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Nitric oxide induces aspects of egg-laying behavior in *Aplysia*

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SUMMARY

Aplysia **egg laying is a complex behavior requiring synchronized activity in many organs. Aspects of the behavior are synchronized *via* the direct effects of peptide bag cell neurohormones and *via* stimuli arising during the behavior. Stimuli synchronizing egg laying were examined by treating *A. fasciata* with a nitric oxide (NO) donor. NO elicited normal appetitive and consummatory behaviors leading to the deposition of cordons containing egg capsules without eggs. The sites at which NO acts were investigated. The latency to egg deposition in response to a NO donor was shorter than that in response to other stimuli, consistent with NO acting at downstream sites from those affected by the other stimuli. The NO donor does not act on neurons in the head ganglia presynaptic to the bag cells or on the bag cells. Ligating the small hermaphroditic duct connecting the gonad to the accessory genital mass blocked egg laying in response to bag cell homogenates, but not in response to exogenous NO, indicating that NO does not act on the gonad. NO is released by transport of eggs along the small hermaphroditic duct, and NO directly acts on the accessory genital mass which packages eggs. NO also acts at a second site, independent of the effect on the accessory genital mass. A NO donor activates appetitive behaviors that normally precede egg laying even in *A. californica* that are unable to lay eggs.**

Key words: egg-laying, NO, nitric oxide, *Aplysia*, feed-forward, feed-back, motor coordination.

INTRODUCTION

Egg laying in *Aplysia* is a complex behavior requiring the coordination of a number of different effector organs. The neuroendocrine bag cells, which secrete egg-laying hormone (ELH) and a variety of additional peptides (Rothman et al., 1983a; Sigvardt et al., 1986; Stuart et al., 1980), are a central component coordinating egg laying. There have been conflicting reports on how different aspects of egg laying are coordinated. It has been suggested that the various peptides secreted by the bag cells have different functions, and egg laying is coordinated by the simultaneous release of the different peptides (Scheller and Axel, 1984; Scheller et al., 1983). This view arises from the supposition that egg laying is a fixed act (Kandel, 1976) in which all of the components occur in a fixed sequence. A group of peptides released from the site at which a decision is made to initiate the behavior could coordinate the sequencing of the behavior. The direct effects of a variety of bag cell peptides on a number of tissues has been shown, supporting this idea. For example, ELH directly affects the gonads and causes egg release (Choate et al., 1993; Coggeshall, 1970; Rothman et al., 1983b), and directly affects central neurons in the buccal and cerebral ganglia and thereby inhibits feeding (Ram, 1982; Ram, 1983; Stuart and Strumwasser, 1980; Teyke et al., 1991), whereas additional peptides cause depolarization of the bag cells as part of a positive feedback loop (Brown and Mayeri, 1989; Kauer et al., 1987; Rothman et al., 1983a). It has also been suggested that egg laying is coordinated by feed-forward and feedback loops (Cobbs and Pinsker, 1982b; Ferguson et al., 1989b; Ter Maat and Ferguson, 1996). The performance of certain aspects of egg-laying behavior act as stimuli that in turn affect other aspects of the behavior (Cobbs and Pinsker, 1982b; Ter Maat and Ferguson, 1996). Thus, injection of only one of the bag cell peptides into the animals, ELH, induces fully-fledged egg-laying behavior (Bernheim and Mayeri, 1995), presumably because it directly activates some aspects of egg laying, and the aspects directly triggered by ELH in turn act as stimuli that trigger the additional aspects of egg laying.

This report focuses on the effects of the unconventional neurotransmitter nitric oxide (NO) on egg-laying behavior. We have found that NO mediates some aspects of egg-laying behavior. Treating *Aplysia* with an NO donor induces parts of the coordinated behavior, while leaving out a crucial aspects of egg laying. The finding that an NO donor induces abnormal egg laying provides insight into how some of the different aspects of egg laying may be coordinated.

Egg-laying is thought to be initiated by the activity of neurons in the cerebral and pleural ganglia (Brown et al., 1989; Ferguson et al., 1989b; Shope et al., 1991) which have axons that reach the neuroendocrine bag cells *via* the pleural-abdominal connectives. The bag cells are generally silent, except preceding egg laying (Dudek et al., 1979; Pinsker and Dudek, 1977). Electrical or chemical stimulation of cerebral and pleural ganglion neurons can trigger a bag cell discharge (Brown et al., 1989; Painter et al., 1988). Brief electrical stimulation of the pleural-abdominal connectives also initiates a bag cells discharge (Ferguson et al., 1989a; Kupfermann and Kandel, 1970; Pinsker and Dudek, 1977; Wayne and Wong, 1994), presumably because the stimulus excites axons from the head ganglia that activate the bag cells. Bag cell activity is characterized by sustained low-frequency synchronous firing of the cells, which are electrically coupled to one another (Kupfermann and Kandel, 1970). Synchronous bag cell activity is readily recorded *via* an extracellular electrode on the plural-abdominal connective, close to the bag cells (Ferguson et al.,

1989a; Kupfermann and Kandel, 1970; Wayne and Wong, 1994). Bag cell firing releases a number of peptide hormones into the hemolymph (Stuart et al., 1980). The peptides are synthesized on a single precursor protein, which is then cleaved (Nagle et al., 1989; Scheller et al., 1983). Homologues of the bag-cell peptides are also synthesized in and released from the atrial gland of the genital tract (Arch et al., 1978; Heller et al., 1980; Nagle et al., 1989; Painter et al., 1988; Scheller et al., 1982). In some *Aplysia* species, including in *A. fasciata* that was used in this study, a distinct atrial gland is not present, and tissue homologous to the atrial gland is present in the anterior portion of the large hermaphroditic duct (Painter et al., 1985). Homogenates of the bag cells or of the atrial gland elicit egg-laying when injected into *Aplysia* (Arch et al., 1978; Bernheim and Mayeri, 1995; Heller et al., 1980; Kupfermann, 1967), in part because some peptides act on the gonad and cause the release of yolk-containing eggs. Eggs are then transported *via* the small hermaphroditic duct from the gonad to the to the fertilization chamber, where they are fertilized by previously stored sperm cells. Eggs are then packaged into capsules and cordons. The packaging is dependent on the albumen and winding glands (the accessory genital mass), which secrete the packaging material (Cummins and Nagle, 2005; Thompson and Bebbington, 1969). The egg cordons are transported *via* the large hermaphroditic duct to the exterior. Eggs then travel down the genital groove toward the head. Egg cordons are then deposited onto the substrate *via* weaving and tamping movements of the head and lips (Bernheim and Mayeri, 1995; Cobbs and Pinsker, 1982a; Ferguson et al., 1989a). Appetitive behaviors called waves and undulations precede the extrusion of the egg cordon. The appetitive behaviors are thought to be produced partially by the effects of bag cell peptides on neural circuits in the head ganglia that control the neck (Ferguson et al., 1986) and partially by feed-forward excitation elicited by egg cordons stimulating parts of the reproductive system (Cobbs and Pinsker, 1982b). The consummatory behaviors are thought to be elicited *via* feed-forward excitation elicited by egg cordons stimulating parts of the reproductive system (Cobbs and Pinsker, 1982b; Ferguson et al., 1986).

Our data indicate that treating *Aplysia* with a NO donor elicits the formation of egg cordons containing capsules that usually lack eggs. NO is likely to act on the accessory genital mass which packages eggs. In addition, NO activates appetitive behaviors that normally precede egg laying, even in *Aplysia* that are unable to lay eggs. This effect is likely to be on pedal ganglion neurons organizing the appetitive behaviors, and are independent of the effect on the accessory genital mass.

MATERIALS AND METHODS

Animals

Aplysia fasciata Poiret 1789 weighing 20–250 g were collected along the Mediterranean coast of Israel. Animals were stored two or four together in plastic mesh cages immersed in 600 l tanks of aerated, filtered Mediterranean seawater at either 18°C or 21°C with a 12 h:12 h light:dark cycle. The animals were fed one or two times weekly with *Ulva lactuca* Linnaeus 1753 which was gathered along with animals, and was stored frozen. Experiments were performed 1–4 weeks after the animals were collected, during the light phase of the daily cycle.

All of the *A. fasciata* used were sexually mature, as evidenced by previous egg laying. One experiment examined aspects of egg-laying behavior in presumably sexually immature animals. Because *A. fasciata* mature very quickly, it is difficult to have a large sample of immature animals for experiments. This experiment was performed on *A. californica* Copper 1863 (Santa Barbara Marine Bio, Santa Barbara, CA, USA) during the winter months (February to March), when they are often sexually immature (Audesirk, 1979; Strumwasser et al., 1969).

Experimental treatments and observations

Twenty-four hours before an experiment, animals were placed one to a container in aerated 5 l containers of Mediterranean seawater kept at 21°C. Animals that laid eggs during this 24 h period were not used. To induce egg laying, animals were injected through the foot into the hemocoel with a variety of substances. Bag cell (BC) and large hermaphroditic duct (LHD) preparations from a single donor animal were homogenized in 1 ml artificial seawater (ASW), and this volume was injected into a single animal. The NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP; Sigma, Rehovot, Israel) was prepared to reach a concentration within the animal of 45 μmol l^{-1}. This concentration depolarizes the giant metacerebral cell (MCC) of the *Aplysia* cerebral ganglion (Jacklet and Tieman, 2004), and also affects aspects of *Aplysia* feeding behavior (Katzoff et al., 2006).The effect of 45 μmol l^{-1} *N*-acetylpenicillamine (NAP; Sigma, Israel), which lacks the NO donating *S*-nitroso group, was also examined. ELH (Peninsula Laboratories, Belmont, CA, USA) was prepared to reach a concentration of 0.15 μmol l^{-1} within the animal. The NOS (nitric oxide synthase) inhibitor *N*ω-nitro-L-arginine methyl ester (L-NAME; Sigma, Israel) was prepared to reach a concentration of 1.85 mmol l^{-1} within the animal. The dosage used (1.85 mmol l^{-1}) was five times larger than that used in previous experiments (Katzoff et al., 2002; Katzoff et al., 2006) that examined the effects of L-NAME on feeding behavior. Methylene Blue, an inhibitor of guanylyl cyclase, was prepared to reach a concentration of 100 μmol l^{-1} within the animal. This concentration affects *Aplysia* feeding behavior (Katzoff et al., 2006), as well as persistent sensitization of nociceprors as a result of a noxious stimulus (Lewin and Walters, 1999). Animals were observed for 2 h following treatment, and the start and duration of egg-laying behavior was noted: the person observing the animals was not informed about the nature of the preceding treatment.

Recording

Extracellular recordings were used to monitor bag cell after-discharge. Recordings were performed from an isolated abdominal ganglion with a suction electrode placed on a pleural-abdominal connective which was cut just anterior to the bag cells. A bag cell after-discharge was initiated by brief electrical stimulation *via* the suction electrode. The recordings were made with a Model 1700 AC differential amplifier (A-M Systems, Carlsborg, WA, USA).

Surgery

Animals were anesthetized and relaxed with isotonic $MgCl_2$ (30% of their body mass). After the animals were completely flaccid, one of two surgical procedures was performed. In one procedure, after an initial incision, the pleural-abdominal connectives were severed bilaterally. In the other procedure the small hermaphroditic duct was tied with silk braided thread from a 6.0 suture (Assut Sutures, CE 0482, Pully-Lausanne, Switzerland). The duct was tied in two places, close to its origin at the gonad, and close to its terminus at the winding gland. The incision was then sewn with silk thread. The surgical procedures were performed at 4°C. After the surgery, animals were returned to 10 l containers of Mediterranean seawater kept at 21°C. The water was changed twice before the start of an experiment, 24 h after the surgery.

RESULTS

NO induces egg laying

Animals were treated with the NO donor SNAP. In 14 of 14 animals, SNAP elicited aspects of egg-laying behavior. Control animals (N=6) were treated with artificial seawater (ASW). ASW never elicited egg laying.

To exclude the possibility that SNAP induces egg laying *via* effects that do not depend on NO release, we tested the effects of SNAP after it had already released most of its NO. SNAP releases NO with a half time of 5 h (Ignarro et al., 1981). We allowed the SNAP solution to sit for 36 h, and then treated the animals with this solution. Egg-laying behavior was elicited in only one of five tested animals. To test whether egg laying might be caused by a much lower concentration of NO, such as that which might be released by SNAP after 36 h of exposure, we injected into one animal a 10 μmol l^{-1} concentration of SNAP (less than a quarter of the concentration used previously). This treatment induced egg laying, suggesting that the small quantities of NO released by SNAP after 36 h could cause egg laying in the single animal that laid eggs in response to the stimulus.

We also tested the possible effect on egg-laying of NAP, an analogue of SNAP that lacks the *S*-nitroso group and therefore does not release NO. Egg-laying was not seen in any of the six animals that were tested.

The physiological effects of NO often occur through its activation of soluble guanylyl cyclase, which in turn causes an increase in cyclic GMP (cGMP) (Ahern et al., 2002; Davis et al., 2001). However, NO can also act through other mechanisms (Davis et al., 2001), such as *S*-nitrosylation (Ahern et al., 2002). We tested whether blocking guanylyl cyclase by treatment with Methylene Blue blocked the effects of the NO donor in inducing egg-laying. Methylene Blue was injected into five animals 20 min before treatment with the NO donor. All five animals laid eggs, similar to those given the NO donor alone, indicating that the effect of NO on egg-laying is not *via* the cGMP second messenger pathway.

Appearance of egg cordons

Egg-laying behavior in response to the NO donor was compared with that in response to treatment with purified ELH (N=3) a BC homogenate (N=10) or an LHD homogenate (N=4), which in a number of *Aplysia* species contains tissue homologous to the atrial gland in *A. californica* (Painter et al., 1985). Such is the case in *A. fasciata* (Susswein and Benny, 1985). The NO donor produced changes in behavior that were similar or identical to those elicited by the other stimuli. Egg deposition was preceded by appetitive behaviors, waves and undulations. Eggs were deposited by weaves and tamps, consummatory behaviors that distribute and attach the egg cordon to the substrate. The similarities in egg-laying behavior during bouts elicited by the NO donor and by ELH or bag cell homogenates led to a similarly raveled appearance of the deposited egg cordons (see Fig. 1A). Other aspects of egg-laying behavior, such as mouth puckering, genital groove swelling, crawling preceding egg deposition and its cessation, and weight of the cordon, were not examined.

The egg cordons deposited in response to the NO donor were compared to those deposited in response to treatment with purified ELH or BC or LHD homogenates. Egg cordons produced in response to ELH and BC or LHD homogenates contained capsules, with yolky eggs packaged within the capsules (Fig. 1B). By contrast, cordons in response to the NO donor usually lacked eggs (Fig. 1C,D). The cordons varied somewhat in their appearance. The most commonly observed pattern (approximately 80%) was that of cordons filled with empty capsules (Fig. 1C). The cordons lacked the typical bright yellow appearance of *A. fasciata* cordons, which arises from the yolky eggs. In most cases, animals continued to produce cordons with no eggs throughout the bout of egg laying. However, in approximately one-third of these bouts, the empty cordon gradually became filled with eggs, with the number of eggs in a capsule gradually increasing. In two cases, the cordons began as a clear string with no capsules (Fig. 1D). As the animal continued depositing the egg cordon, the empty cordon became filled with empty capsules, or with an undifferentiated yellow, yolky material, but with no capsules. The cordons containing only the yolky substance gradually lost the yolk, and in its place partially filled egg capsules appeared (Fig. 1A,E). Unpackaged yolk and empty capsules were not seen together.

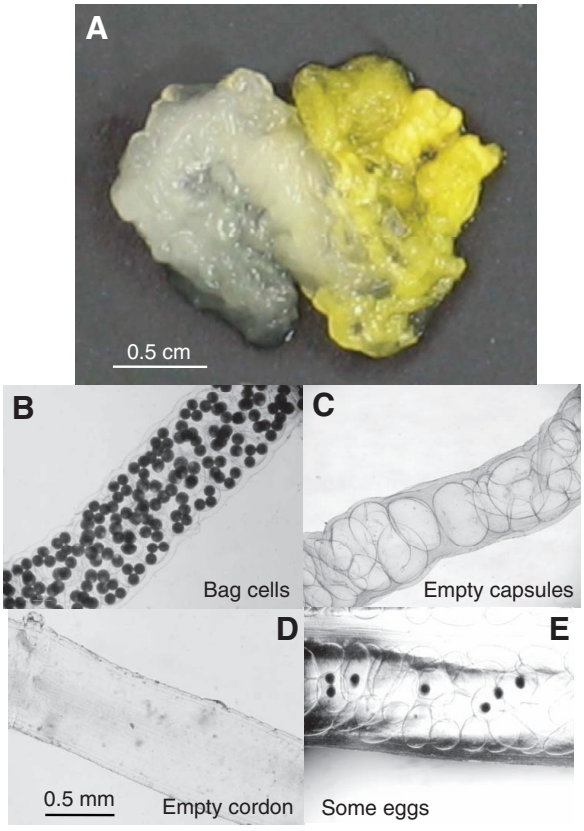

Fig. 1. Examples of egg cordons deposited after treatment. (A) An egg cordon produced in response to the NO donor SNAP (*S*-nitroso-*N*-acetyl-penicillamine) that began with empty egg capsules and ended with capsules filled with eggs. The eggs are filled with yolk, which is yellow. The portions of the egg cordon deposited earlier are white, reflecting the lack of yolk and eggs in the capsules. Note that both the white and the yellow portions of the cordon are knotted and raveled, reflecting identical tamping and weaving behaviors that accompany egg deposition. (B) A section of an egg cordon produced in response to BC homogenate, showing capsules filled with numerous eggs. (C–E) Sections of egg cordons laid in response to NO. (C) The cordons contain empty capsules; this is the most common appearance of the cordons. (D) At the start of egg-laying, cordons sometimes lacked capsules. (E) As deposition of a cordon similar to C progressed, eggs sometimes began to appear in some capsules. Scale bar in D is for B–E.

Latency and duration of egg laying

The latency to egg laying (defined as the first observation of an egg cordon) in response to the NO donor was compared to the latency in animals treated with purified ELH, as well with BC and LHD

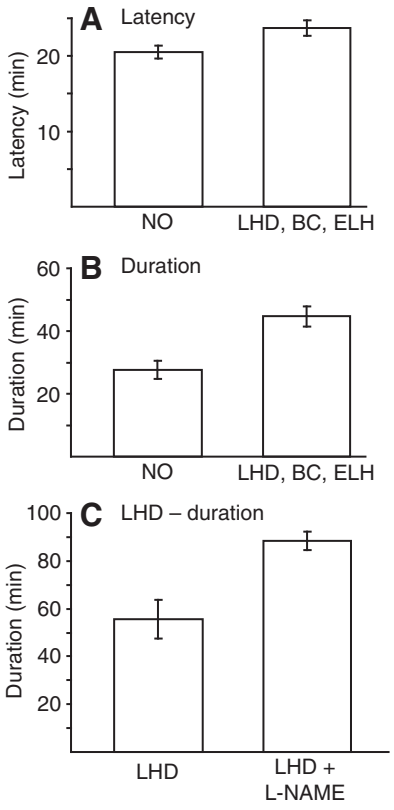

Fig. 2. Latency and duration of egg-laying in response to a number of stimuli. (A) Latency to the start of egg-laying in response to the NO donor, and in response to large hermaphroditic duct (LHD) homogenate, bag cell (BC) homogenate, and egg-laying hormone (ELH). Data for these treatments were combined. Latency in response to the NO donor was significantly shorter than in response to LHD homogenate, BC homogenate and ELH. (B) The duration of egg laying in response to the NO donor *versus* the other treatments. The duration of egg-laying in response to the NO donor is significantly shorter than in response to the BC homogenate. (C) Application of the NOS (nitric oxide synthase) inhibitor L-NAME (*N*ω-nitro-L-arginine methyl ester) significantly increased the duration of egg-laying in response to the LHD homogenate.

homogenates (Fig. 2A). There were no significant differences in latency between animals treated with ELH, or with BC or LHD homogenates ($F_{2,14}$=0.83, P=0.46; one-way analysis of variance), and therefore data from these three treatments were combined. There was a small but significant decrease in the latency to the deposition of egg cordons in animals treated with the NO donor, with respect to the latency in response to other stimuli (P=0.03, t_{29}=2.28; two-tailed t-test). The duration of egg laying was also measured (Fig. 2B). Data from animals treated with ELH and with LHD and BC homogenates were combined, as there were no significant differences between them ($F_{2,14}$=2.58, P=0.12; one-way analysis of variance). There was a significant decrease in the duration of egg laying in response to the NO donor (P=0.001, t_{26}=3.66; two-tailed t-test), with respect to the duration in response to the other three treatments.

Blocking NO transmission does not block egg laying

Since a NO donor elicits aspects of egg laying, we reasoned that blocking NO transmission might block aspects of egg-laying behavior. To test this possibility, animals that were treated with BC or LHD homogenates, or with ELH, were also treated with L-NAME, a competitive inhibitor of L-arginine for nitric oxide synthase. The L-NAME was injected 5 min before treatment with the stimuli eliciting egg laying, since treatment with L-NAME elicits changes in feeding behavior within 5 min (N.M. and A.J.S., unpublished). In 11 of 11 animals normal egg cordons were seen, indicating that NO release is not necessary for egg laying. Latency to egg laying was not affected by L-NAME for either animals treated with BC or LHD homogenates, or for animals treated with ELH. Treatment with L-NAME did not cause significant effects on the duration of egg laying in animals treated with a bag cell homogenate or with ELH. However, L-NAME caused a significant increase in the duration of egg laying in response to the LHD homogenate (P=0.02, t_5=3.19; two-tailed t-test) (Fig. 2C). These data suggests that NO release may shorten the duration of egg laying in response to the LHD homogenate, and treatment with a blocker of NO transmission uncovers this regulation.

It is possible that L-NAME blocks the effects of ELH, or of BC or LHD homogenates, but itself elicits egg laying. As a control for the possible independent effects of 1.85 mmol l^{-1} L-NAME, we examined the effect of treatment with this concentration of L-NAME alone (N=5). The treatment did not elicit egg laying.

Sites of NO action

We examined the possible sites (Fig. 3A) of NO action in initiating egg-laying behavior.

Possible action on head ganglia

We tested the possibility that NO acts by activating neurons in the head ganglia, which in turn excite the bag cells and cause them to discharge. The pleural-abdominal connectives were bilaterally cut, thereby separating the head ganglia from the bag cells. If NO operates on the head ganglia, the surgical procedure should block egg-laying behavior in response to NO.

Egg-laying behavior similar to that described above was elicited by the NO donor in five of seven animals in which the pleural-abdominal connectives had been bilaterally severed. The mean latency from treatment with the NO donor to egg-laying behavior was 20.7±0.5 min (mean±s.e.m.), which is comparable to the latency with the NO donor in unoperated animals (see Fig. 2A). The lack of egg-laying behavior in two of the animals treated with the NO donor is probably due to the effects of the surgery. These data indicate that NO is unlikely to act on neurons in the head ganglia that activate the bag cells and thereby induce egg laying.

Possible action on bag cells

We tested the possibility that NO induces egg-laying behavior by acting directly on the bag cells and causing them to discharge and release peptides. Bag cell activity was monitored in an isolated abdominal ganglion preparation using a suction electrode placed on the cut pleural-abdominal connective, close to the bag cells. Application of the NO donor (N=5) did not cause a bag cell after-discharge (Fig. 3Bi). To be certain that the bag cells were capable of responding to stimuli with an after-discharge, the connective was electrically stimulated after treatment with the NO donor. In five of five cases, electrical stimulation of the connective elicited a bag cell after-discharge (Fig. 3Bii). These data indicate that NO does not cause egg-laying behavior *via* possible effects on the bag cells. NO is likely to act downstream from the bag cells.

Possible action on gonads and on packaging glands

The ovotestis is one site of action of ELH. NO could act on the gonad, causing it to release material into the small hermaphroditic

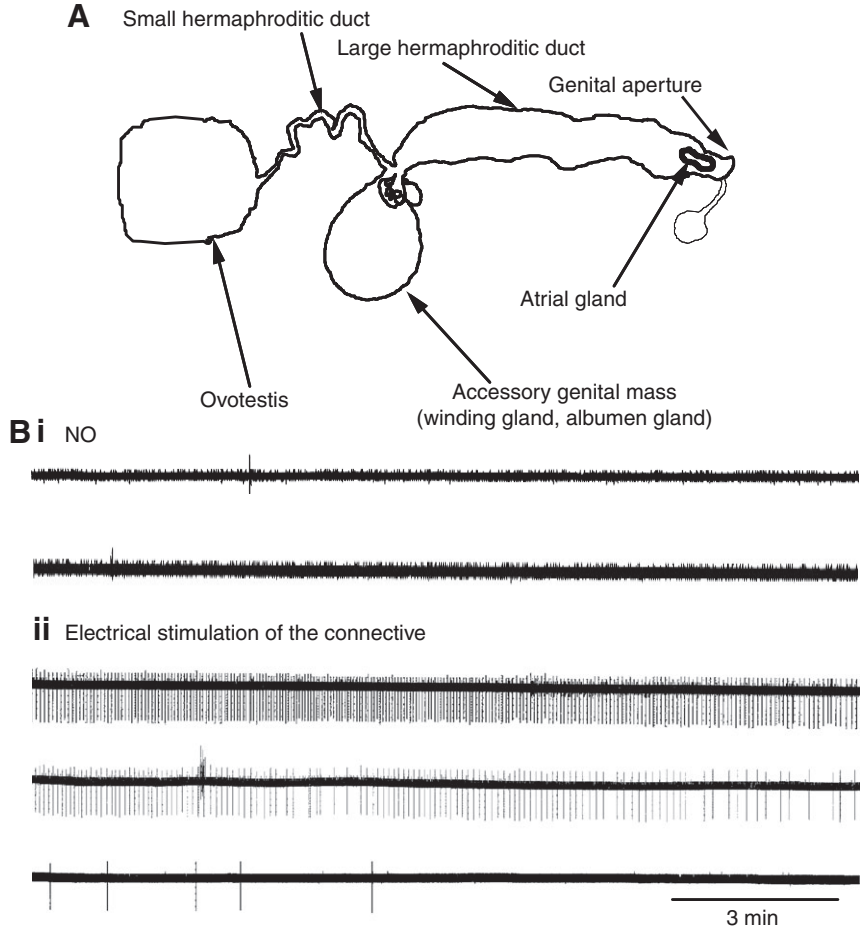

Fig. 3. (A) Diagram of the reproductive system of *Aplysia* showing the sites tested for localization of the effect of NO. (B) Analysis of the effect of the NO donor application to the abdominal ganglion. (i) Effect of application of the NO donor on the activity recorded extracellularly using a suction electrode placed on the cut pleural-abdominal connective, close to the bag cells. The two traces are continuous. The stimulus did not elicit a bag cell after-discharge. (ii) Electrical stimulation of the connective (in the example shown, 7 V, 7 Hz, 5 ms pulse duration, maintained for 5 s) induced an after-discharge. The three traces are continuous. The recordings began a few seconds after the stimulus, since the same suction electrode was used for stimulation and recording. The stimulating electrode was removed from the tubing and the recording electrode was then inserted.

duct, and the presence of material in the duct and distal to it could act as a stimulus for packaging the contents into capsules and cordons. A second possibility is that NO acts directly on the accessory genital mass, and elicits the production of capsules and cordons without the presence of material secreted from the gonad. We tested these two possibilities by examining the effects of the NO donor on *Aplysia* in which the small hermaphroditic duct had been ligated, thereby preventing material secreted by the gonad from reaching the accessory genital mass. If this treatment blocks egg laying in response to the NO donor, one could conclude that NO acts on the gonad. By contrast, if NO still induces egg laying one could conclude that NO acts directly on the packaging machinery.

In six of six animals with a ligated small hermaphroditic duct, treatment with the NO donor elicited egg-laying behavior and the deposition of egg cordons. The latency to the start of egg laying was 21.0±0.44 min (± s.e.m.), which is comparable to the latency in intact animals that were treated with NO. Egg cordons in all 6 animals contained empty capsules, similar to those observed in most cases in which intact animals were treated with the NO donor. These data indicate that NO probably works directly on the accessory genital mass that package the eggs, and thereby produces egg cordons and capsules.

The data above suggest that NO may be released during natural egg laying, and acts on the accessory genital mass, inducing packaging of eggs. NO could be released as a result of a direct effect of bag cell peptides acting on the accessory genital mass. An alternative possibility is that transport of eggs *via* the small hermaphroditic duct, or perhaps a neurally mediated stimulus activated by egg transport, causes the release of NO and the recruitment of packaging by the accessory genital mass. To test between these two possibilities, we examined the effects of a bag cell homogenate on egg-laying behavior in animals in which the small hermaphroditic duct was ligated. If bag cell peptides cause NO release and subsequent egg-laying behavior, this treatment should cause animals to deposit egg cordons. By contrast, if transport of eggs or other neural signals elicit NO release and the deposition of egg cordons, the ligature should prevent the deposition. In five of five ligated animals no egg cordons were deposited in response to the bag cell homogenate.

Direct action on neurons organizing appetitive behavior

We examined whether NO initiates either appetitive or consummatory behaviors in the absence of egg deposition. The effects of the NO donor were examined in *Aplysia* in which egg-laying generally does not occur, probably because they are sexually immature. Because most *A. fasciata* are sexually mature throughout the season in which they are found, this experiment was performed on *A. californica* during the winter months, when many animals do not respond to bag cell homogenates (Strumwasser et al., 1969). We reasoned that treatment with the NO donor might not cause winter animals to deposit an egg cordon, but might still elicit aspects of appetitive or consummatory behaviors.

A. californica were treated with either ASW or with the NO donor and egg-laying behaviors were observed (Fig. 4). In five of nine *A. californica* treated with the NO donor, no egg cordons were produced, whereas the NO donor led to the production of egg

cordons with empty capsules in the other four animals. Behavior during the hour following the treatment was compared in the five animals that did not produce egg cordons and in the animals treated with ASW. The total time devoted to appetitive behaviors (combined head waves and undulations) was significantly greater in animals treated with the NO donor than in animals treated with ASW (P=0.01, t_9=3.03; two-tailed t-test), indicating that NO is able to induce appetitive behaviors even in animals that do not deposit egg cordons. The appetitive behaviors gradually decreased over the hour of observation, as shown by a significant difference in appetitive behaviors between the first and second halves of the observation in immature animals treated with the NO donor (P=0.04, t_4=3.75; two-tailed paired t-test with Bonferroni correction). These data indicate that appetitive behaviors elicited by the NO donor are not dependent on the deposition of egg cordons.

It was of interest to compare the appetitive behaviors in animals that laid eggs in response to the NO donor with appetitive behaviors in animals that did not lay eggs. There was no significant difference in appetitive behaviors between the two groups (P=0.36, t_7=0.97; two-tailed t-test) in the first half hour of the observation, during the period preceding egg laying. This finding indicates that the production of egg cordons does not amplify the response to NO, and that all of the appetitive behaviors could be accounted for by the effects of NO release.

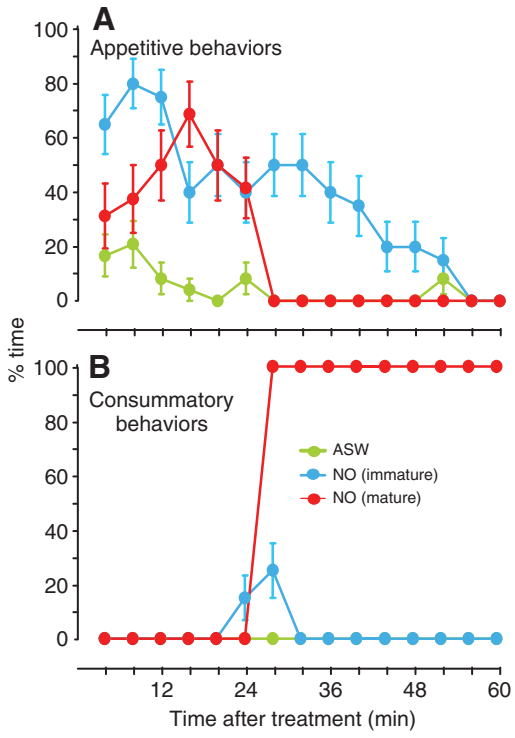

Fig. 4. Appetitive and consummatory behaviors elicited by artificial sea water (ASW), and by the NO donor, in *A. californica* during the winter months, when most of them are immature. Data are shown separately for animals that responded to the NO donor by depositing an egg cordon (mature), and for animals in which no egg cordon was produced (immature). (A) The NO donor elicited a large increase in appetitive behaviors, with respect to that observed in response to ASW. In mature animals the appetitive behaviors stopped abruptly at the start of the consummatory behaviors, whereas in immature animals appetitive behaviors gradually declined over the hour of observation. (B) Consummatory behaviors were observed in mature animals.

In animals that laid eggs the cordons began to appear 22–25 min after the treatment with the NO donor. Consummatory behaviors (tamps and head weaves) also began to appear at this time. Two of the five animals in which the NO donor did not elicit egg-laying displayed a brief increase in consummatory behaviors from 23–27 min after being treated, indicating that NO may initiate consummatory behaviors, but their maintenance requires stimuli provided by egg cordons.

DISCUSSION

Egg laying in *Aplysia* and similar animals (Ferguson et al., 1993; Hermann et al., 1997; Jansen and Ter Maat, 1985) is a complex behavioral sequence under partial neuroendocrine control. It has been widely used as a model system for examining mechanisms underlying the sequencing of behavior, and the interactions between behaviors. Eggs are released from the gonad and then packaged into capsules within cordons, which are eventually transported externally to the head (Ter Maat and Ferguson, 1996; Thompson and Bebbington, 1969), and then deposited by consummatory behaviors affecting body wall muscles, as well as the mouth and lips. Egg laying has widespread interactions with other behaviors, such as feeding and withdrawal reflexes, which are inhibited during egg laying (Cobbs and Pinsker, 1982b; Goldsmith and Byrne, 1993; Ram, 1982; Stuart and Strumwasser, 1980). It has been suggested that the sequencing of egg laying, and its interactions with other behaviors, arises through the actions of a number of neuroendocrine bag cell peptides (Scheller et al., 1983; Scheller and Axel, 1984). Support for this hypothesis comes from the finding that different bag cell peptides directly affect a variety of neural targets (Branton et al., 1978; Sigvardt et al., 1986; Stuart and Strumwasser, 1980), as well as directly affecting the gonad and causing the release of eggs (Choate et al., 1993; Coggeshall, 1970; Rothman et al., 1983b). However, other experiments also showed that aspects of egg-laying behavior are initiated or regulated neurally, as feed-forward or feedback responses to stimuli originating in other portions of the sequence (Cobbs and Pinsker, 1982b; Ter Maat and Ferguson, 1996).

We have found that treating *Aplysia* with an NO donor induces aspects of egg-laying behavior. Exogenous NO presumably calls into play aspects of egg laying that are normally elicited either by neurally mediated signals or by bag cell hormones, which in turn cause the release of NO as an intermediate signal in the context of normal egg laying. Our data indicate that NO acts at specific sites in the egg laying cascade. However, it may also activate aspects of egg laying that it does not directly control, since the sites directly affected may act as stimuli that control other downstream and upstream sites.

Sites of NO action

Our findings rule out a number of sites at which NO could affect egg-laying behavior. In principle, NO could be activated by peptides or by electrical signals affecting neurons in the head ganglia. Bag cell or atrial gland peptides, as well as electrical stimulation, have direct effects on neurons in the head ganglia that excite the neuroendocrine bag cells and thereby cause egg laying (Brown et al., 1989; Ferguson et al., 1989b; Shope et al., 1991). NO is unlikely to be an intermediary for these effects, since cutting the pleural-abdominal connectives by which neurons in the head ganglia communicate with the bag cells did not block the effect of the NO donor. Bag cell and atrial gland peptides also directly excite the bag cells (Heller et al., 1980; Kauer et al., 1987). NO is unlikely to be an intermediary for these effects, since stimulating the bag cells with the NO donor did not cause a bag cell after-discharge (Fig. 3A).

One of the hormones secreted by the bag cells, ELH, acts directly on the gonad to effect egg release. NO is also unlikely mediate this effect, since most cordons lacked eggs or yolk, which are released from the gonad. In addition, egg cordons were elicited by the NO donor even after the small hermaphroditic duct was ligated, thereby preventing egg transport from the gonad to the accessory genital mass. In unoperated animals treated with the NO donor, egg-filled capsules were sometimes present late in a bout of egg laying, suggesting that feedback to the gonad from a downstream site directly affected by NO can cause egg release. However, we cannot eliminate the possibility that NO acts directly on the gonad, although with weaker, less consistent and longer latency effects than at downstream sites.

Our data provide support for three separate sites at which NO affects egg laying.

Egg packaging

One site of action is likely to be the accessory genital mass, composed of the albumen and winding glands, which package the eggs into capsules and cordons. A direct effect on the accessory genital mass would account for the release of cordons without eggs, which are released from the gonad, upstream from the accessory genital mass. Empty cordons were also laid in response to NO when the small hermaphroditic duct connecting the gonad to the accessory genital mass was ligated, indicating that the formation of egg cordons is not dependent on stimuli provided by eggs released from the gonad and transported to the accessory genital mass.

Under natural conditions, the accessory genital mass could be activated by bag cell peptides acting directly on these tissues, which then release NO. In our experiments, application of exogenous NO would bypass the hormones that cause the NO release. A second possibility is that the release of eggs from the gonad and their transport to the packaging machinery is a feed-forward stimulus that causes the release of NO and the packaging of the eggs. Our data provide strong support for the latter hypothesis. First, we found that bag cell homogenates did not induce egg deposition after the small hermaphroditic duct was ligated, indicating that the peptides cannot elicit the release of cordons in the absence of egg transport. This finding is consistent with previous results (Cobbs and Pinsker, 1982b) showing that ligation of the small hermaphroditic duct blocks egg laying in response to a bag cells homogenate. Second, the latency to deposition of a cordon in response to NO was shorter than in response to other stimuli (Fig. 2), suggesting that NO bypasses upstream sites whose activation takes time that adds to the latency. In response to ELH, or BC or LHD homogenates, eggs would be released from the gonads, and then transported to the accessory genital mass, where NO would be released. In response to exogenous NO, release of eggs is not necessary to activate the accessory genital mass, accounting for the shorter latency.

Although NO is likely to act on the accessory genital mass and cause packaging of eggs, it is not necessary for this action, since blocking nitrergic transmission did not block egg laying. Many physiological systems use multiple transmitters as messengers, with each transmitter sufficient but not necessary to elicit an effect. NO is likely to act in tandem with another transmitter in activating the packaging system. This is consistent with the finding that neuron C2 in the feeding circuit, which uses NO as a transmitter, also releases histamine. NO and histamine together depolarize follower cells of C2 (Jacklet and Tieman, 2004).

Appetitive behaviors

A second site of NO action is the neural circuitry that organizes appetitive behaviors preceding egg deposition. Thus, application of the NO donor elicited appetitive behaviors even in *Aplysia* that were unable to produce egg cordons (Fig. 4A). Previous data on gonadectomised animals that are unable to lay eggs showed that appetitive behaviors are still initiated by bag cell hormones (Ferguson et al., 1986), indicating that these behaviors can be directly induced by the hormones. Our data suggest that the effects of bag cell peptides on appetitive behaviors are mediated in part by NO release. Appetitive behaviors are effected by neurons in the pedal ganglia that innervate body wall muscles in the neck (Ferguson et al., 1989b). Moroz (Moroz, 2006) found nitrergic neurons in the pedal ganglia, suggesting that these might be activated by stimuli initiating the appetitive behaviors that then release NO, which would activate adjacent neurons that organize appetitive behaviors.

Previous data (Ferguson et al., 1986; Ferguson et al., 1989a; Ter Maat and Ferguson, 1996) also indicated that the consummatory behaviors arise by feed-forward excitation as a result of egg transport along the genital groove. Activation of consummatory behaviors causes feedback inhibition of the preceding appetitive behaviors (Ter Maat and Ferguson, 1996). Our data are consistent with these findings. First, consummatory behaviors in response to the NO donor were normal, in spite of the anomalies in the contents of the egg cordon. Second, consummatory behaviors were not maintained in non egg-laying animals treated with the NO donor, although they were briefly initiated in some animals (Fig. 4B). The lack of consummatory behaviors in the presumably immature *Aplysia* led to a prolongation of the appetitive behaviors that was not seen in animals that laid eggs (Fig. 4A), in which the consummatory behaviors apparently inhibited the appetitive behaviors.

Feeding behavior in *Aplysia* is inhibited during egg laying (Ram, 1982; Stuart and Strumwasser, 1980). This inhibition is partially caused by feed-forward inhibition mediated by the direct effects of bag cell peptides on neurons controlling feeding (Ram, 1982; Ram, 1983; Sossin et al., 1987; Teyke et al., 1991), as well as by lateral inhibition, in which the performance of consummatory egg-laying behaviors inhibit feeding (Cobbs and Pinsker, 1982b; Ter Maat and Ferguson, 1996). Previous data from our laboratory indicated that the application of inhibitors of NO transmission, or of a NO donor, can modulate aspects of feeding behavior (Katzoff et al., 2006). Increase in NO makes animals less interested in food (N.M., R. Saada, I. Hurwitz and A.J.S., unpublished), and treatment with L-NAME induces food arousal (Hurwitz et al., 2006), suggesting that NO may be involved in the control of feeding during egg laying.

Bout duration

NO may also act at a third site, and action at this site affects aspects of bout duration. The duration of egg laying is related to length of the egg cordon, and presumably to the number of eggs laid. Animals that have a larger number of mature eggs within the gonads would lay more eggs, and the duration of egg laying would therefore be longer. Egg laying duration in response to the NO donor is shorter than in response to the other stimuli (Fig. 2B), presumably because there are few or no eggs released, and egg laying is therefore presumably terminated prematurely. However, our data suggest that the duration of egg laying may be regulated in a complex manner. Although there were no differences in the duration of egg laying in response to ELH, or to BC or LHD homogenates, treatment with the NO blocker L-NAME produced a large increase in the duration of egg laying in response to the LHD homogenate. These data suggest that application of different exogenous substances may result in similar durations of egg laying by calling into play different transmitter systems. The LHD homogenate may specifically call

into play NO release, which shortens the duration of egg laying. Additional studies will be needed to determine the factors governing the duration of egg laying.

Latency of egg laying

The latency of egg laying in our experiments was generally shorter than previously reported in response to stimuli such as shocks to the pleural-abdominal connectives (Ferguson et al., 1989a; Pinsker and Dudek, 1977), which in *A. californica* produced egg laying with latencies of 30 min or longer. For *A. fasciata* it has been reported that latency to egg laying is 27–36 min after the start of the bag cells discharge (Ter Maat and Ferguson, 1996). Part of the latency in response to these stimuli is presumably the time needed to produce an after-discharge in the bag cells, and for the bag cells to release peptides, which in turn then cause egg release and transport, which also take time. Application of a NO donor would shorten the latency by bypassing many of these processes. Our data suggest that a major portion (approximately 20 min) of the latency to egg laying stems from the time needed for NO and other transmitters to act on the accessory genital mass to produce capsules and cordons.

Multiple effects of NO

Previous data have suggested that a number of different sites in the *Aplysia* nervous system affecting a single behavior may use a common transmitter. Thus, neurons in the cerebral and pleural ganglia containing peptides of the bag cells family apparently function in egg laying (Brown et al., 1989; Painter et al., 1988; Pulst et al., 1988). In addition, peptides related to those expressed in identified neuron R15 are also expressed in other neurons active in autonomic control (Romanova et al., 2007). Serotonin release from a number of loci may also contribute to the sensitization of defensive behaviors (Marinesco et al., 2004). The use of NO at a number of sites affecting egg laying may be an additional example of this phenomenon. Moroz (Moroz, 2006) demonstrated that nitrergic neurons are present in all of the central ganglia, as well as in many peripheral sites. It is likely that NO has other functions unrelated to egg laying. However, many of the nitrergic neurons may be activated during the control of different aspects of egg laying.

Feed-forward and feedback control

Complex behavioral sequences in higher animals are controlled by both feed-forward and feedback mechanisms (Ghez and Krakauer, 2000). The finding that *Aplysia* egg laying is also controlled in this manner indicates that this is a general principle organizing behavior, even in relatively simple systems. The *Aplysia* egg laying system is somewhat atypical in that the feed-forward components of the system are partially hormonal and partially neural. The hormonally induced components are relatively stereotyped: once an after-discharge in the bag cells is elicited, and the hormones are released, there will be little ability to regulate the subsequent components of egg laying behavior. However, many aspects of egg laying are variable. The latency to egg deposition as well as its duration are variable, as are the substrates on which the eggs are deposited. Coordination of the egg laying sequence will have to be modulated to take into account the variable aspects of the behavior. Our data, as well as those of others, indicate that activity initiated by various aspects of egg laying coordinate the sequence, and probably allow a particular phase of the sequence to be properly timed. At many levels of the sequence, a neural signal will act on targets that have already been primed by a preceding hormonal effect. Our data indicate that specific neural and hormonal signals may be partially mediated by NO.

LIST OF ABBREVIATIONS:

ASW	artificial seawater
BC	bag cells
ELH	egg laying hormone
L-NAME	*N*ω-nitro-L-arginine methyl ester
LHD	large hermaphroditic duct
MCC	metacerebral (giant) cell
NO	nitric oxide
SNAP	*S*-nitroso-*N*-acetyl-penicillamine

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