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Regulation of early embryonic behavior by nitric oxide in the pond snail *Helisoma trivolvis*

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Summary

Helisoma trivolvis **embryos display a cilia-driven rotational behavior that is regulated by a pair of serotonergic neurons named ENC1s. As these cilio-excitatory motor neurons contain an apical dendrite ending in a chemosensory dendritic knob at the embryonic surface, they probably function as sensorimotor neurons. Given that nitric oxide (NO) is often associated with sensory neurons in invertebrates, and has also been implicated in the control of ciliary activity, we examined the expression of NO synthase (NOS) activity and possible function of NO in regulating the rotational behavior in *H. trivolvis* embryos. NADPH diaphorase histochemistry on stage E25–E30 embryos revealed NOS expression in the protonephridia, buccal mass, dorsolateral ciliary cells and the sensory dendritic knobs of ENC1. At stages E35–40, the pedal ciliary cells and ENC1's soma, apical dendrite and proximal descending axon were also stained. In stage E25 embryos, optimal doses of the NO donors SNAP and SNP increased the rate of embryonic rotation by twofold, in contrast to the fourfold increase caused by 100 μmol l^{-1} serotonin. The NOS inhibitors L-NAME (10 mmol l^{-1}) and 7-NI (100 μmol l^{-1}) decreased the rotation rate by approximately 50%, whereas co-addition of L-NAME and SNAP caused a twofold increase. In an analysis of the surge and inter-surge subcomponents of the rotational behavior, the NO donors increased the inter-surge rotation rate and the surge amplitude. In contrast, the NO inhibitors decreased the inter-surge rotation rate and the frequency of surges. These data suggest that the embryonic rotational behavior depends in part on the constitutive excitatory actions of NO on ENC1 and ciliary cells.**

Key words: Embryonic behavior, nitric oxide, pond snail, *Helisoma trivolvis*, serotonin, ciliary beating, gastropods, NADPH diaphorase.

Introduction

Nitric oxide (NO) is an unstable gas that acts as a messenger molecule in a variety of systems. It has been shown to have neurotransmitter-like functions in both vertebrates (Arancio et al., 1996) and invertebrates (for reviews, see Colasanti and Venturini, 1998; Jacklet, 1997; Martinez, 1995; Moroz, 2000), and is often associated with sensory systems (Bicker, 2001; Breer and Shepherd, 1993; Gelperin et al., 2000; Schmachtenberg and Bacigalupo, 1999). As NO freely crosses cell membranes, acting to modify the activity of a number of intracellular substrates, it has also been suggested that it functions as a signal transduction element (Bicker, 2001; Clementi, 1998; Jacklet, 1997), independent of transcellular signaling.

Gastropod molluscs, with their simple behaviors, well-characterized neural circuits and giant identifiable neurons, are ideal for investigating NO function at cellular, circuit and behavioral levels. In adult *Lymnaea stagnalis*, for example, sensory cells of the lips, as well as a number of neurons within the CNS, have been shown to contain nitric oxide synthase (NOS), the enzyme that catalyzes NO production (Elphick et al., 1995; Moroz et al., 1994). Functional studies on this system indicate that NO is necessary for the transmission of sensory information to the central pattern generator for feeding behavior (Elphick et al., 1995) and activation of buccal motor patterns (Moroz et al., 1993). Furthermore, cellular experiments on *L. stagnalis* neurons have clearly demonstrated neurotransmitter-like actions of NO, including its release from presynaptic cells in an activity-dependent manner and production of slow excitatory postsynaptic potentials on a target neuron (Park et al., 1998; Philippides et al., 2000).

In contrast to studies on adult systems, there is less opportunity to gain an integrated understanding of the physiological and behavioral roles of NO during embryonic development. However, recent studies on embryos of the pond snail, *Helisoma trivolvis*, suggest that this system is well suited to examine in depth the function of a simple neural circuit and its associated behavior during early embryonic development. *H. trivolvis* embryos are individually compartmentalized in egg capsules within egg masses that contain 5–50 sibling embryos (Goldberg, 1995). As they develop from zygote to juvenile stages inside these transparent structures, they display a well-characterized rotational behavior (Diefenbach et al., 1991) that

serves to mix the capsular fluid and enhance oxygen diffusion to the embryo (Kuang et al., 2002). The embryonic rotation is driven by three bands of constitutively beating cilia that are innervated by a bilateral pair of early developing serotonergic neurons (ENC1s). These embryonic neurons periodically release serotonin onto their target ciliary cells, producing increases in ciliary beat frequency and surges in the rate of embryonic rotation (Diefenbach et al., 1991; Kuang and Goldberg, 2001). Anatomical studies revealed that each ENC1 has a single apical dendrite that extends to the embryo surface and is tipped with a putative chemosensory specialization (Diefenbach et al., 1998). Thus, ENC1 appears to be a sensorimotor neuron that interacts with postsynaptic ciliary cells to regulate the embryonic rotation behavior.

Since NO has been demonstrated in embryonic and larval gastropods (Lin and Leise, 1996; Serfösö et al., 1998), and is present and has physiological action in both sensory and ciliary systems (Li et al., 2000; Sisson, 1995; Uzlaner and Priel, 1999), we hypothesized that NO is present and plays an important role in the ENC1-ciliary neural circuit. Lin and Leise (1996) looked at the expression of NOS using NADPH diaphorase (NADPH-d) histochemistry through metamorphosis in the marine prosobranch *Ilyanassa obsoleta*. Expression was restricted to the ganglionic neuropils, with the most intense staining occurring in the apical ganglion. Serfözö et al. (1998) analyzed NADPH-d activity during embryogenesis and in early juveniles in *L. stagnalis*, and showed that the first expression is within the developing protonephridia, and later it occurs in cells of the developing pedal ganglia, developing eye and peripheral sensory neurons of the lips. To test for the expression of NO in *H. trivolvis* embryos in the present study, we used NADPH-d histochemistry on whole embryos. In addition, rotation behavior was analyzed in embryos that were exposed to pharmacological treatments that either increase or decrease levels of NO. We now report that ENC1 and dorsolateral ciliary cells are among a variety of tissues that express NO during early stages of *H. trivolvis* embryogenesis. Furthermore, pharmacological experiments indicated that NO has physiological actions in both ENC1 and ciliary cells. In light of previous *in vivo* and cell culture studies on the ENC1-ciliary neural circuit (Christopher et al., 1996, 1999; Diefenbach et al., 1991; Goldberg et al., 1994; Kuang and Goldberg, 2001), these results suggest that *H. trivolvis* provides a unique model system for investigating NO function at the cellular, circuit and behavioral levels during embryonic development.

Materials and methods

Animals

Helisoma trivolvis (Say, 1816) embryos were collected from a laboratory-reared albino colony housed at the University of Alberta, Canada. Snails were raised in flow-through aquaria containing de-chlorinated water at approximately 26°C, an oyster shell substratum and large plastic Petri plates for egg mass collection (Diefenbach et al., 1991). Snails were fed lettuce and trout pellets (NU-WAY; United Feeds, Calgary, Canada) and maintained on a 12 h:12 h light:dark cycle. Egg masses were collected the morning of their deposition onto collection plates as previously described (Diefenbach et al., 1991). They were transferred to fresh artificial pondwater (APW; 0.025% Instant Ocean, Aquarium Systems, Mentor, Ohio, USA) and maintained at room temperature until they developed to the desired stage. Embryos between stages E25 and E40 were used in this study (Diefenbach et al., 1998; Goldberg et al., 1988).

NADPH diaphorase staining

Whole-mount embryos were removed from egg masses and fixed in 4% paraformaldehyde dissolved in 0.01 mol l^{-1} phosphate-buffered saline for 1–3 h. Embryos were then washed 2× 20 min with Tris-buffered saline (TBS; 1.0 mol l^{-1} Tris in HCl, pH 8.4). NADPH-d reaction product was achieved through incubation of embryos for 3–5 h in 1 mmol l^{-1} NADPH, 0.1 mmol l^{-1} Dicoumarol, 0.5 mmol l^{-1} Nitroblue Tetrazolium salts and 0.4% Triton X-100 (Sigma, St Louis, MO, USA), all diluted in 0.05 mol l^{-1} TBS. At the end of the incubation time, the reaction was stopped with ice-cold TBS. In control experiments, NADPH was omitted from the staining solution. To best preserve cellular integrity, whole embryos were mounted on glass slides in TBS and viewed immediately using Nomarski differential interference contrast (DIC) optics on a Nikon Diaphot TMD inverted microscope with a 40× objective.

Rotational behavior

All rotational experiments were performed on stage E25 embryos. The outer membranes on the basal surface of the egg mass were cut open without disturbing the egg capsules in order to minimize diffusion barriers during drug treatments. Egg masses were allowed to recover for 60 min in filtered APW and then transferred to 35 mm Petri dishes (Falcon 1008) containing 2 ml of either APW or drug-containing APW. Embryos within egg capsules were monitored with a CCD video camera (JVC model TK-860U) mounted on a dissection microscope (Zeiss model SR). After 10 min of incubation, the embryonic rotational behavior was recorded at 2.5 frames s^{-1} for 10 min using a time-lapse video cassette recorder (Panasonic model AG-6720). This procedure was modified when treating embryos with the NO donor sodium nitroprusside (SNP) because of its high light sensitivity. When using SNP, data was collected for only 2 min. Given the similarity between the results of the SNP and *S*-nitroso-*N*-acetylpenicillamine (SNAP) treatments, this change did not appear to affect the results. In every experiment, all drug treatments and controls were done in parallel dishes on the same day. A maximum of five embryos were recorded from a single egg mass, and each treatment group contains data from at least three different egg masses over two separate experiments.

The rotational behavior was analyzed off-line by playing back the recordings at 30 frames s^{-1}, 12 times faster than the

recording rate. This faster playback speed facilitated the observation of very slow rotational movements. Rotations were counted over each recording period and the data was expressed in rotations min^{-1} (r.p.m.). To assess the inter-surge rotation rate and the frequency and amplitude of rotational surges, rotation rate was quantified every 5 s by measuring the degrees of rotation achieved in sequential 5 s intervals for 1 min (Diefenbach et al., 1991). Surges were operationally defined as sequential periods of increasing rotation rate that result in a total rise of at least 0.5 r.p.m., followed by a falling phase in which the rotation rate drops by at least 0.5 r.p.m.

Statistics

All values are means ± S.E.M. In experiments involving multiple treatment groups (Figs 2, 3), statistical significance was determined using an analysis of variance (ANOVA) followed by a Fisher's protected least-significant difference (PLSD) test. In experiments where a single treatment group was compared to a control group (Figs 5–7), the unpaired Student's *t*-test was used. Differences were considered statistically significant at $P<0.05$.

Chemicals

Serotonin (creatine sulphate complex; Sigma), sodium nitroprusside dihydrate (SNP; Sigma), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma) and N^G-nitro-D-arginine methyl ester hydrochloride (D-NAME; Sigma) were dissolved in *Helisoma* saline (HS; 51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 4.1 mmol l^{-1} $CaCl_2$, 1.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes, pH 7.35), and then diluted in APW to the final working concentration. *S*-Nitroso-*N*-acetylpenicillamine (SNAP; Tocris) and 7-nitroindazole (7-NI; Tocris) were dissolved in dimethyl sulfoxide (DMSO), and then diluted with APW so that the level of DMSO did not exceed 0.1%. This concentration of DMSO has previously been shown to have no effect on ciliary beat frequency (Christopher et al., 1999) or embryo rotation rate (Goldberg at al., 1994). All drug solutions were prepared on the same day that they were used.

Results

Localization of NOS in Helisoma *embryos*

NADPH-d histochemistry was used to indicate the expression of NOS activity in whole-mount *H. trivolvis* embryos. At stage E25, reaction product was consistently observed in a variety of structures (Fig. 1A), including the dorsolateral ciliary cells, ciliary cells lining the dorsal buccal cavity and the ciliated protonephridia. This did not represent nonspecific staining of all ciliary cells, as the pedal ciliary band usually contained no reaction product at this stage (Fig. 1A). In addition to ciliary staining, nuclear-localized reaction product was found in a small number of unidentified cells spread throughout the dorsal body wall and primordial mantle. There was also diffuse staining throughout the developing buccal mass and associated radular sac (Fig. 1B).

As ENC1s were easily visualized in unstained stage E25–E30 embryos using DIC optics (Diefenbach et al., 1998), they could be specifically examined for the expression of NOS activity. Surprisingly, NADPH-d reaction product usually occurred only in the distal portion of the apical dendrite, including the chemosensory-like dendritic knob that penetrates the outside surface of the embryo (Fig. 1A). Out of 113 stage E25 and E30 embryos mounted in the appropriate orientation to assess the soma and dendritic knob for NADPH-d staining, 97 (86%) had staining that was restricted to the dendritic knob. Eight embryos (7%) displayed staining in the ENC1 soma, apical dendrite and dendritic knob, whereas in another eight embryos (7%), ENC1 appeared unstained. By stage E35, reaction product was observed in the soma, throughout the apical dendrite and in the proximal descending neurite of ENC1 (Fig. 1B) in most embryos. In contrast, reaction product was never resolved in the distal portions of the descending primary neurite and its branches. The pedal ciliary cells also exhibited latent expression of NOS activity, as reaction product was often observed in these cells by stage E40.

The results of the NOS activity localization experiments are summarized diagrammatically in *H. trivolvis* embryos in front and side view (Fig. 1C,D). Since ENC1 neurons and ciliated cells that drive the rotation behavior were some of the structures that showed consistent and robust staining, these results prompt the hypothesis that NO plays an important role in mediating the chemosensory and ciliary activities that occur in stage E25–E40 embryos.

Regulation of embryo rotation behavior by NO

To assess whether NO may be involved in mediating or regulating embryonic rotation behavior, time-lapse videomicroscopy was used to record stage E25 embryos after exposure to NO donors, NO inhibitors, serotonin or vehicle solutions. In most experiments, a serotonin (100 μmol l^{-1}) treatment group was included as a positive control to indicate the expected maximal rotation response (Diefenbach et al., 1991; Goldberg et al., 1994).

Addition of the NO donors SNAP (Fig. 2A) and SNP (Fig. 2B) for 10 min induced a twofold increase in rotation rate, with the maximal response occurring at a concentration of approximately 10 μmol l^{-1}. In both cases, the increases produced by higher concentrations of NO donor (100 μmol l^{-1} SNAP and 250 μmol l^{-1} SNP) were slightly lower than that produced by the optimal concentrations, with the difference being statistically significant only for SNAP ($P<0.05$, 10 μmol l^{-1} SNAP *versus* 100 μmol l^{-1} SNAP, ANOVA). In contrast to the twofold maximal response to NO donors, 100 μmol l^{-1} serotonin (5-HT) induced a fourfold increase in rotation rate (Fig. 2A,B), similar to the three- to fivefold increase seen in previous studies (Diefenbach et al., 1991; Goldberg et al., 1994). To control for the possibility that the responses to the NO donors were induced by the NO donors themselves or NO by-products, rather than NO, 10 μmol l^{-1} SNAP and 25 μmol l^{-1} SNP solutions were incubated at 20°C for 24 h in the light before being exposed to embryos. After this treatment, which would have completely exhausted the NO-producing activity of these drugs (Van

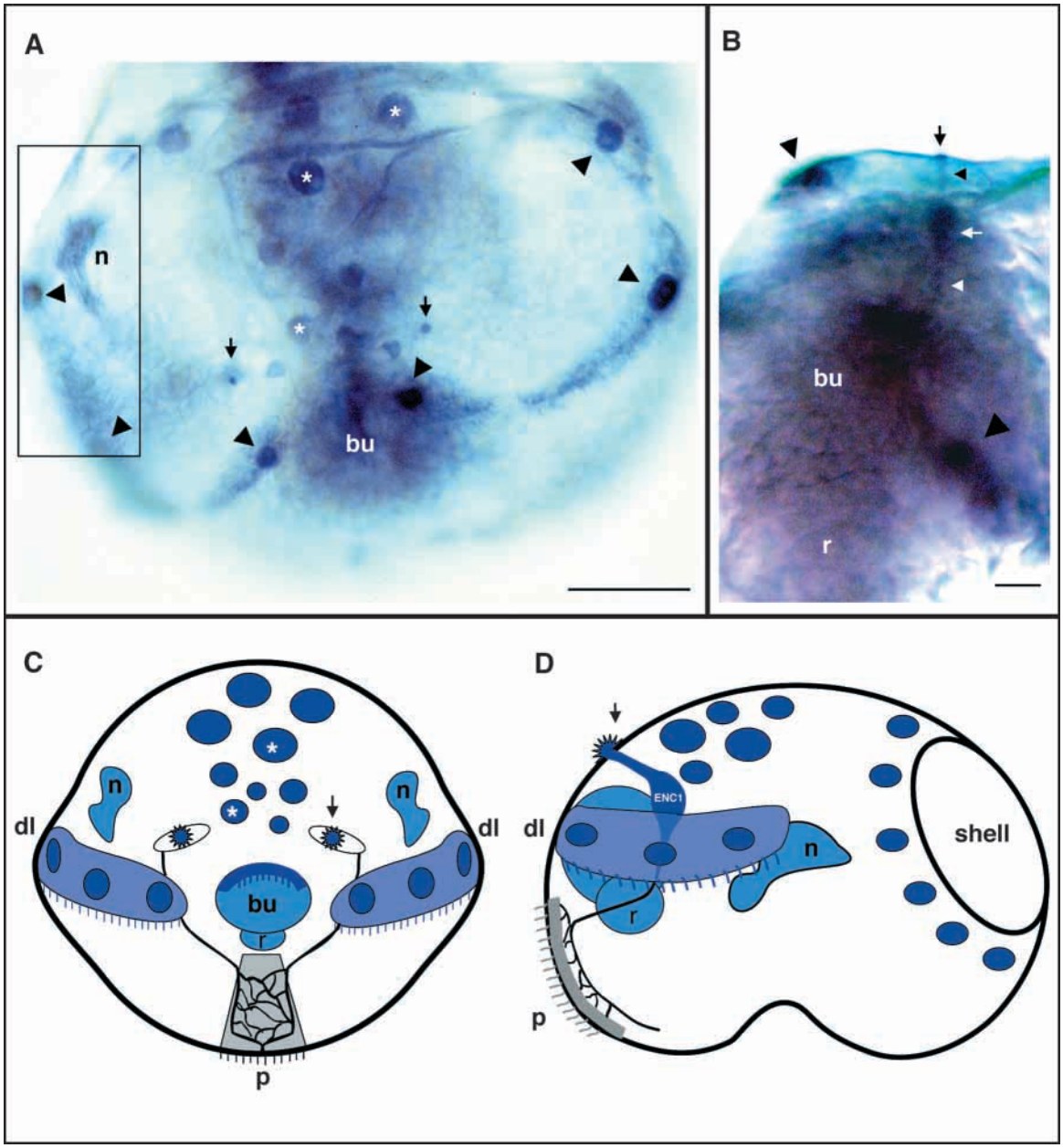

Fig. 1. Localization of NOS in *H. trivolvis* embryos as revealed by NADPH-d staining. (A) Dorsal-anterior view of stage E25 embryo. NOS was expressed bilaterally in the dendritic knobs of ENC1 (arrows), the ciliary cells of the dorsolateral ciliary bands (arrowheads) and the protonephridia (n). The entire buccal mass (bu) was diffusely stained throughout, with greatest intensity occurring in the ciliated dorsal region. Staining also occurred in several unidentified cells near the embryonic surface (asterisks) that were widely spaced throughout the dorsal half of the embryo, including the region of the primordial mantle. The box outlines a deeper focal plane of the same embryo that was spliced in to reveal staining in dorsolateral ciliary cells (arrowheads) and the protonephridium on the right side. Scale bar, 40 μm. (B) Longitudinal profile of stage E35 embryo at the level of the left ENC1 (anterior at left). The dendritic knob (black arrow), apical dendrite (small black arrowhead), soma (white arrow) and proximal descending axon (white arrowhead) of ENC1 were all stained at this stage. In addition, intense staining occurred in a dorsolateral ciliary cell (upper black arrowhead), the dorsal buccal region (bu) and the protonephridium (lower black arrowhead). The diffusely stained radular sac (r) is also shown. Scale bar, 30 μm. (C,D) Summary diagrams of NAPHD-d staining in *H. trivolvis* embryos corresponding to the stages and orientations presented in A (C) and B (D). Only structures depicted in blue were stained. At stage E25 (C), only ENC1's dendritic knob was reliably stained (arrow), whereas by stage E35 (D), its apical dendrite, soma and proximal axon were also stained. At both stages, the dorsolateral cilia (dl), buccal region (bu), radular sac (r), protonephridia (n) and unidentified dorsal superficial cells (asterisks) were stained. Surprisingly, the ciliary cells comprising the pedal ciliary band (p) were not reliably stained at either stage.

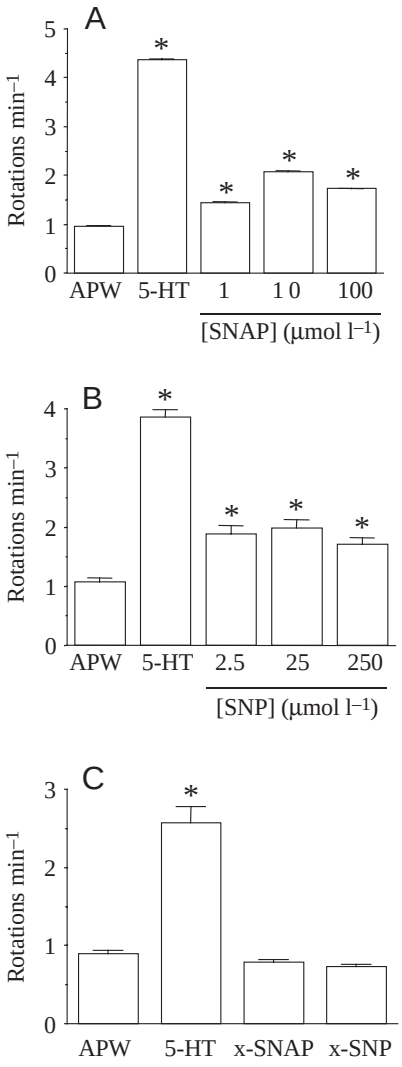

Fig. 2. Effect of NO donors on the embryonic rotation behavior. Application of SNAP (A) or SNP (B) at various concentrations resulted in a significant increase in the rate of embryonic rotation. In both cases, the most effective dose produced a twofold increase in rotation rate, in contrast to the fourfold increase produced by 100 μm serotonin (5-HT). (A) *N*=252 embryos; (B) *N*=147 embryos. (C) SNAP (10 μmol l^{-1}) and SNP (25 μmol l^{-1}) solutions that were pre-exposed to light for 24 h at room temperature (x-SNAP; x-SNP) had no effect on rotation rate. The NO-donating capability of SNAP and SNP are exhausted by this treatment, leaving only stable NO by-products. *N*=68 embryos. Asterisks represent statistically significant differences compared to the control (artificial pondwater, APW) treatment ($P<0.05$).

Wagenen and Rehder, 1999), they were no longer able to induce an increase in rotation rate (Fig. 2C). These results suggest that NO may play a stimulatory role in the rotational behavior of *H. trivolvis* embryos.

The effects of two different NOS inhibitors were assessed to determine more directly whether NO is involved in regulating rotational behavior under normal conditions. Exposure of embryos to the arginine analog L-NAME (10 mmol l^{-1}) induced a nearly 50% reduction in rotation rate ($P<0.01$, L-NAME *versus* APW; ANOVA), whereas the same concentration of D-NAME, a less active enantiomer, had no effect (Fig. 3A). Likewise, the NOS inhibitor 7-NI (100 μmol l^{-1}) caused a similar reduction in rotation rate, from 0.97±0.05 r.p.m. to 0.43±0.02 r.p.m. ($P<0.001$; Fig. 3B). In both these experiments, serotonin produced the expected three- to fivefold increase in rotation rate. To rule out the possibility that the effects of the NOS inhibitors were due to a non-specific action that disables the rotation behavior, we tested whether addition of a NO donor could bypass the NOS inhibition, and produce a normal stimulatory response (Fig. 3C). Addition of 10 mmol l^{-1} L-NAME alone produced the expected decrease in rotation rate. In contrast, when either 1 μmol l^{-1} or 10 μmol l^{-1} SNAP was combined with 10 mmol l^{-1} L-NAME, the rotation rate increased in a dose-dependent manner. Taken together, these results indicate that in stage E25 *H. trivolvis* embryos, normal rotation behavior depends in part on the presence and stimulatory action of NO.

Effects of NO on subcomponents of rotation behavior

Previous studies elucidated two subcomponents of the rotation behavior displayed by stage E25 embryos (Diefenbach et al., 1991). Superimposed upon a tonic slow rate of rotation are phasic bursts of rapid rotation, called surges. Pharmacological and laser-ablation studies suggested that surges result from the periodic release of serotonin from ENC1, causing cilio-excitation and accelerated rotation (Diefenbach et al., 1991; Kuang and Goldberg, 2001). Basal rotation, on the other hand, is probably due to the constitutive activity of dorsolateral and pedal ciliary cells (Goldberg et al., 1994). To determine whether NO manipulations affect the basal rotation rate or rotational surges, embryo rotation rate was measured every 5 s to reveal the temporal pattern of rotation.

Fig. 4 displays the results from two control embryos exposed to APW (Fig. 4A) and two experimental embryos exposed to 10 mmol l^{-1} L-NAME (Fig. 4B). Surges were operationally defined as sequential periods of increasing rotation rate that result in a total rise of at least 0.5 r.p.m., followed by a falling phase in which the rotation rate drops by at least 0.5 r.p.m. From this type of data, the rate of slow tonic rotation, amplitude of rotational surges and frequency of rotational surges were quantified to indicate the likely cellular sites of NO action (Fig. 5–7). For example, effects restricted to the mean rate of slow tonic rotation would indicate NO action directly on the ciliary cells. In contrast, changes in frequency of surges would suggest that ENC1 is the site of NO action, whereas changes in the amplitude of surges would indicate that either cell type may be involved.

In embryos exposed to 10 μm SNAP, the mean rate of slow tonic rotation was significantly elevated in comparison to those exposed to the control vehicle (SNAP, 1.70±0.05 r.p.m. *versus* APW, 0.89±0.05 r.p.m.; $P<0.001$; Fig. 5A). Similar results were obtained with a second NO donor, 25 μmol l^{-1} SNP (Fig. 5B). In contrast, exposure of embryos to the NOS inhibitors L-NAME (10 mmol l^{-1}) or 7-NI (100 μmol l^{-1}) had

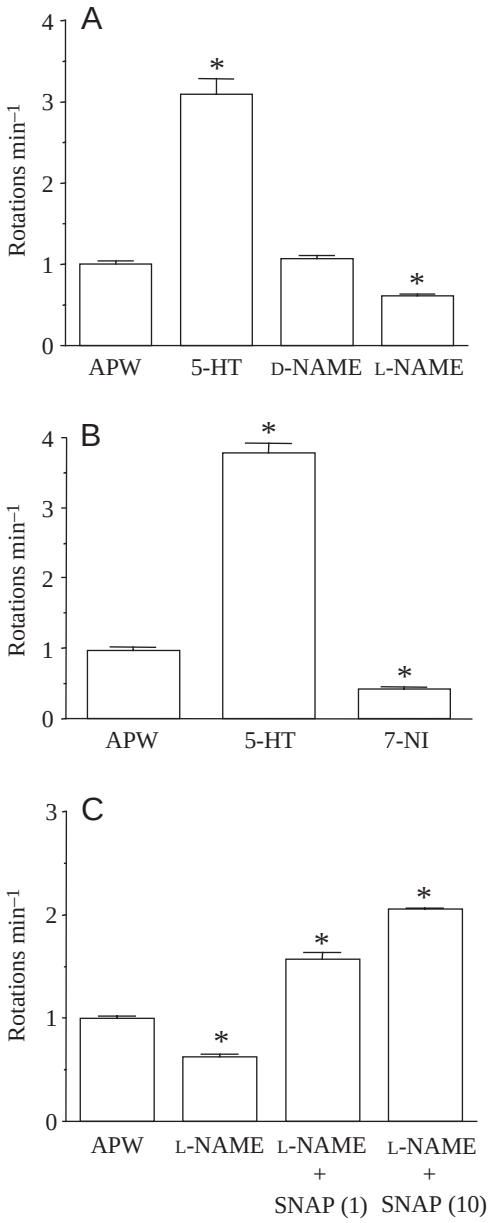

Fig. 3. Effect of NOS inhibitors on the embryonic rotation behavior. (A) Application of 10 mmol l^{-1} L-NAME resulted in a significant decrease in the rate of embryonic rotation (P<0.01, N=100 embryos), whereas 10 mmol l^{-1} D-NAME had no effect. (B) Application of 100 µmol l^{-1} 7-NI also produced a significant inhibition of rotation rate (P<0.001, N=63 embryos). In A+B, 100 µmol l^{-1} serotonin (5-HT) produced the expected three- to fivefold increase in rotation rate. (C) NO donors bypass the inhibitory effect of NOS inhibitors. Coapplication of 10 mmol l^{-1} L-NAME with either 1 µmol l^{-1} (1) or 10 µmol l^{-1} (10) SNAP resulted in a significant increase in embryonic rotation rate, rather than the decrease produced by L-NAME alone (P<0.001, N=137 embryos). Asterisks represent statistically significant differences compared to the control (artificial pondwater, APW) treatment.

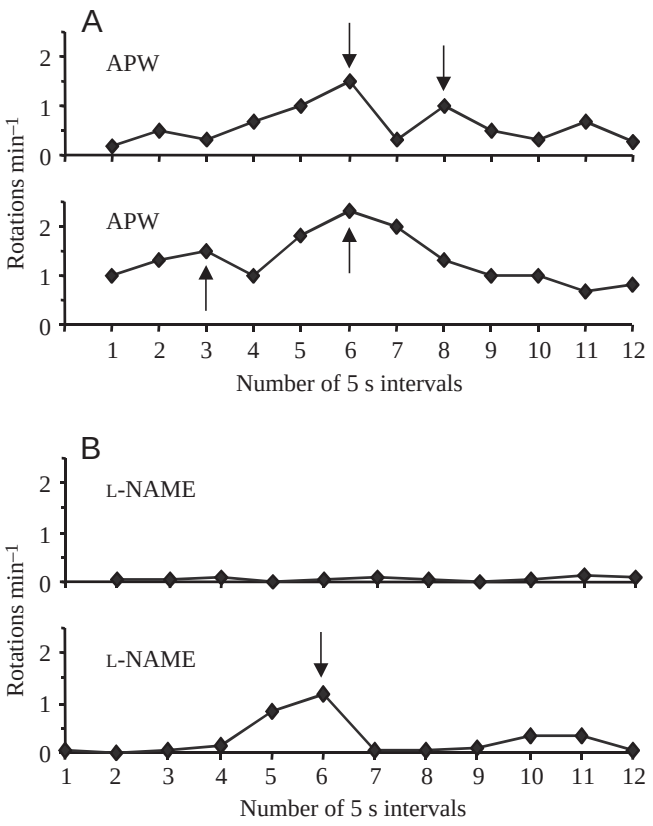

Fig. 4. Effect of L-NAME on subcomponents of embryonic rotation. Changes in rotation rate were measured over 5 s intervals. (A) In the presence of APW, two representative embryos displayed low basal rotation rates (0.3–1.2 r.p.m.) that were interrupted by periodic surges of faster rotation. (B) In the presence of 10 mmol l^{-1} L-NAME, two representative embryos displayed lower basal rotation rates (0–0.05 r.p.m.) and fewer surges. Arrows indicate the peak rotation rate during each surge in A and B.

inhibitory effects, with the mean rate of slow tonic rotation dropping to 0.08±0.01 r.p.m. (P<0.001) and 0.25±0.02 r.p.m. (P<0.0001), respectively (Fig. 5C,D). These results suggest that at least one main site of NO action is on ciliated cells, whereby tonic ciliary beating depends in part on constitutive NO activity.

Examination of the frequency and amplitude of rotational surges may reveal whether ENC1 is also a main target of NO activity. Whereas the NO donors SNAP (Fig. 6A) and SNP (Fig. 6B) did not significantly affect the frequency of rotational surges, the NOS inhibitors L-NAME (P<0.001; Fig. 6C) and 7-NI (P<0.001; Fig. 6D) did significantly reduce surge frequency. In the presence of these drugs, surges only occurred rarely during 1 min observation periods. In contrast to the results on surge frequency, NO donors were more effective than NOS inhibitors in regulating surge amplitude. SNAP (P<0.05; Fig. 7A) and SNP (P<0.05; Fig. 7B) both significantly enhanced the amplitude of surges, whereas L-NAME (Fig. 7C) and 7-NI (Fig. 7D) had no significant effects. However, since surges were only rarely expressed in the presence of NOS inhibitors (Fig. 7A,B), this conclusion is based on a relatively low sample size (N=3 for L-NAME; N=3 for 7-NI). Since surges are generated by the activity of the cilioexcitatory motor neurons, ENC1s (Kuang and Goldberg,

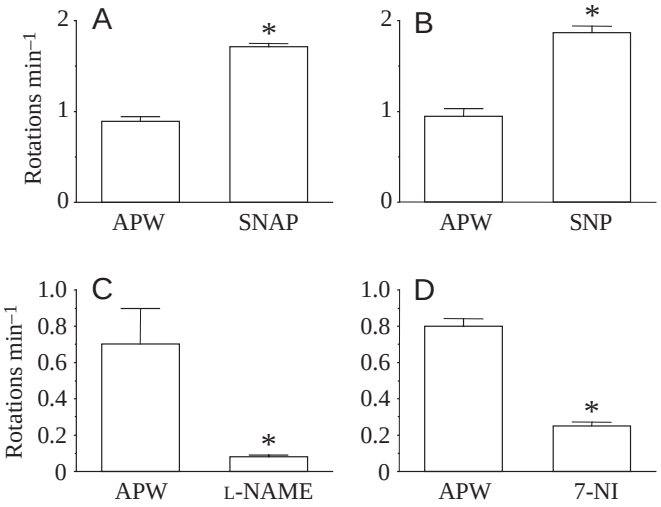

Fig. 5. Effect of NO donors and NOS inhibitors on the inter-surge rate of rotation. Both 10 μmol l^{-1} SNAP (A) and 25 μmol l^{-1} SNP (B) caused a significant increase in the mean rate of slow tonic rotation. In contrast, 10 mmol l^{-1} L-NAME (C) and 100 μmol l^{-1} 7-NI (D) caused a significant reduction in the mean rate of slow tonic rotation. Asterisks represent statistically significant differences compared to the control (artificial pondwater, APW) treatment. (A) P<0.001, N=32 embryos; (B) P<0.001, N=26 embryos; (C) P<0.001, N=14 embryos; (D) P<0.0001, N=22 embryos.

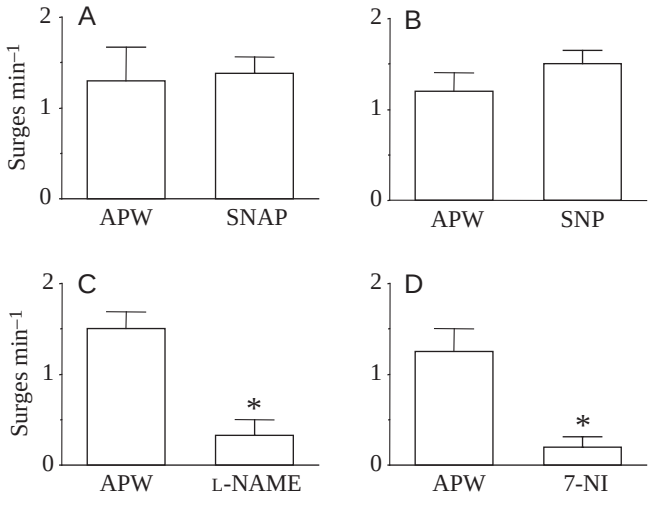

Fig. 6. Effect of NO donors and NOS inhibitors on the frequency of rotational surges. Both 10 μmol l^{-1} SNAP (A) and 25 μmol l^{-1} SNP (B) had no effect on the frequency of rotational surges. In contrast, 10 mmol l^{-1} L-NAME (C) and 100 μmol l^{-1} 7-NI (D) caused a significant reduction in the frequency of rotational surges. Asterisks represent statistically significant differences (P<0.001) compared to the control (artificial pondwater, APW) treatment. (A) N=34 embryos; (B) N=30 embryos; (C) N=17 embryos; (D) N=23 embryos.

2001), the reduction in surge frequency observed in the presence of NOS inhibitors suggests that ENC1s are also a main site of NO action in *H. trivolvis* embryos.

Discussion

H. trivolvis embryos have been used as a model system to study the diverse roles played by neurotransmitters during early stages of neuroembryogenesis. Studies to date have focused on serotonin, as its expression in the paired ENC1 neurons soon after gastrulation represents one of the earliest events of neural development in the entire embryo. Serotonin functions in the early embryo both as a regulator of neural development (Diefenbach et al., 1995; Goldberg and Kater, 1989) and an excitatory neurotransmitter in the ENC1-ciliary neural circuit (Kuang and Goldberg, 2001). This early embryonic circuit is thought to monitor environmental oxygen through ENC1's sensory dendritic knob and stimulate ciliary beating in response to hypoxia, producing an increased rate of cilia-driven embryo rotation (Kuang et al., 2002). Since studies on other systems have demonstrated that NO is a common regulator of neuronal development (Cheung et al., 2000; Ernst et al., 2000; Gendron et al., 2002; Hindley et al., 1997; Seidel and Bicker, 2000; Van Wagenen and Rehder, 1999, 2001; Wright et al., 1998), sensory signaling (Bicker, 2001; Breer and Shepherd, 1993; Gelperin et al., 2000; Schmachtenberg and Bacigalupo, 1999) and ciliary function (Li et al., 2000; Sisson, 1995; Uzlaner and Priel, 1999), the present study addressed whether NO is also present and active in *H. trivolvis* embryos. Our histochemical and pharmacological results indicate that, like serotonin, NO is an important signaling molecule in the ENC1-ciliary neural circuits of *H. trivolvis* embryos.

Localized expression of NOS-like activity in H. trivolvis *embryos*

The pattern of expression of NOS-like activity was examined in stage E25–E40 *H. trivolvis* embryos using NADPH-d histochemistry, a technique with potential limitations in specificity (Beesley, 1995; Blottner et al., 1995). Whereas a parallel analysis using NOS immunoreactivity could help confirm this pattern, NOS isoforms expressed in *H. trivolvis* embryos are not reliably recognized by the antibodies that we have tested to date. However, since pharmacological effects of NOS inhibitors have been observed exclusively in cells and tissues that are NADPH-d positive (Goldberg et al., 1999) (see below), the NADPH-d technique appears to be a reliable indicator of NOS activity in *H. trivolvis* embryos. Non-neuronal reaction product was observed initially in the paired protonephridia, dorsolateral ciliary bands, ciliary band lining the dorsal buccal cavity and assorted unidentified cells scattered throughout the dorsal body wall and primordial mantle. By contrast, reaction product within neurons was limited to the dendritic knob of each ENC1, chemosensory-like structures situated on the dorsolateral surface of the embryo (Diefenbach et al., 1998). By stage E40, reaction product was also observed in the pedal ciliary band, as well as the apical dendrite, soma and proximal descending neurite of ENC1.

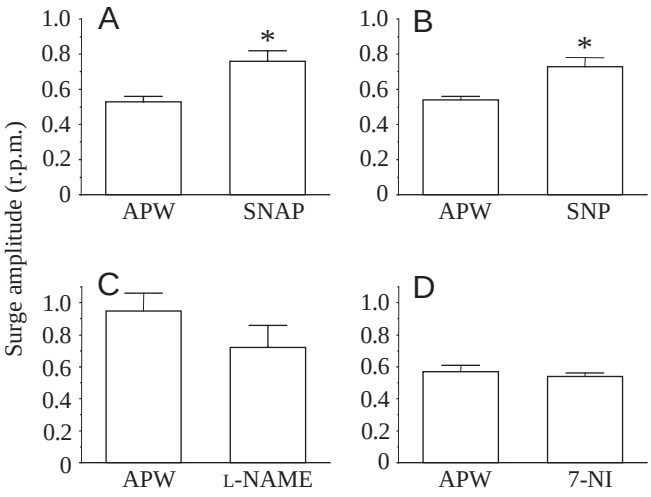

Fig. 7. Effect of NO donors and NOS inhibitors on the amplitude of rotational surges. Both 10 μmol l^{-1} SNAP (A) and 25 μm SNP (B) caused a significant increase in the mean amplitude of rotational surges, expressed in rotations per minute (r.p.m.). In contrast, 10 mmol l^{-1} L-NAME (C) and 100 μmol l^{-1} 7-NI (D) had no effect on the mean amplitude of rotational surges. Asterisks represent statistically significant differences compared to the control (artificial pondwater, APW) treatment. (A) $P<0.05$, $N=44$ surges from 34 embryos; (B) $P<0.05$, $N=39$ surges from 30 embryos; (C) $N=15$ surges from 17 embryos; (D) $N=13$ surges from 23 embryos.

In an NADPH-d analysis of *Lymnaea stagnalis* embryos, the staining pattern observed at corresponding developmental stages was very different from that described above (Serfösö et al., 1998). Whereas the protonephridia were stained in both species, *L. stagnalis* embryos had no staining in any of the other structures found to be positive in *H. trivolvis*. Detection of NADPH-d reaction product first occurred in peripheral and central neurons of *L. stagnalis* at much later stages of development. Although both *H. trivolvis* and *L. stagnalis* are pulmonate basommatophoran pond snails, their respective position within different families suggests that significant differences in embryonic development, structure and function are to be expected.

ENC1s have been proposed to be derived from the apical sensory organ described in a variety of marine gastropod larvae (Diefenbach et al., 1998; Kempf et al., 1997; Page and Parries, 2000). The expression of NOS-like activity in ENC1s is further evidence of this relationship, as NADPH-d reaction product has been found in the apical sensory organ of *Ilyanassa obsoleta* (Lin and Leise, 1996) and *Phestilla sibogae* (Meleshkevitch et al., 1997), the only larval gastropods tested so far. Pharmacological experiments suggested that NO inhibits metamorphosis in *I. obsoleta* (Froggett and Leise, 1999; Leise et al., 2001), yet promotes metamorphosis in *P. sibogae* (Meleshkevitch et al., 1997). Whereas direct-developing gastropods such as *H. trivolvis* do not have a true larval stage terminated by an environmentally induced metamorphosis, our preliminary studies suggest that NO does regulate developmental processes during *H. trivolvis* embryogenesis (Goldberg et al., 1999).

A surprising outcome of the present study was that NADPH-d staining in ENC1 first occurred in the distal apical dendrite and dendritic knob at stage E25, whereas staining in the soma and proximal axon was not consistently expressed until stage E35. Since the dendritic knob is located on the embryo surface and has structural features typical of chemosensory receptors (Diefenbach et al., 1998; Koss et al., 2002), the presence of NOS-like activity suggests that NO is involved in chemoreception. Recent experiments have suggested that ENC1 is an oxygen sensor that excites postsynaptic ciliary cells in response to hypoxia (Kuang et al., 2002). Thus, NO may participate in oxygen sensing in ENC1. Alternatively, since NO has been implicated in olfactory signaling in a variety of systems (Bicker, 2001; Breer and Shepherd, 1993; Gelperin et al., 2000; Schmachtenberg and Bacigalupo, 1999), ENC1 may be a multimodal sensory neuron, with NO involved in the detection of unidentified olfactory cues.

The expression of NOS-like activity in ciliated epithelial cells of *H. trivolvis* embryos is consistent with reports of NOS expression (Asano et al., 1994; Xue et al., 1996) and NO production (Li et al., 2000) in mammalian ciliary cells. The differential distribution of NADPH-d reaction product in dorsolateral and pedal ciliary cells at stage E25 correlates well with our earlier finding that serotonin plays a greater role in ENC1-mediated cilioexcitation in pedal *versus* dorsolateral ciliary cells (Kuang and Goldberg, 2001). Furthermore, since ENC1 only directly innervates the most medial cell of each dorsolateral band (Koss et al., 2002), NO signaling may be required to pass the excitation to adjacent lateral cells.

Physiological effects of NO on ENC1s and ciliary cells

The behavioral effects of pharmacological treatments that either raise or lower levels of NO in embryos indicated that NO is tonically active, with sites of action in both ENC1s and their target ciliary cells. The stimulation of embryo rotation rate produced by two different NO donors, and the inhibition produced by two different NOS inhibitors, provided strong evidence that NO plays an excitatory role in generating the normal, ongoing rotation behavior. Previous studies have established that the rotation behavior has two basic components: (i) slow tonic rotation mediated by the constitutive activity of the dorsolateral and pedal ciliary bands; and (ii) periodic episodes of rapid rotation, called surges, that result from the phasic release of serotonin from both ENC1s onto their postsynaptic ciliary cells (Diefenbach et al., 1991; Goldberg et al., 1994; Kuang and Goldberg, 2001). Thus, NO may mediate its excitatory effect by acting on various processes, including ENC1 sensory transduction and integration, ENC1-ciliary synaptic transmission and tonic ciliary beating. These possibilities were assessed by quantitative analysis of the subcomponents of the embryo rotation behavior during responses to pharmacological treatments.

The reduction in frequency of surges caused by NOS

inhibitors is the most direct evidence that ENC1 is a site of NO action. Since NO donors did not cause an opposite increase in surge frequency, NO may act constitutively rather than periodically to promote excitatory bursts in ENC1. Whether NO affects ENC1 activity through a modulation of ion channel activity, as described in identified *Aplysia* neurons (Koh and Jacklet, 2001), or by acting on other elements in the ENC1 sensory transduction pathway, is unknown.

The effect of NO donors on surge amplitude indicates either an ENC1 or ciliary site of NO action. Of these possibilities, presynaptic facilitation of ENC1-ciliary synaptic transmission is most likely because in cell culture experiments on isolated ciliary cells, NO donors did not facilitate the ciliary response to serotonin (Goldberg et al., 2000). However, the possibility of NO acting postsynaptically as a neurotransmitter (Park et al., 1998) or modulator of the serotonin response pathway cannot be ruled out.

The effects of NOS inhibitors and NO donors on the non-surge component of embryo rotation are the easiest to interpret. They strongly suggest that NO is a constitutive regulatory element of ciliary beating, providing a tonic excitatory influence under basal conditions. Furthermore, since these results were consistent with the effects of NOS inhibitors and NO donors on isolated ciliary cells (Goldberg et al., 2000), where there was no opportunity for ENC1-ciliary interactions, NO must be produced within ciliary cells to act as an endogenous regulator of ciliary beating. These results do not rule out the possibility that NO may also function in a neurotransmitter-like manner, diffusing from ENC1 into ciliary cells to stimulate ciliary beating. In addition, since only some ciliary cells are innervated by ENC1 (Koss et al., 2002), the remaining ciliary cells may depend on the paracrine actions of NO to produce excitatory responses. These transcellular actions of NO could be tested by exposing embryos to extracellular NO scavengers.

Studies on mammalian ciliary cells have not yet revealed a complete understanding of the role of NO in regulating ciliary beating. Stimulation of ciliary beating by NO was reported to occur in tracheal cells of rats (Li et al., 2000) and rabbits (Uzlaner and Priel, 1999), and in bovine bronchial cells (Sisson, 1995). Taken together with the stimulatory action of NO on the non-surge component of embryonic rotation (Fig. 5) and isolated *H. trivolvis* ciliary cells (Goldberg et al., 2000), it appears that ciliary stimulation may be a common action of NO. However, a study on ovine tracheal cells reported that NO donors did not affect the ciliary beat frequency (Salathe et al., 2000). Furthermore, only the present study on *H. trivolvis* embryos indicated that NO is a constitutive regulator of ciliary activity or cilia-driven behavior. Application of a NOS inhibitor on its own, which should indicate the presence of tonic NO activity, had no effect on the ciliary beat frequency of rat tracheal cells (Li et al., 2000). A more extensive investigation of this question in other ciliary systems is required before concluding whether *H. trivolvis* embryonic ciliary cells are typical or unique with respect to NO exerting tonic control of ciliary beating.

In conclusion, both the histochemical experiments on NOS distribution and the pharmacological and behavioral experiments on NO function point to a central physiological role for NO in regulating the ENC1-ciliary neural circuit at multiple sites of action. NO thus joins serotonin as a neurotransmitter system expressed early in *H. trivolvis* embryonic development that participates in controlling the first behavioral response of the animal, a hypoxia-induced stimulation of embryonic rotation (Kuang et al., 2002). Although not directly addressed by this study, it is highly likely that the physiological regulation of this behavior involves intimate interactions between these two neurotransmitters.

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