Constitutive and permissive roles of nitric oxide activity in embryonic ciliary cells.

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Running head: Constitutive NO activity in ciliary cells

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ABSTRACT

Embryos of *Helisoma trivolvis* exhibit cilia-driven rotation within the egg capsule during development. In this study we examined if nitric oxide (NO) is a physiological regulator of ciliary beating in cultured ciliary cells. The NO donor S-Nitroso-N-acetylpenicillamine (SNAP; 1-1000 µM) produced a dose-dependent increase in ciliary beat frequency (CBF). In contrast, the nitric oxide synthase (NOS) inhibitor 7-Nitroindazole (10 and 100 µM) inhibited the basal CBF and blocked the stimulatory effects of serotonin (100 µM).

NO production in response to serotonin was investigated with 4,5-Diaminofluorescein diacetate imaging. While SNAP (100 µM) produced a rise in NO levels in all cells, only 22% of cells responded to serotonin with a moderate increase. The cGMP analogue 8-Br-cGMP (0.2 and 2 mM) increased CBF and the soluble guanylate cyclase inhibitor LY 83583 (10 µM) blocked the cilio-excitatory effects of SNAP and serotonin. These data suggest that NO has a constitutive cilio-excitatory effect in *Helisoma* embryos and that the stimulatory effects of serotonin and NO work through a cGMP pathway. It appears that in *Helisoma* cilia NO activity is necessary, but not sufficient, to fully mediate the cilio-excitatory action of serotonin.
INTRODUCTION

Cilia, cellular organelles that appear as hair-like extensions from the cell membrane, are ubiquitous throughout biology, excluding insects and nematodes (Rivera, 1962). They exhibit a conserved microtubular ultrastructure and are responsible for the generation of fluid currents over the surface of the cell (reviewed in Satir and Sleigh, 1990). Studies on a wide variety of organisms have generated little consensus over the regulation of ciliary beating. It appears that ciliary beat frequency (CBF) is normally controlled by phosphorylation of specific ciliary proteins and changes in intracellular calcium concentration ([Ca$^{2+}$]). A cyclic adenosine monophosphate - protein kinase A (cAMP-PKA) dependent pathway has been implicated in the stimulation of CBF in human nasal epithelium (Di Benedetto et al., 1991), hamster oviductal epithelium (Morales et al., 2000), frog esophagus (Braiman et al., 1998) and gill cilia of Mytilus edulis (Murakami, 1987). Alternatively, protein kinase C (PKC) has been shown to increase CBF in frog esophagus through activation of calcium influx (Levin et al., 1997) and decrease CBF in sheep tracheal epithelium through ciliary protein phosphorylation (Salanthe et al., 1993). Increases in [Ca$^{2+}$], have been found to stimulate CBF and change the ciliary beat mechanics in Paramecium (Bonini et al., 1991), Chlamydomonas (Witman, 1993) and ctenophores (Tamm and Terasaki, 1994). In frog esophagus, elevated Ca$^{2+}$ is necessary but not sufficient to enhance CBF (Zagoory et al., 2001), whereas in ovine tracheal epithelium, the data suggests that Ca$^{2+}$ is having a direct effect at the level of the cilium (Salanthe and Bookman, 1999). Further, in rabbit airway epithelium it
appears that CBF is altered by frequency modulated changes in $[\text{Ca}^{2+}]$. Thus, no singular unified mechanism for CBF regulation has emerged.

Nitric oxide is a gaseous signalling molecule that has recently been implicated in the regulation of ciliary function. It is produced in the conversion of L-arginine into L-citrulline by the enzyme NO synthase (NOS) and may function as both a neurotransmitter and an intracellular signal transduction molecule in vertebrates and invertebrates (Arancio et al., 1996; Bentz et al., 2000; Moroz, 2000; Jacklet, 1997). In rabbit airway epithelium, a $\beta$-adrenoceptor-mediated pathway causes NO synthesis, which in turn increases CBF (Tamoki et al., 1995). Likewise in human airway epithelium, muscarinic receptor activation stimulates an increase in CBF through NO production (Yang et al., 1996). Furthermore, ethanol stimulates NO production and a subsequent increase in CBF in bovine airway epithelium (Sisson, 1995). In a variety of mammalian systems examined, the cilioexcitatory actions of NO are mediated by the cyclic guanine monophosphate (cGMP) second messenger system (Wyatt et al., 1998; Uzlaner and Priel, 1999; Li et al., 2000).

While there is mounting evidence supporting the role of NO in mammalian CBF regulation, there has been little examination of the role of NO within invertebrate ciliated cells, with the exception of *Paramecium* (reviewed in Bonini et al., 1991). This is surprising given the apparent conservation of this signaling molecule throughout the animal kingdom (reviewed in Colasanti and Venturini, 1998). Prior to the development of the central nervous system in *Helisoma trivolvis*, a bilateral pair of serotonergic sensory-
motor neurons, embryonic neurons C1 (ENC1s), innervates the paired dorsolateral ciliary bands and a single pedal ciliary band (Diefenbach and Goldberg, 1991; Goldberg, 1998; Kuang and Goldberg, 2001; Koss et al., in press.). These simple neural circuits regulate the first embryonic behavior, cilia-driven embryo rotation within the egg capsule (Kuang et al., 2002). The rate of embryonic rotation is regulated according to the oxygen content within the egg capsule, whereby hypoxia causes faster rotation that stirs the capsular fluid and promotes oxygen diffusion to the embryo (Kuang et al., 2002). This response is mediated in part by the release of serotonin onto pedal and dorsolateral ciliary cells, which stimulates CBF through a novel PKC isoform and Ca\textsuperscript{2+} influx (Christopher et al., 1996; 1999). In the present study, we examined whether NO may also contribute to the regulation of ciliary activity.

In a recent study, NADPH diaphorase staining suggested the presence of NOS in the dorsolateral ciliary cells and ENC1s' sensory apparatus at stage E25, with additional expression in the pedal ciliary cells and ENC1’s soma and descending neurite at later stages (stage E35-E40) (Cole et al., 2002). Additionally, pharmacological manipulations of NO content altered embryonic behavior. Whereas the application of NO donors increased the rate of embryonic rotation, NOS inhibitors decreased the rate of embryonic rotation (Cole et al., 2002). Furthermore, the behavioral data suggested that NO had effects on both ENC1 and ciliary cells. Taken together these results prompted us to test the hypothesis that embryonic ciliary cells are major sites of NO activity. In the present study on cultured dissociated ciliary cells, we now report that endogenous NO constitutively stimulates ciliary beating and this cilioexcitatory action is mediated through
soluble guanylate cyclase (sGC) and cGMP. Additionally, the cilioexcitatory effects of serotonin depend on a NO, sGC and cGMP pathway.
MATERIALS AND METHODS

Animals

*Helisoma trivolvis* embryos were collected from a laboratory-reared albino colony raised at the University of Alberta. Snails were raised in flow-through aquaria containing de-chlorinated water at approximately 25 °C, an oyster shell substratum and maintained on a 12 hr – 12 hr light – dark cycle. Snails were fed Romaine lettuce and trout pellets (NU-WAY, United Feeds, Calgary, Canada). Egg masses were collected daily, as previously described, from large plastic petri plates placed in the aquaria for the purpose of egg mass deposition (Diefenbach et al., 1991). These egg masses were transferred to a 35 mm petri dish containing artificial pond water (APW; 0.025% Instant Ocean, Aquarium Systems, Mentor, Ohio) and maintained at room temperature through development. Embryos between stages E20 and E40 were used in this study (Diefenbach et al., 1998; Goldberg et al., 1988).

Chemicals and Solutions

Embryonic cells were cultured in *Helisoma* defined medium (HDM: 50% Liebovitz-15 (Gibco, Burlington, ON), 40.0 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 5.0 mM Hepes, 50 µg mL⁻¹ gentamicin, 150 L-glutamine µg mL⁻¹ (Sigma, St. Louis, MI), pH 7.3-7.35). Serotonin (5-hydroxytryptamine creatine sulphate complex, 5-HT; 100 µM; Sigma, St. Louis, MI) was dissolved in *Helisoma* saline (HS: 51.3 mM NaCl, 1.7 mM
KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 5.0 mM Hepes; pH 7.35). S-Nitroso-N-acetylpenicillamine (SNAP; 1-1000 µM; Tocris, Ellisville, MO), 7-Nitroindazole (7-NI; 10 and 100 µM; Tocris, Ellisville, MO) and 8-Bromo-cGMP (8-Br-cGMP; 0.2 and 2 mM; Sigma, St. Louis, MI) were dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MI), and then diluted to working concentrations in HS so that the DMSO level did not exceed 0.1%. This DMSO concentration has previously been demonstrated to have no effect on ciliary beat frequency (Christopher et al., 1999). LY 83583 (LY; 10 µM; Tocris, Ellisville, MO) was dissolved in ethanol (EtOH) and diluted in HS so that the EtOH concentration did not exceed 0.1%. Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.1 and 1 mM; Sigma, St. Louis, MI) and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; 300 µM; Calbiochem, San Diego, CA) were dissolved in water and then diluted to working concentrations in HS. 4,5-Diaminofluorescein diacetate (DAF-2 DA; Calbiochem, San Diego, CA) was dissolved in DMSO. All drugs were prepared the day of use.

Ciliated Cell Culture

Embryonic ciliated cells were cultured as previously described (Christopher et al., 1996, 1999). Briefly, intact egg masses (stage E20 – E35) were disinfected in 35% ethanol and the embryos were removed. Isolated embryos were treated with 0.2% trypsin (Sigma, St. Louis, MI) for 30 min and then mass dissociated by repeatedly passing them through a 63 µm nylon mesh (Small Parts Inc., Miami, FL). Dissociated cells were cultured in poly-L-lysine-coated (hydrobromide, mol. wt. 4000-15000, 1 µg
mL⁻¹, Sigma, St. Louis, MI) culture dishes (Falcon 3001, Becton Dickinson, Franklin Lakes, NJ) containing HDM. For NO imaging studies, cells were cultured on poly-L-lysine-coated glass-bottom culture dishes. The cultures were maintained in a humidified box at room temperature (20-22 °C) for 18-24 hours to allow for cell adhesion to the substrate.

**Time-Lapse Videomicroscopy**

Ciliary beating was monitored with a CCD video camera (JVC, TK-860U) mounted on a compound microscope (Nikon, Diaphot) with phase contrast optics, and was recorded with a time-lapse videocassette recorder (VCR; Panasonic AG-6720). Ciliary activity was recorded over a 5-10 sec interval immediately prior to and 10 min after a maintained application of the vehicle control or drug solution. Off-line analysis consisted of slowing the playback speed to 1/24 normal and manually counting ciliary beats over a one minute interval. For each treatment, CBF was presented as a percentage of the pre-treatment measurement. Each experiment included a parallel control culture that received only the vehicle solution. Error bars represent the standard error of the mean (s.e.m.). Statistical significance was determined by an analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD) test. Each bar graph contains an inset that indicates the statistical comparison between all treatment groups. Statistically significant differences occurred between treatment groups that do not share the same underscore (p < 0.05). Within each inset the treatment groups are arranged in decreasing amplitude of the response.
NO Imaging

Embryonic ciliated cells were loaded with 5 µM DAF-2 Diacetate in HS for 45 min at room temperature. Culture dishes were rinsed with HS three times before imaging. In the presence of NO, DAF-2 is converted to the fluorescent product DAF-2 triazole (Kojima et al., 1998). Given that this fluorescent product accumulates within the cell, no attempts were made to washout the serotonin-induced fluorescence. However, in every experiment SNAP (100 µM) was applied following serotonin to ensure that the indicator was responding to NO. Ciliated cells were imaged with a 100X oil-immersion objective (1.3 N.A. Fluor) on an inverted microscope (Zeiss, Axiovert 135) with excitation at 495 nm from an Hg-Xe arc lamp (Hamamatsu, Hamamatsu, Japan). Emission fluorescence, at 515 nm, was collected using an intensified charge-coupled device (ICCD) video camera (Paultek Imaging, Grass Valley, CA). Neutral density filters (Omega Optica, Battleboro, VT) were used to ensure that fluorescent images were within the sensitivity range of the camera. Data were collected using custom software courtesy of Dr. S. Kater (University of Utah) as 8-bit images. Captured images were digitized through a QuickCapture frame grabber board (Data Translation, Mississauga, ON) and saved to a computer for off-line analysis (Macintosh Quadra 950). Images were analyzed for fluorescence intensity on a Macintosh G4 computer using the public domain program NIH Image (version 1.61) and a macro for DAF Imaging custom written by C.J.H. Wong. Fluorescence was thresholded in a region of interest that included most of the surface of the cell and fluorescence was measured as the average pixel intensity and
background subtracted. Fluorescence was measured as changes in arbitrary fluorescence units (ΔF) relative to the average of fluorescence measured 30 and 60 seconds before the start of the experiment. A response was defined as a sustained increase in fluorescence of 3 or more fluorescent units within 5 time points after the addition of a drug. In no cases did perfusion with HS stimulate an increase in fluorescence. Given that DAF-2 is a non-ratiometric indicator, no attempts were made to quantify NO.
RESULTS

Effect of NO on Ciliary Beating

Evidence from a variety of systems suggests that NO has cilio-excitatory actions. To determine if NO produces similar effects on *Helisoma* ciliated cells, the NO donor SNAP was applied to cultured ciliated cells and CBF was measured with time-lapse videomicroscopy. Addition of SNAP for 10 min increased CBF in a dose–dependent manner (Fig. 1A). The 100 µM SNAP concentration produced a maximal increase of 118.1 ± 4.1% (p < 0.05 vs. HS control, n=10), whereas application of 1000 µM SNAP had no effect (Fig 1A). The SNAP-induced increase in CBF was compared to a serotonin treatment, a known endogenous cilio-excitatory factor in *Helisoma* embryos (Fig 1B). A 10 min application of 100 µM serotonin, a dose known to maximally increase CBF (Christopher et al., 1996), stimulated a significant increase in CBF to 132.5 ± 3.2% (p < 0.05 vs. HS control, n=11) (Fig 1B). Co-application of 100 µM SNAP and 100 µM serotonin produced a significant increase in CBF to 125.7 ± 5.6% (p < 0.05, n=14), not statistically different from the independent treatments of either SNAP or serotonin alone (Fig 1B). These data suggest that there was no additivity between serotonin and SNAP responses.

To further investigate the involvement of an NO pathway in the regulation of ciliary activity, the effects of a NOS inhibitor on CBF were examined (Fig. 2). Application of 7-NI produced a dose-dependent decrease in CBF after 10 min (Fig. 2A). Application of
10 µM 7-NI significantly reduced CBF to 80.8 ± 3.3% of the pretreatment rate (p < 0.05, n=10) and the 100 µM dose of 7-NI significantly decreased CBF to 75.4 ± 1.3% of the pretreatment rate (p < 0.05, n=13). There was no significant difference between the 10 µM 7-NI and 100 µM 7-NI treatments. Application of an additional NOS inhibitor, L-NAME, also produced a significant decrease in the rate of ciliary beating at the concentrations tested, 0.1 mM (p < 0.05; n=7) and 1 mM (p < 0.05; n=7; data not shown). These findings suggest that endogenous NO may have a constitutive excitatory action on ciliary beating in *Helisoma* embryos.

To determine whether NOS inhibition affects the cilio-excitatory action of serotonin, 100 µM 7-NI was co-applied with 100 µM serotonin. Application of serotonin stimulated CBF to increase to 132.6 ± 3.0 % when applied on its own, but no significant change in CBF when co-applied with 7-NI (Fig. 2B). As expected, 100 µM 7-NI alone inhibited CBF to 85.3 ± 3.5% (p < 0.05, ANOVA; Fig. 2B). Thus, it appeared that the NOS inhibitor blocked the cilio-excitatory action of serotonin.

To determine if the site of NO activity was strictly intracellular we examined the effects of the extracellular NO scavenger c-PTIO on the ciliary activity of isolated ciliary cells. Application of 300 µM c-PTIO had no effect on basal CBF (control vs. c-PTIO, p > 0.05) or the cilio-excitatory response to serotonin (5-HT vs. c-PTIO + 5-HT, p > 0.05; Fig. 3A). As a positive control, we tested whether c-PTIO could attenuate the ciliary response to the NO donor SNAP, which should elevate NO extracellularly. Indeed, 300 µM c-PTIO completely blocked the cilio-excitatory response to SNAP (SNAP vs. c-PTIO + SNAP, p
< 0.05; Fig. 3B). These data suggest that the NO produced within ciliary cells acts intracellularly rather than in an autocrine or paracrine fashion.

**Effect of serotonin on NO Production**

Since 7-NI inhibited ciliary beating when presented alone, its blockage of the serotonin response does not prove that NO participates in the signal transduction of serotonin’s effect. Therefore, we used the fluorescent NO indicator DAF-2 DA to determine if serotonin directly stimulates NO production. In most cells examined, the baseline fluorescence increased gradually under basal conditions in HS (Fig. 4). This increase of approximately 5-8 units over 30 min represents basal NO production and is consistent with the idea of constitutive NO activity. To ensure that solution changes did not alter fluorescence, the HS was replaced with fresh HS before applying the drug treatment (Fig. 4). Application of 10 µM and 100 µM SNAP revealed that only the higher dose of the NO donor produced a detectable increase in DAF-2 fluorescence (Fig. 4A). Thus, 100 µM SNAP was added after each serotonin treatment as a positive control for the effectiveness of DAF-2. 78% of the cells (28 of 36 cells) examined did not show an increase in NO in response to application of 100 µM serotonin for 15 min, as represented in Fig. 4B. In contrast, the remaining cells displayed a marginal step-like increase of 3-8 fluorescent units in response to 100 µM serotonin (Fig. 4C). In all cases, this response was smaller than that produced by 100 µM SNAP. Taken together, these results suggest that serotonin does not effectively stimulate NO production in ciliated cells.
Involvement of cGMP in Excitatory Ciliary Responses

One of the most recognized signal transduction pathways utilized by NO is the cGMP pathway (Bredt and Snyder, 1992; Hanafy, K.A. et al., 2001). We decided to use a membrane permeable cGMP analogue to determine if this pathway participates in ciliary regulation in Helisoma embryos (Fig. 5). Application of 0.2 mM and 2.0 mM 8-Br cGMP caused significant increases in CBF to 116.6 ± 1.5% (p < 0.05 vs. control, n=18) and 125.5 ± 2.2% (p < 0.05, n=14) of pretreatment values, respectively (Fig. 5). Neither dose of 8-Br cGMP produced an increase in CBF that was significantly different from the serotonin positive control (100 µM). These data demonstrate that activation of a cGMP pathway produces an increase in ciliary activity.

To examine if a cGMP pathway is involved in the cilio-excitatory effects of NO and serotonin, an inhibitor of the enzyme soluble guanylate cyclase was used (Fig. 6). Application of 10 µM LY83583 alone produced no change in CBF after 10 min, but significantly decreased CBF to 86.7 ± 4.8% of the pretreatment value when co-applied with 100 µM SNAP (Fig. 6A). Similarly, the stimulatory effect of serotonin was blocked by 10 µM LY when co-applied with LY (Fig. 6B). These findings suggest that a cGMP pathway may be necessary to mediate the cilio-excitatory effects of both NO and serotonin.
DISCUSSION

Evidence from mammalian systems suggests an important role for NO in the regulation of ciliary activity. These findings prompted us to examine embryos of the pond snail *Helisoma trivolvis* for the involvement of NO in ciliary regulation. We determined that the application of the NO donor SNAP produced an increase in CBF that was not additive to the stimulatory effects of serotonin. The NOS inhibitor 7-NI decreased the basal rate of ciliary beating and blocked the stimulatory effect of serotonin. Serotonin did not appear to stimulate a significant increase in NO production as revealed through fluorescent imaging. Finally, a cGMP analogue stimulated ciliary beating and a sGC inhibitor blocked the stimulatory effects of both serotonin and SNAP.

A novel result from this study was that 7-NI significantly reduces the rate of unstimulated ciliary beating. This result suggests that NO has a constitutive action in *Helisoma* ciliated cells. The rising baseline of fluorescence in the DAF-2 experiments supports this idea of basal NO production. Furthermore, constitutive NO activity is also supported by whole animal examinations of embryonic behavior, where both 7-NI and the arginine analog L-NAME caused nearly a 50% reduction in the embryonic rotation (Cole et al., 2002). Studies on other systems have not revealed a similar constitutive role for NO. In both human adenoid tissue and rat airway epithelium, application of NOS inhibitors did not result in a significant inhibition of CBF (Yang et al., 1996; Li et al., 2000). In both these mammalian systems, NO is recruited as a signal transduction element rather than being continually produced to drive basal ciliary activity. More
ciliary systems need to be examined to determine whether the novel constitutive action of NO observed in the present study is unique or widespread among many species.

It is interesting to note that in contrast to 7-NI, application of the sGC inhibitor, LY 83583, did not significantly affect CBF when applied alone. This suggests that the constitutive NO activity may not act through the sGC-cGMP pathway. NO has been shown in other cellular responses to generate physiological activity through direct protein interactions (reviewed in Broillet, 1999). For example, S-nitrosylation, the transfer of an NO group to cysteine residues on proteins, has been proposed to be as prevalent a regulatory mechanism as phosphorylation events. Given that NO is a highly conserved signaling molecule that is being increasingly identified to modulate ciliary activity, it may be that direct interactions at the level of ciliary proteins is a common regulatory mechanism. Further experiments are required to determine whether the constitutive action of NO in embryonic ciliary cells from *Helisoma* operates through such a mechanism.

Serotonin, the endogenous cilio-excitatory neurotransmitter in *Helisoma*, is released from a pair of bilateral neurons that innervate two subpopulations of embryonic ciliary cells (Kuang and Goldberg, 2001; Koss et al., in press). Previous studies have revealed that calcium influx and activation of a unique PKC isoform are necessary to produce the serotonin induced changes in CBF (Christopher et al., 1996, 1999). The cilio-excitatory actions of NO identified in the present study, together with the expression of NOS in both ENC1 neurons and ciliary cells (Cole et al., 2002), prompts the hypothesis that NO
plays a role in the ciliary response to serotonin. Furthermore, the ability of the extracellular NO scavenger c-PTIO to block the SNAP-induced rise in CBF without affecting basal CBF or the response to serotonin, suggests that NO acts intracellularly rather than in an autocrine or paracrine fashion.

The lack of additivity in the ciliary response to co-application of serotonin and SNAP, together with the effectiveness of the NOS inhibitor 7-NI in blocking the response to serotonin, suggest that NO and serotonin interact. However, our results do not suggest that NO acts as an independent signal transduction element in the serotonin response. The most effective dose of SNAP was unable to stimulate as large an increase in CBF as serotonin. This is consistent with evidence from whole animal studies that NO is less effective than serotonin in stimulating embryonic rotation (Cole et al., 2002).

Furthermore, the partial effectiveness of NO is similar to our previous finding that activation of PKC is necessary, but not sufficient, to mediate the serotonin response (Christopher et al., 1999). Perhaps NO and PKC operate as cofactors in the response pathway, whereas a rise in intracellular calcium, which is able to fully mimic the serotonin response, represents a different step in the cascade.

The most recognized intracellular target for NO is sGC, which catalyzes the production of cGMP. NO, working through a cGMP pathway, has been shown to both increase CBF (Uzlaner and Priel, 1999) and decrease CBF (Tamoki et al., 1995) depending on the species examined. In Helisoma ciliary cells, a cGMP analogue produced an increase in CBF similar in magnitude as that of serotonin (100 µM) stimulation. Given
that the most effective dose of SNAP did not maximally stimulate ciliary beating, this result suggests that NO only partially activates the cGMP second messenger system in *Helisoma* ciliated cells. The sGC inhibitor LY 83583 blocked the stimulatory effects of both exogenous NO and serotonin, further implicating this pathway in cilio-excitation.

Ciliary stimulation through the upregulation of NO production is supported by findings from rabbit trachea (Uzlaner and Priel, 1999; Tamaoki et al., 1995), rat airway epithelium (Li et al., 2000), bovine airway epithelium (Sisson, 1995) and human adenoid explants (Yang et al., 1996). In contrast to these systems, NOS inhibitors reduced the CBF of *Helisoma* ciliated cells when presented alone. It is not known, therefore, whether constitutively expressed NO or NO generated as part of the serotonin signal transduction pathway is active in the serotonin response. To examine if serotonin recruits NO, we used DAF-2 imaging, and our results indicate that serotonin does not stimulate a significant increase in NO production, suggesting that the serotonin stimulatory pathway functions in parallel to the NO stimulatory pathway. However, these experiments were performed on cultures of mixed cell populations, where the three known subpopulations of *Helisoma* ciliary cells could not be identified (Kuang and Goldberg, 2001). It is possible that these different subpopulations exhibit different regulatory profiles and that the 22% of the weakly responding cells are from one subpopulation in which serotonin does recruit NO.

Alternatively, it is possible that serotonin stimulation results in a small localized production of NO that is sufficient to stimulate an increase in CBF but is below the
sensitivity of the dye. Concern for the specificity and accuracy of DAF-2 imaging has been raised, specifically that divalent cations in the medium were thought to alter detected fluorescence (Broillet, M.C., et al., 2001). However, these concerns were waylaid when it was shown that gaseous NO reacted with DAF-2 to produce fluorescent product independent of Ca\(^{2+}\) and Mg\(^{2+}\) at physiological concentrations (Suzuki et al., 2002). In our hands, the indicator revealed that 100 µM serotonin and the lower dose of NO donor (10 µM SNAP) did not reliably produce a detectable change in fluorescence, whereas 100 µM SNAP did alter the fluorescent signal. It is possible that serotonin stimulates the production of an amount of NO that is below the amount liberated by 100 µM SNAP. This follows from the finding that the 10 µM dose of SNAP did not produce a detectable increase in NO with DAF-2 but did produce a slight increase in CBF. Thus, if serotonin does stimulate the generation of NO, it may be in physiological amounts below the lower limits of detection for the indicator.

NO has been identified to play a diverse number of roles within cells, including the regulation of invertebrate development (Froggett and Leise, 1999; Heck et al., 2000; DiGregorio et al., 2001). Although molluscan metamorphosis is incompletely understood, a picture is emerging that implicates both NO and serotonin (Leise et al., 2001). *Helisoma* undergoes direct development in the egg capsule without a metamorphosis event in its life cycle. However, the serotonergic and nitrergic systems remain very important in regulating embryonic development (Goldberg, 1998; Goldberg et al., 1999). It may be that coordinated ciliary activity is central to normal embryonic development in *Helisoma* and that the function of both NO and serotonin are derived
from marine ancestors that exhibited different life histories. The nitrergic system appears to regulate basal ciliary activity to support embryonic rotation in the egg capsule, which may facilitate development. In contrast, the serotonergic system may have developed to upregulate ciliary beating in response to new challenges faced by encapsulated embryos, such as metabolically or environmentally derived hypoxia (Hunter and Vogel, 1986; Kuang et al., 2002). Most importantly, these two systems interact, with NO playing a permissive role in the cilio-excitatory response to serotonin.

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REFERENCES


FIGURE LEGENDS

Figure 1. Effect of the NO donor SNAP on CBF in cultured embryonic Helisoma cells.
A) Concentration-dependent effects of SNAP. n= 5-12 cells for each treatment group.
B) CBF was measured following application of serotonin (5-HT; 100 µM; n=11 cells),
SNAP (100 µM; n=9 cells) and co-application of serotonin and SNAP (n=14 cells). A &
B: Inset: Statistically significant differences occurred between treatment groups that do
not share the same underscore (p < 0.05).

Figure 2. Effect of the NOS inhibitor 7-NI on CBF. A) Dose-dependent effects of 7-NI.
10 µM 7-NI: n=10 cells and 100 µM 7-NI: n=13 cells. B) CBF was measured following
application of serotonin (100 µM; n=10 cells), 7-NI (100 µM; 10 cells) and co-application
of serotonin and 7-NI (n=14 cells). A & B: Inset: Statistically significant differences
occurred between treatment groups that do not share the same underscore (p < 0.05).

Figure 3. Effect of NO scavenger c-PTIO on CBF. A) CBF was measured in response
to c-PTIO (300 µM; n=8 cells), serotonin (100 µM; n=7 cells) and co-application of
serotonin and c-PTIO (n=12 cells). B) CBF was measured in response to SNAP (100
µM; n=8 cells) and co-application of SNAP and c-PTIO (300 µM; n=9 cells). DMSO was
a vehicle control (n=5 cells). A& B: Inset: Statistically significant differences occurred
between treatment groups that do not share the same underscore (p < 0.05).
Figure 4. Effect of serotonin and SNAP on NO production. Cells loaded with DAF-2 were imaged during 15 min incubations with saline, serotonin, or SNAP. Images were captured every 1 min and data is presented as the change in fluorescence ($\Delta F$) in arbitrary units. A) A representative trace of a ciliated cell responding to only the 100 $\mu$M dose of SNAP. B) A representative trace of a ciliated cell not responding to the application of serotonin but showing an increase in fluorescence following a solution change to 100 $\mu$M SNAP. C) A representative trace of a ciliated cell showing a marginal increase in fluorescence in response to serotonin. A & B: n=35 cells, C: n=4 cells.

Figure 5. Effect of the membrane permeable cGMP analogue 8-Br-cGMP on CBF. CBF was measured following application of 100 $\mu$M serotonin (n=14 cells), 0.2 mM 8-Br-cGMP (n=18 cells), and 2 mM 8-Br-cGMP (n=14 cells). Inset: Statistically significant differences occurred between treatment groups that do not share the same underscore (p < 0.05).

Figure 6. Effect of the soluble guanylate cyclase inhibitor LY 83583 on CBF. A) CBF was measured following application of SNAP (100 $\mu$M; n=4 cells), LY 83583 (LY; 10 $\mu$M; n=10 cells) and co-application of SNAP and LY (n=8 cells). B) CBF was measured following application of serotonin (100 $\mu$M; n=8 cells), LY 83583 (LY; 10 $\mu$M; n=9 cells) and co-application of serotonin and LY (n=10 cells). A & B: Inset: Statistically significant differences occurred between treatment groups that do not share the same underscore (p < 0.05).
Figure 1- Doran et al.
Figure 3- Doran et al.
Figure 4- Doran et al.
Figure 5- Doran et al.

- Control
- 5-HT
- 0.2 mM
- 2 mM

CBF (%) vs. 8-Br cGMP

- 2 5-HT 0.2 Cont.
Figure 6- Doran et al.

A

SNAP, EtOH, LY, SNAP+LY

EtOH  SNAP  LY  SNAP+LY

B

5-HT, EtOH, 5-HT, LY, LY+5-HT

EtOH  5-HT  LY  LY+5-HT