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Review

Integrative biology of an embryonic respiratory behaviour in pond snails: the 'embryo stir-bar hypothesis'

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Summary

Embryos of freshwater snails undergo direct development from single cell to juvenile inside egg masses that are deposited on vegetation and other substratum in pond, lake and stream habitats. *Helisoma trivolvis*, a member of the Planorbidae family of basommatophoran snails, has served as a model for studying the developmental and physiological roles for neurotransmitters during embryogenesis. Early studies revealed that *H. trivolvis* embryos from stage E15 to E30, the period between gastrulation and the trochophore–juvenile transition, display a cilia-driven behaviour consisting of slow basal rotation and transient periods of rapid rotation. The discovery of a bilateral pair of early serotonergic neurons, named ENC1, which project an apical process to the embryo surface and basal neurites to ciliated cells, prompted the hypothesis that each ENC1 is a dual-function sensory and motor neuron mediating a physiological embryonic response. This article reviews our past and present studies and addresses questions concerning this hypothesis, including the following. (1) What environmental signal regulates ENC1 activity and rotational behaviour? (2) Does ENC1 function as both a primary sensory and motor neuron underlying the rotational behaviour? (3) What are the sensory transduction mechanisms? (4) How does ENC1 regulate ciliary beating? (5) Do other basommatophoran species have similar neural–ciliary pathways and behavioural responses? (6) How is the behaviour manifest in the dynamic natural environment? In this review, we introduce the 'embryo stir-bar hypothesis', which proposes that embryonic rotation is a hypoxia-sensitive respiratory behaviour responsible for mixing the egg capsule fluid, thereby enhancing delivery of environmental oxygen to the embryo.

Introduction

The phylum Mollusca includes an exceptionally wide range of organisms, with members from eight extant and two extinct classes as divergent in form and function as the octopuses, clams and snails. The vast majority of the roughly 250 000 species live in marine environments; however, two classes of mollusc, the bivalves and gastropods, have members that are adapted to a freshwater existence. Within the gastropods, freshwater species include various taxa containing snails with gills, as well as the order Pulmonata that contains snails with lungs.

Pulmonate snails lay egg masses containing encapsulated embryos whose life history includes lecithotrophic direct development, whereby the embryos develop directly into juveniles within the egg mass before hatching and relying on external food sources. In contrast, most other molluscs display a planktotrophic life history, whereby veliger larvae emerge from egg masses as free-swimming planktotrophs, and remain in that stage until the presence of a suitable substrate signals a settlement behaviour, followed by metamorphosis into a benthic juvenile. While there is a rich record of studies on the marine biology of molluscan embryos and larvae from planktotrophic species dating back at least 60 years (Hadfield et al., 2000), the very nature of their life history limits their usefulness as experimental models. On the other hand, the lecithotrophic direct development and transparent egg mass structures of bassomatophoran pulmonates are features that make these pond snails experimentally tractable.

Lymnaea stagnalis and *Helisoma trivolvis* [also known as *Planorbella trivolvis* (Brown, 1991)] are both bassomatophoran pulmonates that have served as important model organisms in a broad range of neurobiological studies. *L. stagnalis* has been used primarily to study the neural mechanisms underlying feeding and respiratory behaviours (Vavoulis et al., 2007; Bell et al., 2007; Haque et al., 2006), as well as a variety of questions on learning and memory (Martens et al., 2007), neural plasticity and cell biology (Dunn and Syed, 2006; Jimenez et al., 2006). While *H. trivolvis* has also contributed in some of these areas (Murphy, 2001; Torreano et al., 2005), it has become a particularly good model to address a broad range of developmental questions because of the advantageous morphological and biomechanical properties of its egg mass. The egg mass is a flat circular structure containing 5 to 50 sibling embryos, each housed individually in an egg capsule (Fig. 1) (Goldberg et al., 1988). The organization of egg capsules on a single plane, combined with the transparency of the egg mass and egg capsule membranes, allows for easy observation of embryonic development and behaviour. Moreover, egg capsule membranes are easily penetrated by relatively small compounds (Beadle, 1969), such that developing embryos can be subjected to *in situ* experimental treatments. Finally, live embryos can be

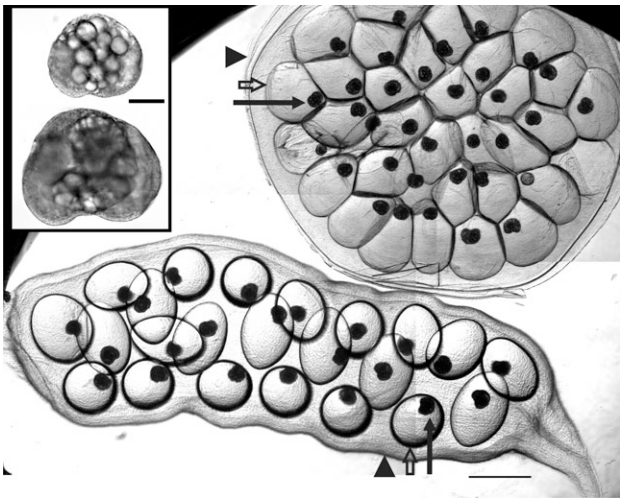

Fig. 1. Morphological comparison of egg masses and embryos from *H. trivolvis* (stage E25, top) and *L. stagnalis* (stage E39, bottom). Whole egg masses and decapsulated embryos (inset) were imaged 3 days after egg masses were laid by young adult snails. The characteristic tight planar arrangement of egg capsules (open arrow) inside the egg mass (arrowhead) and the flatter translucent embryo (arrow, also see inset) facilitate imaging of early development and behaviour in *H. trivolvis*. The *L. stagnalis* egg mass shown is considerably smaller than egg masses typically laid by older snails, whereas the *H. trivolvis* egg mass is more representative of the typical size. In the inset, embryos are shown in left-side view under differential interference contrast (DIC) optics. The stages shown represent the period of robust rotational behaviour for each species. The thin scale bar represents 500 μm and the thick scale bar in the inset represents 50 μm.

efficiently removed from their egg capsules and isolated from the egg mass for various procedures, including *in vitro* treatments, microsurgery and laser ablations, and, when necessary, embryos can be re-implanted into egg capsules for developmental or behavioural analyses (Kuang and Goldberg, 2001; Kuang et al., 2002a; Kuang et al., 2002b). *L. stagnalis* embryos are still accessible to many of these same types of procedure; however, the three-dimensional arrangement of egg capsules in an oblong egg mass, as well as the instability of egg capsules within the egg mass, make it considerably more difficult to conduct these kinds of experiment (Fig. 1).

A serendipitous result from one of the earliest studies on *H. trivolvis* embryos was that *in situ* exposure to a neurotoxin intended to deplete embryonic serotonin and affect normal development (Goldberg and Kater, 1989) caused an immediate and pronounced stimulation of a rotational behaviour. The compound 5,7-dihydroxytryptamine (5,7-DHT) is thought to deplete serotonergic terminals by its uptake through serotonin transporters, oxidization and production of free radicals that cause intracellular damage of the terminal (Baumgarten et al., 1982). Whereas 5,7-DHT produced the expected depletion and developmental abnormalities over the long term, its immediate and unexpected effect was the stimulation of embryonic rotation through the stimulation of serotonin receptors (Diefenbach et al., 1991). This one accidental finding prompted a series of studies that continues to the present day on the integrative biology of rotational behaviours in pond snail embryos. Earlier studies focused mainly on the neurocircuitry, neuropharmacology and signal transduction mechanisms underlying the cilia-driven rotational behaviour, whereas questions about behavioural relevance, activating environmental cues and underlying sensory pathways have been addressed more recently. With some of the advances made in these various areas of investigation, we are now in a position to extend this model system into the ecological and evolutionary arena, exploring both how the behaviour is manifested under the complex dynamic environment of the snail's natural habitat and how it is manifested in pond snails from three different families of pulmonates.

As reviewed below, our studies have revealed that the embryonic rotational behaviour is mediated by a pair of two-cell neural circuits, each containing a serotonergic sensorimotor neuron named embryonic neuron C1 (ENC1). The cell body of ENC1 senses environmental cues by projecting an apical sensory dendrite to the anterodorsal surface of the embryo and controls the motility of the dorsolateral and pedal ciliary bands by synapsing with the ciliary cells through its neurites. Serotonin stimulates ciliary beating through multiple serotonin receptors, and signal transduction pathways involving calcium, protein kinase C and nitric oxide. One of the natural environmental cues that stimulates the behaviour is hypoxia, prompting the hypothesis that ciliary activity and embryonic rotation are respiratory behaviours that cause stirring of the egg capsule fluid and enhancement of oxygen diffusion to the embryo. Preliminary experiments on *Lymnaea stagnalis* and two species of snail from the family Physidae suggest that hypoxia-induced rotational responses are expressed widely throughout the pulmonates, and may confer an adaptive advantage to embryos faced with environmental fluctuations in dissolved oxygen.

Embryonic rotational behaviour and the underlying physiological machinery

H. trivolvis embryos when inside their egg capsules undergo a constitutive rotational behaviour between the stages of E15 and E30 (Diefenbach et al., 1991), representing the developmental period from immediately after gastrulation until the beginning of the trochophore–juvenile transition. Embryonic stages in *H. trivolvis* are expressed as a percentage of the total development time (Goldberg et al., 1988; Goldberg, 1995; Diefenbach et al., 1998). Stage E0 corresponds to the formation of the zygote, E10 to gastrulation, E25 to the partitioning of the foot primordium from the abdomen with a morphologically distinguishable pedal furrow, and E100 to hatching. Time-lapse video analyses of egg masses imaged under dissection or compound microscopes revealed that the rate of embryo rotation is fastest at stage E25, typically ranging from 0.6 to 1.2 rotations per minute (r.p.m.) at this stage. These measurements represent an overall rate of rotation that combines two underlying components, constitutive rotation at a slow basal rate and periods of accelerated rotation called surges (Diefenbach et al., 1991; Cole et al., 2002).

These early behavioural observations prompted a series of hypotheses about the underlying physiological mechanisms that were strongly supported in subsequent studies. For example, they suggested that the slow basal rotation was due to constitutive ciliary beating, whereas the surges resulted from the stimulation of ciliary beating by cilio-excitatory motor neurons. Finally, the absence of rotational arrests suggested that the cilia mediating the rotational movement were only under excitatory control, contrary to locomotory cilia in marine gastropods that receive both excitatory and inhibitory inputs (Braubach et al., 2006). Finally, the serendipitous result of the 5,7-DHT treatment described above suggested that serotonin is the primary cilio-excitatory neurotransmitter in *H. trivolvis* embryos.

Histological analyses of fixed embryos and differential interference contrast (DIC) observation of live embryos indicated

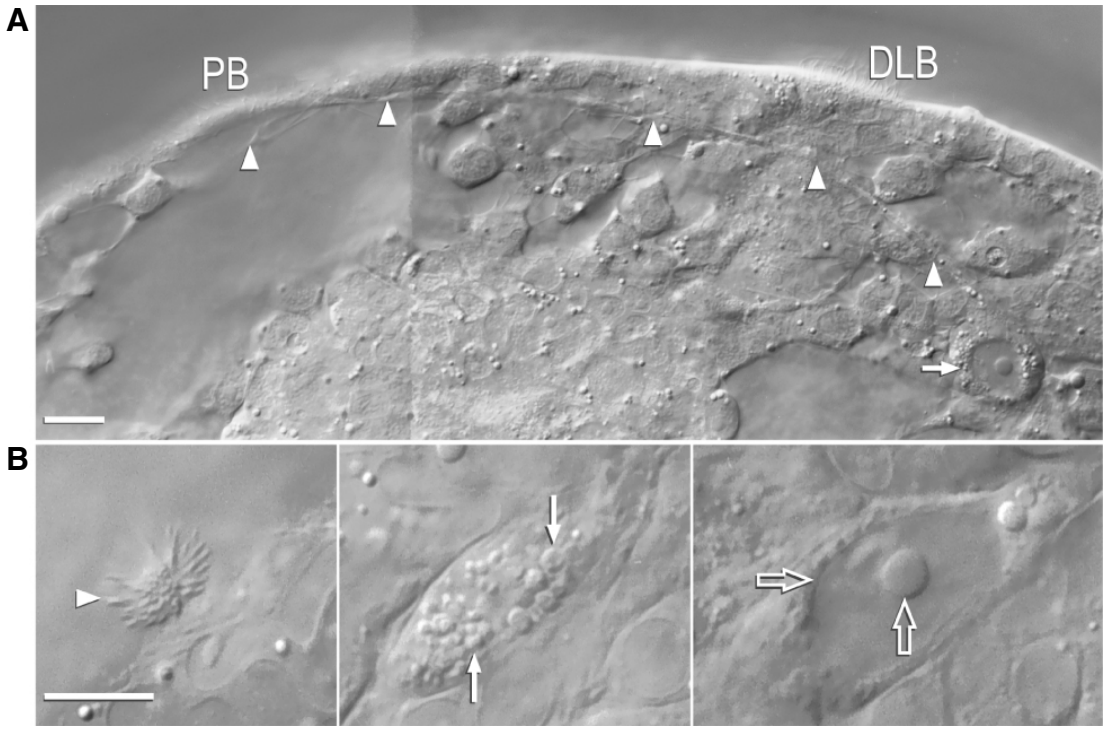

Fig. 2. ENC1 and post-synaptic ciliary cells viewed under DIC optics. (A) ENC1 soma (horizontal arrow) and its primary neurite (vertical arrowheads) projecting towards the dorsolateral (DLB) and pedal ciliary (PB) bands. (B) ENC1 viewed through a series of descending focal planes. The sensory-like dendritic knob (horizontal arrowhead) is located on the embryo surface (left panel), leading to a stubby apical process containing numerous prominent vesicles (vertical arrows) just below the surface (middle panel). Below that sits the soma, with its characteristic large nucleus (horizontal open arrow) and prominent nucleolus (vertical open arrow, right panel). Scale bar, 10 μm.

that organogenesis of the central nervous system does not begin until after stage E20, well after the onset of the rotational behaviour (Goldberg, 1995; Diefenbach et al., 1991; Diefenbach et al., 1998). However, immunofluorescence experiments revealed that serotonin is expressed in a bilateral pair of large neurons as early as stage E13 (Diefenbach et al., 1998). These neurons, named embryonic neuron C1 (ENC1) because they were originally thought to be the metacerebral giant serotonergic neurons studied in a variety of adult gastropods (Granzow and Rowell, 1981), projected their primary neurites ventrally to the region adjacent to the pedal band of cilia. Thus, their morphology and neurotransmitter phenotype were consistent with them being the cilio-excitatory motor neurons. Interestingly, each ENC1 projects a stubby apical dendrite dorsally that is tipped with a specialization that extends through to the surface of the embryo (Fig. 2) (Goldberg and Kater, 1989; Diefenbach et al., 1998). This superficial dendritic knob contains an array of microvilli and non-motile cilia, an anatomical arrangement typical of a sensory specialization. Thus, the early anatomical data suggested that ENC1 was a sensorimotor neuron involved in regulating ciliary activity in response to an unknown environmental cue.

The appearance of ENC1 prior to the formation of the central nervous system, and the absence of any other detectable neurons between stages E13 and E25, suggests that this neuron evolved during pulmonate evolution to play a critical role in embryos specific to encapsulated development. However, the relatively medial location of these neurons on the anterodorsal aspect of the embryos, combined with their stubby apical dendrites and superficial dendritic knobs, led to a more likely interpretation (Diefenbach et al., 1998). There is now general agreement that rather than being the embryonic metacerebral giant neurons as proposed earlier, ENC1s are the evolutionary remnants of the apical sensory organs of marine gastropods, specialized embryonic nervous systems that control locomotory and settlement behaviours in planktonic veligers (Voronezhskaya et al., 1999). These are typically small ganglia containing relatively few neurons, including three to six serotonergic neurons depending on the species (Kempf et al., 1997). Some of these neurons have sensory-like morphologies, including the serotonergic para-ampulary neurons that look strikingly similar to ENC1. We hypothesize that the apical sensory organ became drastically reduced during the evolution of pulmonates because the absence of the planktonic larval stage eliminated the need for the more extensive neural machinery required to control the more complex behaviours associated with the planktonic life history. ENC1s and their possible homologues in other pulmonates were retained to carry out a relatively simple task specific to encapsulated development (see below). Further comparative analyses are required to help confirm such an evolutionary hypothesis.

The synaptic connectivity between ENC1 and ciliary target cells was confirmed through anatomical and physiological analyses. Both DIC microscopy and serotonin immunofluorescence showed ENC1 neurite branching in close apposition to the basal surface of ciliary cells throughout the pedal band of cilia, as well as the most medial of the four ciliary cells that comprise each dorsolateral band of cilia (Koss et al., 2003). Ultrastructural experiments revealed the expected chemical synaptic profiles between ENC1 and ciliary cells. Surprisingly, these synapses often occurred on short projections emanating from the basal surface of the ciliary cells

(Koss et al., 2003). Gap junction profiles between adjacent ciliary cells were also observed, suggesting that ciliary cells not innervated by ENC1 may respond indirectly through gap junction-mediated transfer of electrical or chemical signals between ciliary cells.

In contrast to the circumstantial evidence of ENC1–ciliary communication provided by the anatomical experiments, conclusive evidence could only be obtained through experimental perturbation of ENC1 activity in physiological experiments. Taking advantage of the ability to visualize cellular structures in live intact embryos through DIC microscopy, laser techniques were used to confirm functional connectivity between ENC1 and ciliary cells. A laser stimulation protocol was developed that results in the death of ENC1 5–6 h after laser treatment (Kuang and Goldberg, 2001). Not only did the bilateral laser ablation of ENC1 reduce the rate of embryo rotation to the basal level and eliminate surges (Kuang et al., 2002b), but also the laser treatment caused an initial increase in cilia beat frequency (Kuang and Goldberg, 2001). This suggested that the initial effect of the laser treatment was an injury-induced depolarization leading to transmitter release and postsynaptic activation of ciliary beating. This demonstration of laser-induced stimulation of transmitter release was further confirmed by blocking the cilioexcitatory response with an effective serotonin antagonist (Kuang and Goldberg, 2001).

The signal transduction mechanisms by which serotonin causes an increase in cilia beat frequency have been examined in behavioural and cellular experiments. Behaviourally, serotonin causes up to a fourfold increase in rotation rate through a low affinity receptor that is preferentially blocked by the serotonin antagonist mianserin (Diefenbach et al., 1991; Goldberg et al., 1994). This response to serotonin is also blocked by treatments that interfere with nitric oxide signalling (Cole et al., 2002). While nitric oxide is produced and active in both ENC1 and ciliary cells, it appears to act primarily as an intracellular messenger, rather than in anterograde or retrograde transmission between ENC1 and ciliary cells (Cole et al., 2002; Doran et al., 2003) (see below).

Our cellular studies on signal transduction pathways in ciliary cells were recently boosted by the development of techniques to isolate identified ciliary patches (Doran et al., 2004), thus eliminating the problems encountered in earlier studies on heterogeneous cell populations (Goldberg et al., 1994; Christopher et al., 1996; Christopher et al., 1999). Collectively, these studies have revealed that serotonin-induced cilioexcitation involves a complex network of signal transduction pathways. In the absence of serotonin, ciliary cells display constitutive ciliary beating at low beat frequencies that underlies the basal rate of rotation observed in intact embryos. Addition of serotonin causes an immediate increase in ciliary beat frequency in pedal and dorsolateral ciliary cells that involves activation of serotonin receptors, protein kinase C, nitric oxide and an increase in intracellular calcium (see below).

Two serotonin receptor subtypes have been cloned in *H. trivolvis*, one a member of the 5-HT_1 family of G-protein-coupled serotonin receptors and the other a member of the 5-HT_7 family of G-protein-coupled serotonin receptors. These receptors, named 5-HT_{1Hel} and 5-HT_{7Hel}, respectively, are both expressed in embryonic ciliary cells, as well as in many neurons of the adult central nervous system (Mapara et al., 2008; Doran et al., 2004). A preliminary study of these suggested that 5-HT_{1Hel} receptors probably mediate a transient large-amplitude cilioexcitatory response to serotonin (Gallin et al., 2006). In contrast, the 5-HT_{7Hel} receptors appear to be involved in producing long-lasting cilioexcitatory responses of lower amplitude that are sustained even after serotonin washout. This dual control system may explain why earlier pharmacological experiments identified very few effective blockers of the serotonin response. Mianserin, a compound with poor selectivity for serotonin receptor subtypes (Petrascheck et al., 2007), is the only serotonin antagonist shown to effectively block the cilioexcitatory or rotational responses to serotonin (Goldberg et al., 1994).

Beyond the activation of serotonin receptors, calcium, protein kinase C and nitric oxide have all been implicated in producing the cilioexcitatory response to serotonin (Christopher et al., 1996; Christopher et al., 1998; Doran et al., 2003; Doran et al., 2004; Doran and Goldberg, 2006). In most ciliary systems studied to date, a rise in intracellular calcium is directly responsible for activating the molecular ciliary machinery (Salathe and Bookman, 1999; Lansley and Sanderson, 1999; Zagoory et al., 2001; Doran and Goldberg, 2006). Experiments with calcium buffers, calcium-sensitive dyes and manipulations of extracellular calcium concentration in pedal and dorsolateral ciliary cells suggest that serotonin induces a highly localized acute rise in intracellular calcium that stimulates beat frequency, in addition to a slow dispersed rise in cytosolic calcium that may function in the refilling of intracellular calcium stores (Doran and Goldberg, 2006; Doran et al., 2004; Doran, 2005). Pharmacological experiments indicated that although phospholipase C and protein kinase C contribute partially to the cilioexcitatory response, the calcium necessary for ciliary stimulation is released from an intracellular store through a caffeine-sensitive release mechanism, rather than an inositol triphosphate- or ryanodine-sensitive release mechanism (Doran, 2005). Finally, nitric oxide is constitutively expressed in ciliary cells and plays a permissive role in cilioexcitation. Although serotonin only causes a moderate stimulation of nitric oxide production in some ciliary cells, interference with ongoing nitric oxide production or activity completely prevents the cilioexcitatory response to serotonin (Doran et al., 2003). This profile of signal transduction activities is relatively unique compared with the various other vertebrate or invertebrate systems in which cilioexcitatory responses have been examined. Whereas many of the intracellular messengers are the same in most systems, their specific activities, interactions and relative roles are highly variable [for example, protein kinase C (see Doran and Goldberg, 2006; Morales et al., 2000; Levin et al., 1997; Mwimbi et al., 2002)].

Identification of hypoxia as an environmental regulator of embryonic rotation

Pond snail embryos undergo encapsulated development in the relatively stable environment of the egg mass. The energy expended in producing the rotation behaviour and the precise neural circuitry underlying the rotational surges together suggest that the behaviour must provide some benefit to the embryo. The alternative interpretation that the rotational behaviour is simply a vestige of locomotory behaviours expressed by veliger larvae of ancestral planktonic species seems far less likely.

The highly specialized dendritic knob of ENC1 has an increased surface area through sensory-like cilia and microvilli (Diefenbach et al., 1998). This prompted the hypothesis that the ENC1–ciliary circuits are activated by a specific environmental cue, and the ensuing stimulation of ciliary beating and embryo rotation comprise an adaptive response to the cue. Candidate environmental signals include those that fluctuate independently of embryo metabolism, such as light and temperature, and those that are affected by embryo metabolism, such as oxygen, carbon dioxide, nutrients and metabolic waste products. Early on in experiments evaluating this latter group of signals, we discovered that exposure of egg masses to hypoxic pond water induces a robust stimulation of embryo

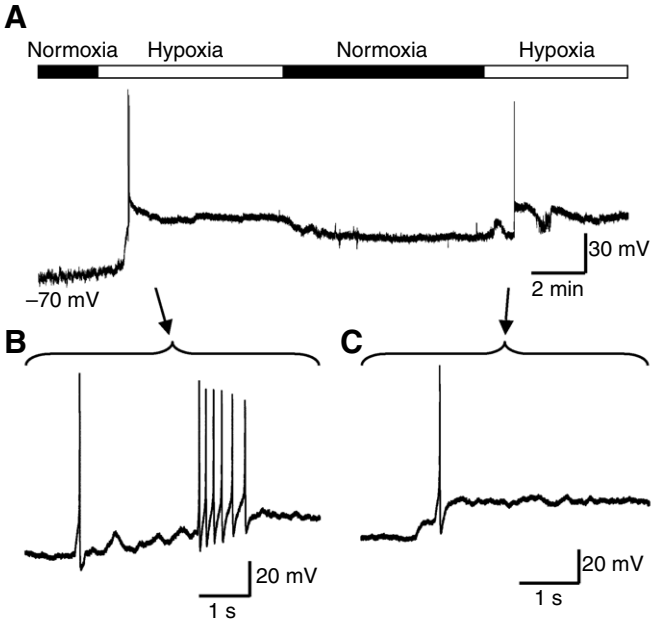

Fig. 3. Electrophysiological responses of ENC1 to hypoxia in an explant culture recorded under whole-cell current clamp. (A) Under normoxic conditions, the membrane potential of ENC1 was around –70 mV. Hypoxia (approximately 90% reduction in oxygen concentration) elicited membrane depolarization and a burst of action potentials, followed by sustained membrane depolarization. Restoration of normoxia partially repolarized the membrane potential. A second cycle of hypoxia again elicited membrane depolarization and an action potential. (B,C) Action potentials elicited by the first and second cycle of hypoxia viewed on an expanded time scale.

rotation similar in magnitude to that produced by maximal concentrations of exogenous serotonin (Kuang et al., 2002a). This came as a great surprise since ciliary beating is highly dependent on the availability of ATP (Woolley, 2000), and thus an adequate supply of oxygen.

The rotational response to hypoxia was immediate and long lasting, with the rate of rotation remaining elevated for up to 3 h during sustained hypoxia (Kuang et al., 2002a). Termination of hypoxic treatments induced a pronounced and transient inhibition of rotation rate below normal levels. Embryos reliably responded during repeated cycles of hypoxia and normoxia, and behavioural sensitization of the hypoxia-induced response was observed by the third cycle. The response was concentration dependent, with threshold, half-maximal and maximal responses occurring at a P_{O_2} of 60, 28 and 13 mmHg, respectively. Finally, in contrast to the potent effects of hypoxia, embryo rotation was only weakly sensitive to hyperoxia or hypercapnia (Kuang, 2002). Together, these characteristics indicate that the rotation behaviour and underlying neural–ciliary circuits function to detect and form adaptive responses to environmental hypoxia (see below).

Whilst the physiological mechanisms underlying the motor components of the rotational response to hypoxia are partially understood as a result of our studies on ENC1–ciliary communication and serotonin-induced cilioexcitation (see above), much less is known about the sensory side of the response. Laser ablation and pharmacological experiments confirmed that the response to hypoxia depends on intact ENC1s and serotonin release. As well, ciliary cells isolated in cell culture had no response to hypoxia, further suggesting that ENC1 is directly responsible for hypoxia detection (Kuang et al., 2002a). To confirm this electrophysiologically, the technique developed to isolate identified patches of ciliary cells (Doran et al., 2004) was adapted for ENC1. Whole-cell current-clamp recordings of isolated ENC1s revealed hypoxia-induced action potential activity in four of the four cells tested, indicating that ENC1 contains the sensory apparatus to detect hypoxia (Fig. 3).

Mechanisms of hypoxia sensing have long been a topic of intense debate (Buckler, 2007; Kemp and Peers, 2007). Whilst the hypoxia-sensing pathway in ENC1 has not yet been examined in isolated cells, pharmacological experiments on whole embryos and isolated ciliary cells have implicated the mitochondrial electron transport chain and potassium channel closure (Fig. 4). Treatment

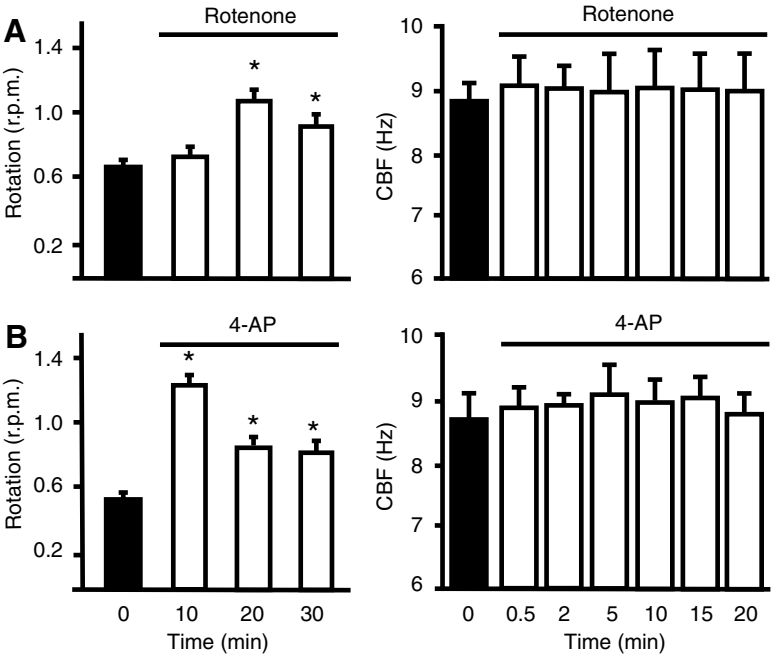

Fig. 4. Involvement of the mitochondrial electron transport chain and potassium channels in embryonic rotation. (A) Rotenone (10 μmol l^{-1}), an inhibitor of the mitochondrial electron transport chain, stimulated embryonic rotation (r.p.m., rotations per minute) after 10 min of incubation (left panel, N=15 embryos), but had no direct effect on the ciliary beat frequency (CBF) of isolated ciliary cells (right panel, N=5 cells). (B) 4-Aminopyridine (4-AP, 5 mmol l^{-1}), a potassium channel inhibitor, induced prolonged increases in embryonic rotation (left panel, N=15 embryos), but had no direct effect on the CBF of isolated ciliary cells (right panel, N=5 cells). *P<0.05 compared with zero time point, ANOVA followed by Fisher's PLSD.

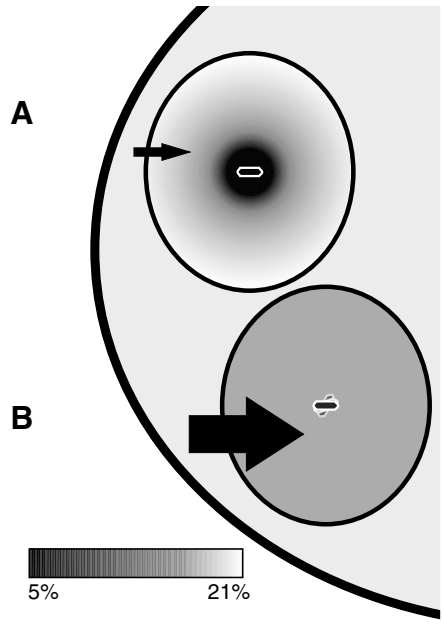

Fig. 5. The embryo stir-bar hypothesis. (A) In the absence of rotation, a large oxygen concentration gradient will form due to the metabolic consumption of oxygen by the embryo (depicted as stationary stir bar) and the unstirred boundary layer of high oxygen concentration below the capsule surface. (B) Embryonic rotation and ciliary activity (depicted as rotating stir bar) function to mix the capsular fluid, causing a reduction in the size of the oxygen gradient inside the capsule, a higher concentration of oxygen at the embryo surface, and enhanced transfer of oxygen into the egg capsule. The arrow size in A and B represents the relative size of the oxygen gradient across the egg capsule membrane. Stir bar length is not representative of embryo size (see Fig. 1).

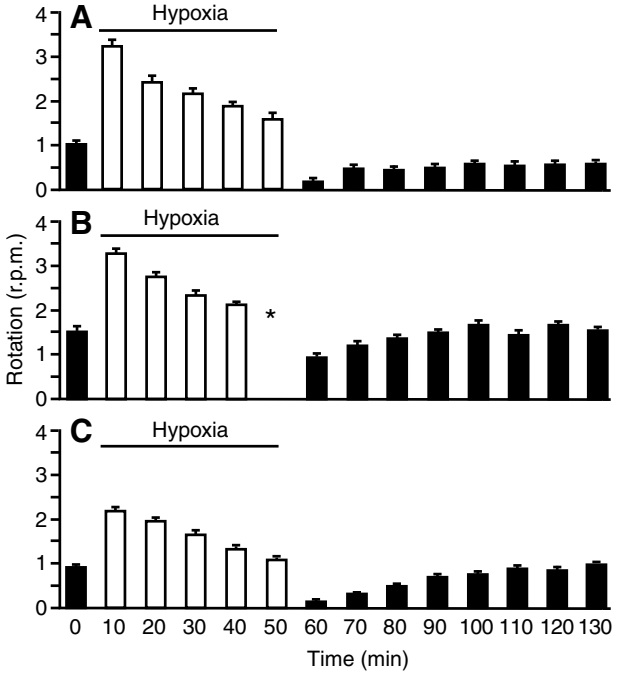

Fig. 6. Hypoxia stimulates embryonic rotation behaviour in three families of freshwater pond snails. *H. trivolvis* (A), *L. stagnalis* (B) and *Physa gyrina* (C), members of the Planorbidae, Lymnaeidae and Physidae families of basommatophoran snails, respectively, demonstrated similar profiles of increased embryonic rotation rates upon prolonged exposure to hypoxia (open bars). Upon return to normoxic conditions (filled bars), an initial inhibition of rotation followed by a return to baseline levels was observed in all three species. Each bar represents mean r.p.m. ± s.e.m. from 22–26 embryos from two stage E25 egg masses (A), 13–17 embryos from one stage E39 egg mass (B) and 23–27 embryos from two stage E35 egg masses (C). Asterisk in B represents loss of a data point due to a corrupted digital file.

of whole embryos with either rotenone or 4-aminopyridine, inhibitors of the electron transport chain and oxygen-sensitive potassium channels (Lopez-Barneo et al., 2001; Haddad and Jiang, 1997), respectively, increased the embryonic rotation rate. In contrast, neither of these affected the cilia beat frequency in isolated ciliary cells, suggesting that the site of action is upstream, most likely in ENC1 cells. While these experiments indicate possible elements of the hypoxia sensor, a more comprehensive analysis of hypoxia sensing in isolated ENC1s is required.

The embryo stir-bar hypothesis

Adult pond snails, which respire both cutaneously in the aquatic environment and through air breathing at the water surface, respond to hypoxia through oxygen-sensing peripheral neurons that activate the air-breathing pathway (Bell et al., 2007). The stimulation of the embryo rotation behaviour by hypoxia suggests that this behaviour also serves a respiratory function that helps to ensure an adequate supply of oxygen to the developing embryo, and thus confers an adaptive advantage to embryos. We hereby call this the ‘embryo stir-bar hypothesis’, whereby embryo rotation, coupled with the underlying ciliary activity, serves to mix the egg capsule fluid (Fig. 5). In this scenario, mixing would diminish the unstirred boundary layer underneath the capsular membrane and reduce the diffusion gradient from the capsule surface to the oxygen-consuming embryo. Not only would this activity increase the oxygen concentration at the embryonic surface, but it would also steepen the diffusion gradient between the outside and inside surfaces of the egg capsule membrane, thus enhancing the transfer of oxygen into the egg capsule.

A variety of studies have recently been initiated to test the validity of the embryo stir-bar hypothesis. These include measurements of oxygen within egg capsules, testing the effects of sustained hypoxia in normal and rotation-compromised embryos, comparative analysis of closely and distantly related species, and evaluation of rotation behaviour in natural environments.

Our initial measurements of capsular oxygen using oxygen-sensitive microelectrodes confirmed the presence of a significant oxygen gradient from egg capsule surface to embryo under normoxic conditions (Kuang et al., 2002a). Furthermore, exposure of egg masses to hypoxic pond water caused rapid reductions in egg capsule oxygen (Kuang, 2002), suggesting that the egg mass and egg capsule membranes form weak diffusion barriers to environmental oxygen. Oxygen measurement experiments testing the mixing effects of embryo rotation and ciliary activity when stimulated by either serotonin or hypoxia are currently underway.

In another direct test of the embryo stir-bar hypothesis, assessing the effects of hypoxia in rotation-compromised embryos would determine whether rotational responses are required for normal development or viability, and thus address the adaptiveness of the behavioural response. Embryos are able to survive long-term exposure to strong hypoxia (<10% normal oxygen concentration)

pond water for 10 h (Shartau and Goldberg, 2007). We are now testing how this survival period might be affected if the rotation behaviour was experimentally attenuated. De-ciliation treatments such as chloral hydrate or hypertonicity (Quarmby, 2004) were partially effective in causing de-ciliation, but not specific enough to clearly determine whether the loss or slowing of rotation has negative consequences on embryo development and viability (Shartau and Goldberg, 2007). In preliminary experiments, pharmacological perturbation of embryo rotation using the serotonin antagonist mianserin affected the progression of embryonic development. Whilst this result helps support the hypothesis, approaches that more specifically attenuate the rotation behaviour, such as molecular knockout of the ciliary serotonin receptors or other ciliary proteins, or double laser ablation of ENC1 (Kuang et al., 2002b), will produce more definitive tests. These experiments may also reveal whether the basal-unstimulated rotation behaviour also plays an adaptive role under normoxic conditions.

A comparative analysis of the embryonic rotation behaviour can also shed light on whether the hypoxia-induced rotational response is a functional adaptation in pond snails. It would be expected that if this is an important survival strategy, it would be expressed in related gastropod species that have encapsulated, directly developing embryos found in diverse fresh water environments. Indeed, in recent experiments on representative species from the three prominent families of freshwater pond snails, the Planorbidae, Lymnaeidae and Physidae, hypoxia-induced rotational responses were observed in all species tested (Fig. 6). Interestingly, the ENC1 homologues in *Lymnaea stagnalis* are the transient apical catecholaminergic (TAC) neurons (Voronezhskaya et al., 1999), in which dopamine has replaced serotonin as the primary cilioexcitatory neurotransmitter in the rotation response (Voronezhskaya et al., 1999; Kuang, 2002). Since physids are thought to be more closely related to lymnaeids and planorbids than these latter two groups are to each other (Jorgensen et al., 2004), it will be interesting to determine whether the ENC1 homologues in physid embryos use serotonin or dopamine as a cilio-excitatory neurotransmitter. Furthermore, testing whether hypoxia is an important cue in more distantly related marine snail species with encapsulated direct embryonic development will reveal whether this is an ancient adaptation, or one that evolved more recently as pulmonates inhabited fresh water environments (Moran and Woods, 2007; Lee and Strathmann, 1998).

A critical final element in validating the embryo stir-bar hypothesis is understanding how the embryonic rotation behaviour functions in nature. Snails reside in diverse freshwater environments, ranging from pristine oligotrophic waters that undergo only limited fluctuations in oxygen concentration to productive eutrophic waters that undergo large seasonal and diurnal fluctuations in oxygen concentration. Our initial field experiments on *H. trivolvis* embryos in a small spring-fed oligotrophic pond revealed that embryonic rotation rates varied according to the daily cycle in temperature, whereas oxygen concentrations were relatively stable throughout the daily temperature and light cycles (R.B.S. and J.I.G., unpublished observations). With the feasibility of these field experiments and a preliminary baseline data set now established, we look forward to testing how the embryo rotation behaviour helps embryos in eutrophic or stagnant habitats weather periods of severe hypoxia induced by algal blooms and heat. Furthermore, studying hypoxia responses under a variety of natural conditions may reveal how embryos balance the advantages gained through active responses that enhance oxygen availability over the short term with the metabolic cost of expending energy during sustained periods of hypoxia. The instigation of these ecological experiments highlights how the rotational respiratory behaviour in pond snail embryos is an ideal model system for integrative biology in the broadest sense, incorporating analyses at molecular, physiological, behavioural, ecological and evolutionary levels.

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