 5-HT (100 μmol l^{-1}) or a 5-HT antagonist (50 μmol l^{-1}). Long-term hypoxia caused delayed embryonic development that appeared morphologically normal. Hypoxia also induced a transient increase in rotation rate in embryos exposed to artificial pond water (APW) or 5-HT that lasted around 3 h. 5-HT-treated embryos had an elevated rotation rate over embryos in APW throughout the long-term exposure to hypoxia. Long-term anoxia also induced a transient increase in rotation rate in embryos exposed to APW or 5-HT. Rotation ceased in embryos exposed to APW by 13 h but persisted in 5-HT-treated embryos for up to 40 h. Fifty per-cent mortality was reached at 9 h of anoxia in embryos in APW and at 24 h in 5-HT-treated embryos. The 5-HT antagonist mianserin partially inhibited the 5-HT enhancement of rotation but not the prolongation of survival in anoxia. The ability of 5-HT to prolong survival in anoxia reveals a 5-HT-activated metabolic pathway that liberates an alternative energy source.

Key words: *Helisoma trivolvis*, energetics, anoxia, mortality, serotonin, embryonic development.

INTRODUCTION

Cilia-driven rotational behaviour in embryos of the pond snail *Helisoma trivolvis* has been established as the primary behaviour by which embryos respond to their environment during early development. Hypoxia is thought to be the primary environmental factor affecting rotational behaviour, acting directly upon the sensory-motor embryonic neuron C1 (ENC1) (Kuang et al., 2002a). Embryonic rotation is stimulated by the intermittent release of serotonin (5-HT) from ENC1 onto target ciliary cells (Diefenbach et al., 1991; Goldberg et al., 1994; Kuang and Goldberg, 2001; Kuang et al., 2002b). Application of the 5-HT antagonist mianserin, as well as laser-stimulation and laser-ablation experiments, helped confirm ENC1's role in regulating rotation through a 5-HT pathway (Goldberg et al., 1994; Kuang and Goldberg, 2001).

Helisoma embryos develop in membrane-bound egg masses that contain multiple fluid-filled egg capsules containing individual embryos. These structures, along with the capsular fluid (CF), create diffusion barriers that impede the movement of gases and metabolic wastes. Large diffusion gradients of O_2 have been found in the egg capsules of *H. trivolvis*, with O_2 highest at the egg capsule membrane and lowest at the embryo (Kuang et al., 2002a). It has been postulated that embryonic rotation is used to stir the CF in order to minimise diffusion gradients (Hunter and Vogel, 1986; Goldberg et al., 2008). As this behaviour is stimulated during hypoxia, it is believed that increased rotation promotes greater mixing of the CF and improved delivery of O_2 to the embryo (Goldberg et al., 2008).

Many aquatic environments containing pond snails undergo large fluctuations in the aquatic partial pressure of O_2 (P_{O_2}) and can remain extremely hypoxic for many hours or even days, depending upon the prevailing conditions (Chang and Ouyang, 1988; Cole, 1994). Embryonic pond snails actively rotate in natural ponds and this behaviour is influenced by fluctuations in environmental P_{O_2} and temperature (Shartau et al., 2010). Therefore, it is likely that the periodic release of 5-HT during hypoxic episodes may enhance embryonic survival through regulation of rotational behaviour. If embryonic rotation is crucial for O_2 mixing, then it is predicted that the stimulation of ciliary-driven rotation by 5-HT will improve the outcome of embryos exposed to long-term hypoxic challenges.

In this study, we address whether 5-HT plays a protective role allowing embryos to withstand extended periods of hypoxia. We hypothesised that the stimulation of ciliary-driven rotation by 5-HT enhances the survival of embryos during prolonged hypoxia. To test the hypothesis, we monitored embryo survival and rotation rates in different levels of O_2 for 24–48 h while in the presence or absence of 5-HT or a 5-HT antagonist.

MATERIALS AND METHODS

Experimental animals

An inbred, laboratory-reared colony of albino *Helisoma trivolvis* Say 1816 was maintained in aquaria containing artificial pond water (APW: 0.025% Instant Ocean, Aquarium Systems, Mentor, OH, USA) at room temperature. Egg masses were removed daily from collecting plates and staged as a percentage of intracapsular development, with stage E0 corresponding to 0% development (zygote) and stage E100 corresponding to 100% development (hatching) (Goldberg and Kater, 1989; Diefenbach et al., 1998); all embryos used in this study were of embryonic stage E20–E30. Embryonic rotation was monitored using an imaging system composed of a dissection microscope (Zeiss, StemiSR, Toronto, ON, Canada) mounted with a digital video camera (Qimaging, Qicam Fast, cooled color 12-bit, Surrey, BC, Canada). Rotation was recorded using the time-lapse function of Northern Eclipse imaging

software (Northern Eclipse 6.0, Empix Inc., Mississauga, ON, Canada). The mean rate of rotation was analysed by counting the number of rotations during an 8–10 min video.

Experimental protocol

Hypoxia treatments

Hypoxia and anoxia treatments consisted of calibrated ratios of O_2 and N_2, or 100% N_2, respectively. Gases were mixed using mass flow controllers (Smart Trak digital mass flow, Sierra Instruments, Monterey, CA, USA) driven by Chinook Scientific software (version 2.0, written by R. J. Wilson, University of Calgary, Calgary, AB, Canada) and verified by an analyser (Illinois Instruments, Johnsburg, IL, USA, Model 3750). To ensure water saturation, gases were bubbled into a sealed Petri dish (150 mm × 25 mm) beginning 10 min prior to the behavioural measurements. Gas mixtures were continually introduced (1.8 l min^{-1}) during the experiments through fluorinated ethylene propylene tubing (Cole Parmer, Montreal, QC, Canada) and exhausted *via* a small opening at the elevated side of the dish. The egg mass was stabilised with dental wax at the lower edge of the dish. In all experiments, baseline measurements of rotation rate in normoxia were taken immediately prior to the introduction of drugs or hypoxia/anoxia. Measurements of embryonic rotation were taken at hourly intervals over 24 h for hypoxia treatments and until at least 75% embryonic mortality was observed for anoxia treatments. Each hourly measurement was made in the first 8–10 min of the hour, and the first measurement was considered as the 0-h time point. Embryos were treated with 5-HT (100 µmol l^{-1} serotonin creatinine sulfate complex, Sigma-Aldrich Co., St Louis, MO, USA) or its antagonist mianserin (50 µmol l^{-1} mianserin hydrochloride, Sigma-Aldrich Co.) 10 min prior to introducing gas mixtures.

Analysis of embryonic death

Embryo mortality was determined by morphological criteria. Live embryos exhibited a pearly shine and easily visible distinct cells. Dead embryos were characterised by a loss of distinct cellular morphology, and an opaque and fuzzy appearance (Fig. 1a,b). We verified visual determinations of embryonic death using a cell death assay performed on a sample of embryos (N=20 embryos). *Helisoma trivolvis* embryos were decapsulated in *Helisoma* saline using forceps and a 30G needle, and then incubated for 30–45 min in the dark in a solution containing 10 µg ml^{-1} propidium iodide (PI) and 10 µg ml^{-1} 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) prepared in *Helisoma* saline. Dead cells are permeable to both PI and DAPI whereas live cells are permeable to DAPI only (Lawry, 2008). The concentrations of PI and DAPI were chosen according to previous studies on intact *Drosophila* embryos (Hime and Saint, 1992; Verheyen and Cooley, 1994). After incubation, the excess stain was aspirated, and the embryos were washed and mounted on a coverslip containing dental wax posts on each corner. Using the ×20 objective on a Nikon TE300 (Mississauga, ON, Canada) and mercury lamp excitation, nuclear uptake of PI and DAPI were observed under the red channel (excitation=535 nm, emission=617 nm) and UV channel (excitation=358 nm, emission=461 nm), respectively.

A live stage E25 embryo under normoxic conditions was heavily stained with DAPI but not PI (Fig. 1aii), confirming the predominance of live cells. Embryos subjected to anoxia for 14 h looked dead (Fig. 1b,bi) and stained almost completely with PI (Fig. 1bii); thus, providing confirmation of extensive cell death. When 5-HT-treated embryos were exposed to anoxia for 14 h they remained alive, although they were morphologically different than normoxic embryos (Fig. 1c,ci). These embryos did not stain positively for PI, indicating an absence of cell death (Fig. 1cii).

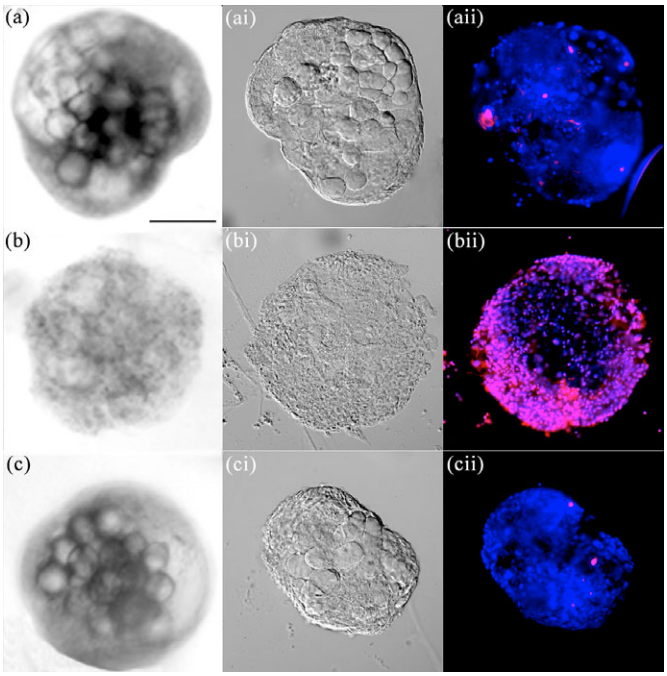

Fig. 1. Validation of morphological determination of embryonic death. Representative examples of stage E25 embryos subjected to various treatments are shown. Embryos were viewed with brightfield optics (a–c), Normarski Differential Interference Contrast optics (ai–ci) or under fluorescence excitation after staining for DAPI (4′,6-diamindino-2-phenylindole-dihydrochloride) and PI (propidium iodide) (aii–cii). Normal stage E25 embryos had large yolk cells, a pedal furrow and stained intensely with DAPI but not PI (a–aii). Embryos exposed to anoxia for 14 h underwent embryonic death (b–bii). Embryos lost recognisable characteristics and had an indistinct surface indicating disintegration of the embryo (b and bi). Both DAPI and PI stained brightly indicating extensive cell death (bii). When serotonin (5-HT)-treated embryos were exposed to anoxia for 14 h, they display altered development; however, yolk cells were still visible and the embryonic surface was distinct (c–ci). Staining with DAPI and PI shows no increased cell death, indicating the embryo was alive (cii). Scale bar: 100 µm.

Data analysis

Data were expressed as means ± standard error of the mean (s.e.m.). For statistical purposes, each embryo was considered an 'N' and embryos from at least three different egg masses were used. Unless stated otherwise, the statistical significance of differences among groups was determined using two-way ANOVA (analysis of variance) followed by a *post-hoc* Bonferroni's Multiple Comparison test or Tukey's test. These statistical tests were performed on the graphics and statistical analysis program GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Prolonged hypoxia elicits increased embryonic rotational activity

The rotational behaviour of *H. trivolvis* embryos in normoxia (140 Torr; 20.9% O_2; at 1110 m above sea level, 1 Torr equals 0.15% O_2) was examined in the presence of 5-HT (100 µmol l^{-1}) or mianserin (50 µmol l^{-1}). Mianserin has been shown to be the most effective antagonist against 5-HT-stimulated rotation (Goldberg et

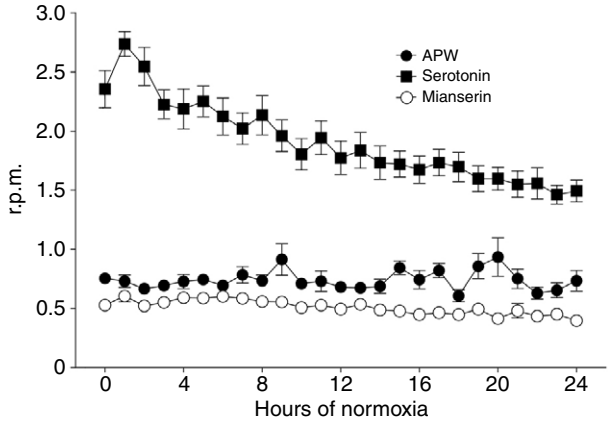

Fig. 2. Effect of serotonin (5-HT) and mianserin on embryonic rotation during normoxia. Stage E20 embryos were subjected to normoxia for 24 h in the presence of artificial pond water (APW) (filled circles; N=15), 100 μmol l^{-1} 5-HT (filled squares; N=15) or 50 μmol l^{-1} mianserin (open circles; N=15). The three groups differed significantly ($F_{2,74}$=228.6, P<0.0001; N=45).

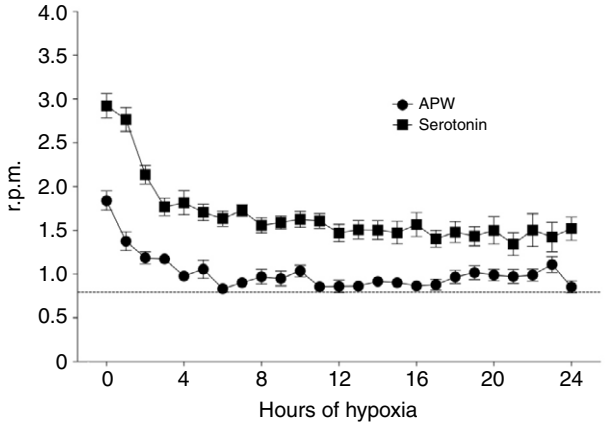

Fig. 3. Effect of serotonin (5-HT) on embryonic rotation during hypoxia. Stage E20 embryos were subjected to hypoxia (11 Torr) for 24 h in the presence of artificial pond water (APW) (filled circles; N=15) and 100 μmol l^{-1} 5-HT (filled squares; N=15). Rotation rates were significantly increased by the presence of 5-HT ($F_{1,719}$=568.02, P<0.0001; N=30). The broken line indicates the basal rate of embryonic rotation in normoxia measured prior to the introduction of 5-HT and hypoxia.

al., 1994). Control and mianserin-treated embryos exhibited steady rotation rates over the 24 h period of 0.74±0.09 (N=15) and 0.51±0.06 r.p.m. (N=15), respectively (Fig. 2). By contrast, 5-HT-treated embryos experienced a sharp, transient increase, typical with exogenous 5-HT application, resulting in a peak rotation rate of 2.74+0.20 r.p.m. (N=15). This increase was followed by a gradual decrease in rotational activity; however, the rotation rate of 5-HT-treated embryos was still 2.2 times higher than control embryos at 24 h of treatment (Fig. 2). Rotation rates differed significantly between the three treatment groups ($F_{2,74}$=228.6, P<0.0001).

During long-term (24 h) exposure to hypoxia (11 Torr; 1.7% O_2), embryos in APW experienced a sharp (approximately 140%) increase in rotation rate upon exposure to hypoxia to a peak rate of 1.86±0.19 r.p.m. (N=15), followed by a return to near basal rotation rates in 6 h (Fig. 3). 5-HT-treated embryos also underwent a sharp increase in rotation rate to a peak rate at 2.60±0.16 r.p.m. (N=15), followed by a decline along a similar time course as the embryos in APW. However, rotation rates remained significantly elevated from the embryos in APW over the duration of the experiment ($F_{1,719}$=568.02, P<0.0001) (Fig. 3).

Anoxia exposure resulted in sudden, transitory increases to rotational rates in all treatment groups. Peak rotation rates were reached within the first hour of exposure to anoxia, with maximal rotation rates of 2.06±0.26 r.p.m. (N=15) in APW, 3.15±1.24 r.p.m. (N=37) in 5-HT, 1.65±0.20 r.p.m. (N=20) in mianserin and 2.31±0.87 r.p.m. (N=20) in 5-HT/mianserin (Fig. 4A). Rotation rates of embryos in APW fell to less than half of the basal rate at 6 h of anoxia, and rotation was nearly absent at 8 h. By contrast, 5-HT-treated embryos underwent a prolonged period of elevated rotation rate that persisted for up to 10 h of anoxia. In these 5-HT-treated embryos that survived the anoxic treatment beyond 10 h, the rotation rates were near basal level for up to 40 h (Fig. 4A). Treatment of embryos with mianserin alone partially inhibited the rotation rate during the initial exposure to anoxia, after which the rotation rates of mianserin-treated embryos were not different than those in APW. Embryos treated with 5-HT and mianserin displayed slower rotation than those treated with 5-HT alone for at least 8 h, after which the rotation rates in the two groups were similar (Fig. 4).

Rotation rates at selected time points were examined to compare the impact of anoxia over time between treatments (Fig. 4B–D). The rotation rates of embryos in APW at 0, 6 and 12 h were 2.06±0.15, 0.29±0.06 and 0.01±0.00 r.p.m., respectively, indicating a rapid decline in rotation rate. 5-HT caused a significant increase in rotation rate at all time points (0 h: $F_{3,85}$=24.88, P<0.0001; 6 h: $F_{3,90}$=27.14, P<0.0001; 12 h: $F_{3,63}$=5.474, P<0.0001) with rotation rates of 3.13±0.12, 1.59±0.12 and 0.69±0.09 r.p.m. at 0, 6 and 12 h, respectively. Mianserin-treated embryos in anoxia displayed rotation rates that were not different from those embryos in APW. Rotation rates of 5-HT/mianserin-treated embryos were significantly different from those treated 5-HT alone for the first 6 h; however, those differences disappeared at 12 h (Fig. 4B–D).

Hypoxia delays embryonic development

As embryos sustained a normal rate of rotation during prolonged exposure to hypoxia, we tested whether embryonic development was also sustained. The development of embryos subjected to hypoxia (11 Torr) for 24 h was delayed compared with the controls (Fig. 5). Four days following hypoxia exposure, embryo morphology appeared normal but development continued to lag behind the control embryos exposed to normoxia (Fig. 5). The delay was sustained throughout embryogenesis as the time to 50% hatching from stage E25 was 32.6±1.9 h longer in hypoxia-treated embryos (132.6±9.9 h in hypoxia *vs* 100.0±8.0 h in normoxia, $F_{5,5}$=1.534, P<0.05).

5-HT has a protective effect against mortality during anoxia

Exposure of *H. trivolvis* embryos to anoxia led to surprising differences in mortality rates between embryos in APW and 5-HT-treated embryos. Egg masses in APW displayed rapid embryonic mortality starting at 5 h of anoxia, and nearly all of the embryos died by 12 h (Fig. 6A). By contrast, 5-HT-treated egg masses displayed delayed and more gradual mortality starting at 6 h of anoxia, with some embryos remaining alive beyond 35 h (Fig. 6A). Treatment of the egg masses with mianserin alone did not dramatically alter these trends, although treatment with 5-HT and

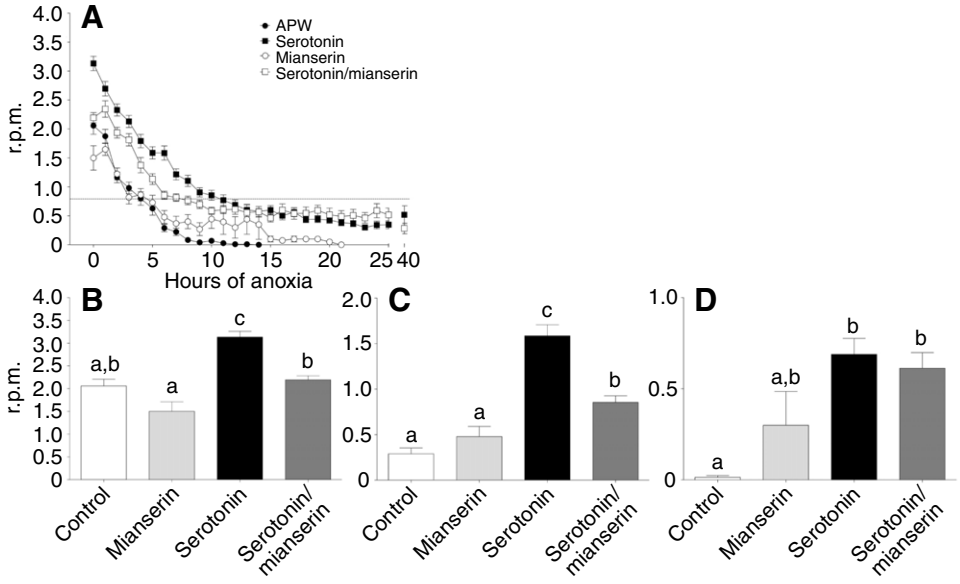

Fig. 4. Effect of serotonin (5-HT) and mianserin on embryonic rotation during anoxia. Stage E20 embryos in either artificial pond water (APW) (N=15), 100 μmol l^{-1} 5-HT (N=37), 50 μmol l^{-1} mianserin (N=20) or both 100 μmol l^{-1} 5-HT and 50 μmol l^{-1} mianserin (N=20) were subjected to anoxia until 75% mortality. (A) Long-term rotation rates of embryos in APW (closed circles), 5-HT (filled squares), mianserin (open circles) and both 5-HT and mianserin (open squares). The broken line indicates the basal rate of embryonic rotation in normoxia measured prior to the introduction of drugs and anoxia. (B) Rotation rates at 0 h, measured during the first 10 min of anoxia. (C) Rotation rates at 6 h of anoxia exposure. (D) Rotation rates at 12 h of anoxia exposure. Significance between treatments was determined by using ANOVA, followed by Bonferroni's Multiple Comparison Test (0 h: $F_{3,85}$=24.88, P<0.0001; 6 h: $F_{3,90}$=27.14, P<0.0001; 12 h: $F_{3,63}$=5.474, P<0.0001). Treatment bars with the same letters were not significantly different whereas those with different letters were significantly different.

mianserin enhanced survival marginally over 5-HT-treated embryos (Fig. 6A).

To quantitatively assess the progression of mortality between the various treatment groups, the times to reach 25%, 50% and 75% mortality were determined (Fig. 6B–D). Embryos in APW reached 25%, 50% and 75% mortality at 8.05±1.26, 9.09±1.25 and 10.16±1.28 h, respectively. 5-HT caused a significant delay in mortality throughout the anoxia treatment, with 25%, 50% and 75% mortality occurring at 9.67±3.5, 24.80±3.80 and 28.89±4.10 h, respectively (25%: $F_{3,17}$=4.477, P<0.05; 50%: $F_{3,17}$=7.651, P<0.01; 75%: $F_{3,17}$=18.58, P<0.0001). Mianserin did not significantly affect mortality during anoxia when added to embryos exposed to APW or 5-HT (Fig. 6B–D).

DISCUSSION

Increased rotation during hypoxia is a short-term behavioural response

The serotonergic ENC1s of *H. trivolvis* embryos mediate an immediate ciliary-driven rotation behavioural response to low P_{O_2}. In this study, we hypothesised that the stimulation of ciliary-driven rotation by 5-HT enhances the survival of embryos during prolonged hypoxia. Embryos subjected to multiple exposures of hypoxia become sensitised and increase their rotational response, indicating that this metabolically taxing behaviour provides a net positive benefit for the embryo through the stirring effects of embryo rotation (Kuang et al., 2002a; Goldberg et al., 2008). The results of the present study confirm that this behavioural response is transient, probably because the metabolic costs required for fast rotation outweigh the benefit gained from its stirring action when hypoxia is sustained. However, this study revealed an additional action of 5-HT that may contribute to the embryo's capacity to withstand extended periods of severe hypoxia. 5-HT caused a significant prolongation of embryo survival during anoxia well beyond the period of increased behavioural rotation, strongly suggesting that 5-HT also activates a metabolic pathway for ATP production.

Two mechanisms probably contribute to the transient time-course of the behavioural response to hypoxia. First, the production and turnover of 5-HT decreases as these processes require O_2, resulting in a reduced amount of available 5-HT (Nilsson, 1989; Diksic et al., 1991). Second, the ENC1 O_2 sensing neuronal pathway may

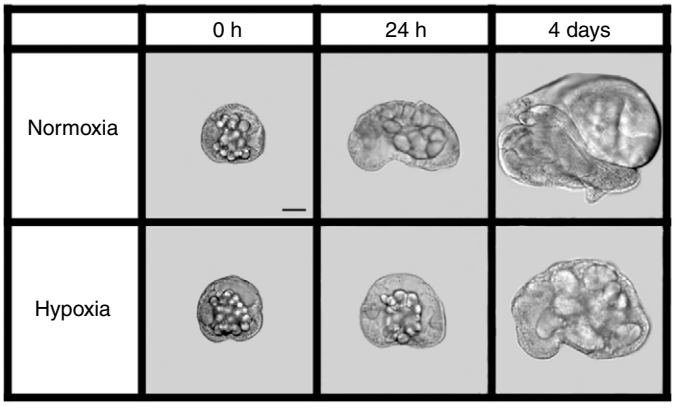

Fig. 5. Long-term exposure to hypoxia delays development. Examples of embryonic development of a control embryo in normoxia and an embryo subjected to 24 h of hypoxia, followed by 96 h of normoxia. At 0 h both embryos were at stage E25. Following 24 h exposure to hypoxia, the hypoxia-treated embryo was at stage E28 whereas the matched embryo under normoxia had reached stage E35. After four days following hypoxia treatment the hypoxia-treated embryo had reached stage E40, while the embryo in normoxia was at stage E60. Scale bar: 100 μm.

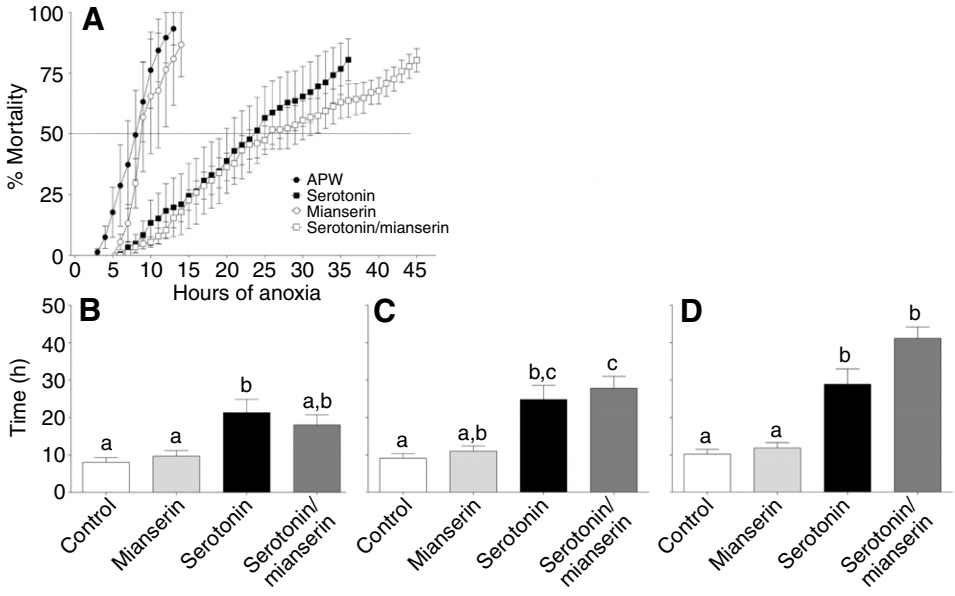

Fig. 6. Serotonin (5-HT) enhances embryonic survival in anoxia. Stage E20 embryos were subjected to anoxia until at least 75% mortality occurred in the presence of artificial pond water (APW) (N=5), 100 μmol l^{-1} 5-HT (N=8), 50 μmol l^{-1} mianserin (N=4) or both 100 μmol l^{-1} 5-HT and 50 μmol l^{-1} mianserin (N=7). (A) Mortality of embryos in APW (closed circles), 5-HT (filled squares), mianserin (open circles) and both 5-HT and mianserin (open squares). The 50% mortality level is indicated by the dashed line. 5-HT-treated embryos took significantly longer to reach 25% (B), 50% (C) and 75% (D) mortality then those in APW. Mianserin did not inhibit the 5-HT prolongation of survival. Significance between treatments is indicated by letters. Treatments bars having the same letters were not significantly different whereas those with different letters were significantly different, as determined by an ANOVA followed by Bonferroni's Multiple Comparison Test was used to assess differences (25%: $F_{3,17}$=4.477, P<0.05, N=24; 50%: $F_{3,17}$=7.651, P<0.01, N=24; 75%: $F_{3,17}$=18.58, P<0.0001, N=24).

become desensitised during prolonged hypoxia, either at the level of the sensory apparatus or ciliary 5-HT receptors (Kuang et al., 2002a). However, the ability of exogenous 5-HT to cause sustained faster rotation during prolonged hypoxia suggests that receptor-desensitisation does not occur (Fig. 3).

Despite their ability to survive prolonged hypoxia and maintain a normal rotation rate, development is affected (Fig. 5). Normal development is delayed during hypoxia, suggesting that these gastropod embryos respond to extended periods of hypoxia in a similar fashion as other encapsulated organisms such as opisthobranch gastropods, sea urchins, zebrafish, *Manduca sexta* and alligator embryos (Strathmann and Strathmann, 1995; Padilla and Roth, 2001; Woods and Hill, 2004; Warburton et al., 1995). Perhaps the available energy resources during prolonged hypoxia are insufficient to fuel the metabolically expensive cellular processes that operate during development.

Absence of O_2 leads to a suppression of metabolic activities

The ability of different species to tolerate anoxia is highly variable, ranging from mere minutes to many months in vertebrates and invertebrates (de Zwaan and Eertman, 1996; Bickler and Buck, 2007). The mechanisms behind anoxia tolerance are not fully understood; however, limiting energy expenditures through metabolic suppression has been demonstrated to be an important survival strategy (de Zwaan and Eertman, 1996; Hochachka et al., 1996; Hochachka and Lutz, 2001; Jackson, 2002; Bickler and Buck, 2007). Metabolic suppression involves some combination of regulating the pathways of ATP production (glycolysis, oxidative phosphorylation) and ATP demand (protein synthesis, ion pumping) (Hochachka et al., 1996; Bickler and Buck, 2007).

Helisoma trivolvis embryos survive anoxia for around 9 h. They do not appear to undergo complete metabolic arrest but rather rely on metabolic suppression during severe hypoxia, as development and growth is slowed. Furthermore, embryonic rotation remains active almost until death, suggesting that embryos do not completely arrest ATP turnover by halting energy intensive activities. Depletion of ATP stores ultimately results in death, as other gastropods unable to halt ATP turnover have been shown to have low tolerance to anoxia (de Zwaan and Eertman, 1996). Our results suggest that *H. trivolvis* embryos are able to minimise ATP consumption and turnover adequately to withstand severe hypoxic conditions that are often found in ponds (Chang and Ouyang, 1988; Cole, 1994; Shartau et al., 2010). Furthermore, it appears that they can tolerate brief periods of anoxia, a condition that is less typical of their natural environment.

Energy regulation through a 5-HT pathway

Two interesting events happened when *H. trivolvis* embryos were subjected to long-term anoxia in the presence of exogenous 5-HT. First, their rotational behaviour persisted for an extended period of time at near basal levels (Fig. 4A) and second, they experienced a tremendous prolongation of survival (Fig. 6). Treatments with exogenous 5-HT suggest that embryos have a large amount of unrealised capacity to withstand anoxic environments. These differences between 5-HT-treated embryos and untreated embryos probably arise from previously unknown effects on energy production and consumption.

O_2 is required not only to fuel aerobic metabolism but also in the synthesis and catabolism of 5-HT (Nilsson, 1989; Diksic et al., 1991). Therefore, a low 5-HT turnover rate is necessary to maintain a functional 5-HT system during anoxia, which can range from an hour in humans to 2.5 days in the highly-anoxia-tolerant crucian carp (Nilsson, 1990). The turnover rate of 5-HT in *H. trivolvis* embryos is unknown but is likely to be in the range of a few hours,

as rotation rates drop quickly in anoxia after the initial period of faster rotation. However, when subjected to high concentrations of exogenous 5-HT, rotation rates remained elevated and survival is prolonged, suggesting a beneficial function that can be attributed to 5-HT. During anoxia, endogenous 5-HT production probably ceases and rotation slows accordingly. Similarly, the loss of 5-HT results in a termination of the proposed metabolic response that helps fuel the embryo during sustained hypoxia.

Anaerobic metabolism during anoxia produces small amounts of ATP through the breakdown of glucose during glycolysis. A common source of glucose is glycogen, which undergoes glycogenolysis, producing glucose-1-phosphate which then enters glycolysis (Garrett and Grisham, 2005). Glycogen and glucose are found in the CF that bathes encapsulated embryos (Taylor, 1973; Stockmann-Boshbach and Althoff, 1989), with glycogen of particular interest for long-term survival during hypoxia as many lower vertebrates produce stores of it to provide a steady, slow release of energy (Bickler and Buck, 2007). In rodents, 5-HT has been shown to induce glycogenolysis by activating 5-HT-senstitive adenylate cyclase (Quach et al., 1982; Darvesh and Gudelsky, 2003), and examples of 5-HT-sensitive adenylate cyclase have also been identified in cockroaches (Nathanson and Greengard, 1974), *Schistosoma* (Kasschau and Mansour, 1982), *Drosophila* (Witz et al., 1990), bivalves (Sanderson et al., 1985) and *Aplysia* (Bacskai et al., 1993; Barbas et al., 2003; Filla et al., 2009). Although the cAMP pathway does not mediate the rotational response to 5-HT (Christopher et al., 1996), it may be involved in the metabolic response that leads to prolonged survival in hypoxia.

Mianserin acts on the 5-HT-type II ($5HT_2$) receptor; however, whether this receptor is present in *H. trivolvis* embryos is unknown as only the 5-HT-type I *H. trivolvis* ($5\text{-}HT_{1Hel}$) and 5-HT-type VII *H. trivolvis* ($5\text{-}HT_{7Hel}$) receptors have been identified (Mitchell et al., 1990; Csaba et al., 2003; Mapara et al., 2008). It is difficult to conclude from the actions of mianserin that the $5\text{-}HT_2$ receptor is present because of its poor specificity and the unreliability of transferring pharmacological profiles of vertebrate receptors to their invertebrate counterparts (Goldberg et al., 1994; Hay-Schmidt, 2000; Mapara et al., 2008). It appears that mianserin is an effective antagonist on whichever receptor is linked to acute 5-HT-stimulated rotation. However, because mianserin did not block the prolongation of survival during anoxia, this response may be mediated by a mianserin-insensitive receptor (Goldberg et al., 1994) (Fig. 6). Darvesh and Gudelsky demonstrated that mianserin blocks 5-HT-induced glycogenolysis through $5HT_2$ receptors (Darvesh and Gudelsky, 2003); however, *H. trivolvis* appears to use a different mechanism. Perhaps, 5-HT mediates increased glycogenolysis through $5\text{-}HT_{7Hel}$ receptors, which have been localised to *Helisoma* ciliary cells (Doran, 2005). As $5\text{-}HT_{7Hel}$ receptors typically are linked to stimulation of adenylate cyclase, we postulate that 5-HT activates energy metabolism and in turn prolongs survival in anoxia through a $5\text{-}HT_7$–cAMP signal transduction pathway.

Dual role of 5-HT release in *H. trivolvis*

Rather than acting solely as a neurotransmitter that enhances embryonic rotation, 5-HT may play a large role in regulating the energy supply in *H. trivolvis* embryos. These results highlight the potential importance of ENC1 5-HT release. The periodic release of 5-HT to induce surge activity may have an additional function during normoxia and hypoxia by enhancing carbohydrate metabolism and ATP production. Thus, in addition to causing faster embryonic rotation to mix the CF, the hypoxia-induced stimulation of ENC1 and release of 5-HT may serve to augment energy production to fuel the behavioural response and ensure an adequate energy supply during periods of hypoxia.

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