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RESEARCH ARTICLE

Nitric oxide affects short-term olfactory memory in the antennal lobe of *Manduca sexta*

Stephanie L. Gage[1,*], Kevin C. Daly[2] and Alan Nighorn[1]

[1]University of Arizona, Tucson, AZ, USA and [2]University of West Virginia, University Avenue, Morgantown, WV, USA

*Author for correspondence (gages@email.arizona.edu)

SUMMARY

Nitric oxide (NO) is thought to play an important neuromodulatory role in olfaction. We are using the hawkmoth *Manduca sexta* to investigate the function of NO signaling in the antennal lobe (AL; the primary olfactory network in invertebrates). We have found previously that NO is present at baseline levels, dramatically increases in response to odor stimulation, and alters the electrophysiology of AL neurons. It is unclear, however, how these effects contribute to common features of olfactory systems such as olfactory learning and memory, odor detection and odor discrimination. In this study, we used chemical detection and a behavioral approach to further examine the function of NO in the AL. We found that basal levels of NO fluctuate with the daily light cycle, being higher during the nocturnal active period. NO also appears to be necessary for short-term olfactory memory. NO does not appear to affect odor detection, odor discrimination between dissimilar odorants, or learning acquisition. These findings suggest a modulatory role for NO in the timing of olfactory-guided behaviors.

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INTRODUCTION

Nitric oxide (NO) is highly expressed in olfactory systems (Bredt et al., 1991; Müller and Hildebrandt, 1995; Elphick et al., 1995; Hopkins et al., 1996; Kendrick et al., 1997; Nighorn et al., 1998; Fujie et al., 2002; Collmann et al., 2004), yet its function remains unclear. The structural organization of the primary olfactory network suggests that diffusible messengers such as NO could be fundamental in olfactory processing (Breer and Shepherd, 1993). Sensory afferents innervate dense, spheroidal neuropils called glomeruli and synapse with secondary cells that facilitate signaling between and within olfactory glomeruli (Price and Powell, 1970; Pinching, 1970). A glomerulus is suggested to function as a unit (Kauer and Cinelli, 1993; Hildebrand and Shepherd, 1997; Mori et al., 1999; Bozza et al., 2002; Wachowiak and Shipley, 2006) and is often surrounded by several layers of glial processes (Tolbert and Oland, 1990; Hildebrand and Shepherd, 1997). As a diffusible messenger, NO may modify signaling within a glomerulus because of its limited diffusion (Breer and Shepherd, 1993).

NO is produced from nitric oxide synthase (NOS), a complex Ca^{2+}-activated enzyme that catalyzes the conversion of L-arginine to form NO. NO affects neurons through multiple signaling cascades, including those triggered by the soluble guanylyl cyclase/cyclic guanosine monophosphate (sGC/cGMP) pathway and through S-nitrosylation. NOS and the NO-target sGC are highly expressed in the AL and olfactory bulb in all species investigated (Bredt et al., 1991; Müller and Hildebrandt, 1995; Elphick et al., 1995; Hopkins et al., 1996; Kendrick et al., 1997; Nighorn et al., 1998; Fujie et al., 2002; Collmann et al., 2004). In *Manduca sexta*, NOS is localized to the olfactory receptor neurons, and sGC is found in almost all projection neurons, some local interneurons and the serotonin-immunoreactive neuron (Collmann et al., 2004). Studies from *M. sexta*, land slugs and mice demonstrate that NO is produced upon odor stimulation and/or electrical stimulation to the olfactory nerve (Collmann et al., 2004; Fujie et al., 2002; Lowe et al., 2008). In the antennal lobe of *M. sexta*, NO production patterns are spatially focused and dependent on the identity and concentration of the odor stimulus (Collmann et al., 2004). In AL neurons, NO affects basal neuronal activity, suggesting a persistent presence of NO (Wilson et al., 2007), and affects whole-cell currents (Higgins et al., 2012). These studies indicate that NO has profound physiological effects in the olfactory system that are likely to influence olfactory processing and olfactory-guided behaviors.

In addition to potentially affecting the primary functioning of the olfactory system, NO is thought to play a role in olfactory learning and memory (for a review, see Susswein et al., 2004). Insights from other animal species have demonstrated that NOS inhibition affects a wide variety of learning and memory paradigms that include contextual fear learning in mice (Kelley et al., 2010), delayed visual recall in monkeys (Prendergast et al., 1997a), negative patterning in turtles (Yeh and Powers, 2005) and spatial navigation in rats and mice (Prendergast et al., 1997b; Mutlu et al., 2011). Specifically in olfaction, NOS inhibition affects odor associations in sheep (Kendrick et al., 1997), newborn rat pups (Samama and Boehm, 1999) and land slugs (Yabumoto et al., 2008). Interestingly, an already-learned association is unaffected by NOS inhibition (Yamada et al., 1995; Müller, 1996; Kendrick et al., 1997; Samama and Boehm, 1999; Yeh and Powers, 2005), suggesting that the role of NO is specific to learning processes and not retrieval. In honeybees, NOS inhibition experiments reveal that learning acquisition is intact, but a specific form of long-term memory is impaired (Müller, 1996). These results support the idea that different forms of memory occur in parallel and are formed by distinct molecular mechanisms. Taken altogether, NO

could underlie molecular substrates needed for learning acquisition, or underlie those that form specific memory traces.

In this study, we explore our working hypothesis that NO is a modulator of olfactory-guided behavior. We first question whether basal levels of NO change during the daily light cycle. To know when NO is produced in the AL provides clues as to how it is utilized in the olfactory system. Like many nocturnal insects, *M. sexta* depends on its olfactory system to find mates, feed and lay eggs during scotophase, or subjective night. If NO production is variable and increases during this active period, it would suggest a potential role for NO in olfactory-guided behaviors. We show that NO concentrations are variable and higher during scotophase. We then combine NOS inhibition in the ALs with a learning paradigm utilizing the proboscis extension reflex (PER) to ask three basic questions: (1) does NO affect odor detection, (2) does NO affect discrimination between dissimilar odorants, and (3) does NO affect the odor association process through learning or memory? We show that NO specifically affects short-term memory. NO does not appear to affect odor detection, odor discrimination between dissimilar odorants, or learning acquisition. Given our results, we speculate that NO may play an important ecological role in the timing of olfactory-guided behaviors.

MATERIALS AND METHODS

Animals

Manduca sexta (Linnaeus 1763) (Lepidoptera: Sphingidae) were reared in the Department of Neuroscience at the University of Arizona. Animals were raised on an artificial diet (see supplementary material Table S1) and maintained under a long-day photoperiod regimen (17 h:7 h light:dark) at 25°C and 50–60% relative humidity. Females at pupae stage 16 were transferred into a biological incubator (Model I-36VL; Percival Scientific, Perry, IA, USA) under a 12 h:12 h light:dark cycle and kept at 25°C at 50–60% relative humidity. Unfed, 4- to 5-day-old females were used for both NO detection and the learning experiments.

NO detection and analysis

NO was measured using the inNO-T system and the IV series of NO sensors (both from Innovative Instruments, Tampa, FL, USA). In this system, the NO sensor records the diffusion of NO from the animal tissue to the sensor surface. The electrical current produced is proportional to the concentration of NO in the tissue and is calibrated for each sensor. For the particular sensor used, 1 pA was equal to 1.89 nmol l^{-1}.

To measure NO in *M. sexta*, brains from 4-day-old females were dissected during the third hour post scotophase or photophase (12 h apart on a 12 h:12 h light:dark cycle). Each brain was divided into ALs, optic lobes and the remaining brain. Each area of the brain was individually placed into liquid nitrogen and stored at –80°C until analysis. Lobes were then placed on dry ice and individually homogenized using a T8.01 Netzgerat IKA Labortechnik homogenizer (Janke and Kunkel, Staufen, Germany) in 50 µl of saline. The homogenized lobe was immediately measured for NO concentration using the inNO-T system. After measuring each lobe, the sensor was replaced into saline to re-obtain a baseline current. Concentrations were determined by measuring the change in concentration immediately prior to the lobe measurement (in saline) to the peak of the NO current.

Pharmacology and microinjection surgery

The NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, St Louis, MO, USA) was dissolved in filtered physiological saline (150 mmol l^{-1} NaCl, 3 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} KCl, 10 mmol l^{-1} TES; pH 6.9) and used at a 15 mmol l^{-1} concentration. This concentration was determined to be the minimal effective dose in extracellular recording in *M. sexta* (Wilson et al., 2007) and approximate to the concentrations used in molluscan preparations (Gelperin, 1994).

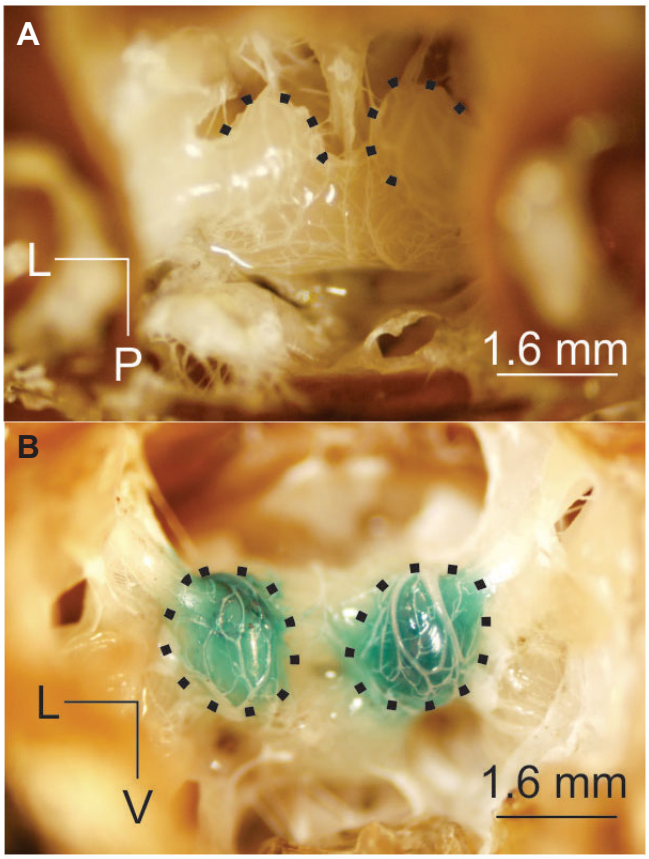

Fig. 1. Views of the antennal lobes (ALs) during surgery and dye injection. (A) Visualization of the ALs through the surgical window (note: connective tissues removed for clarity). (B) Visualization of volume distribution by injection into the ALs. Dashed lines highlight edges of the AL. L, lateral; P, posterior; V, ventral.

Drug delivery into the ALs was performed according to the method described in Lei et al. (Lei et al., 2009). Animals were restrained in a plastic tube and an hourglass window was cut in the head capsule (Fig. 1). The ALs were visualized by moving aside connective tissue with fine forceps. Quartz pipettes (o.d. 1.0 mm, i.d. 70 mm; Sutter Instruments, San Diego, CA, USA) were pulled with a Model P-2000 puller (Sutter Instruments) and clipped to allow solution passage. Pipettes were filled with L-NAME or saline and manually inserted into each AL with 10 drops (total: 33±11 nl, mean ± s.d.; N=3) administered per lobe using a General Valve Picospritzer II (East Hanover, NJ, USA) (volume distribution visualized by injecting undiluted blue food coloring; Fig. 1). The moths were sealed by replacing the cut window and applying myristic acid (Sigma-Aldrich). The identity of the drug *versus* saline control was blind to both the experimenter performing the surgery and the experimenter observing behavior in all experiments.

Olfactory stimuli and delivery

The olfactory stimuli tested include: (1) a synthetic *Datura wrightii* blend that mimics the main components and their proper ratios

emitted from *D. wrightii* (*M. sexta* host plant) (Riffell et al., 2008b; Riffell et al., 2009); (2) hibiscus oil blend (diluted 1:1000; Select Oils, Tulsa, OK, USA); (3) linalool (5 μg μl^{-1}; Sigma-Aldrich); (4) methyl salicylate (5 μg μl^{-1}; Sigma-Aldrich) and (5) control air (blank). Mineral oil (Sigma-Aldrich) was the vehicle for all odors/odorants used. Concentrations were chosen based upon maximal cellular responses in the *M. sexta* AL during multi-channel recording (Dacks et al., 2008). Olfactory stimuli were delivered by a solenoid-controlled air stream into an odor-containing glass syringe. Each syringe contained 10 μl of the odor/odorant on a piece of filter paper.

The odors/odorants chosen as the conditioned stimulus (CS+) were selected based upon ecological significance and studies in the literature. We initially used *D. wrightii* to assess the role of NO in odor detection. *Datura wrightii* is the preferred host plant of *M. sexta*, and is known to illicit innate responses (Raguso and Willis, 2002; Raguso and Willis, 2005; Riffell et al., 2009). We instead found an effect on learning or memory and confirmed our findings using a hibiscus oil blend. Hibiscus is not a reported host plant of hawkmoths and serves as a novel odor to gauge learning and memory. Linalool and methyl salicylate represent two commonly encountered chemical classes in plant headspaces: terpenoids and aromatics. Terpenoids such as linalool comprise upwards of 70% of all volatiles emitted from *D. wrightii*, and aromatics such as methyl salicylate are another major component (Riffell et al., 2008b).

Learning and memory assays

Appetitive conditioning

The PER is an unconditioned feeding reflex that was first employed for olfactory conditioning in honeybees (Takeda, 1961) [for a review of olfactory conditioning in honeybees, see Giurfa and Sandoz (Giurfa and Sandoz, 2012)]. The neuroanatomy underlying the proboscis extension in *M. sexta* is well characterized (Davis and Hildebrand, 2006) and the PER learning paradigm is a modified version of the method described in Daly and Smith (Daly and Smith, 2000). Animals were restrained in a plastic tube with eyes covered (wax) prior to surgery and conditioning. A clear plastic tube was situated over the elongated proboscis to secure a uniform position of the proboscis and to observe maximum pumping motion and extension. Five-day-old moths were trained in a forward-paired conditioning paradigm to associate an odor with a sucrose reward [1 μl, 25% sucrose solution, the latter chosen by sucrose-dominant sugar concentrations present in *D. wrightii* nectar (Raguso et al., 2003; Guerenstein et al., 2004; Farkas et al., 2011)]. A 5-s odor pulse was delivered to the odor-containing syringe positioned 5 cm from the right antenna. Three seconds into the pulse, the sucrose was applied to the tip of the proboscis with a pipette. This sequence was repeated in all assays for a total of six trials spaced 4 min apart.

Learning and memory

Animals were removed from the biological incubator (Percival Scientific) 1.5 h into scotophase and kept in dark conditions under red light. Moths were restrained and injected with L-NAME, 15–30 min prior to conditioning. Conditioning began 2.5 h into scotophase. One hour after conditioning completion, moths were tested for learning by the presentation of odor alone and recording proboscis extension. Each animal was tested three times with a 5-s odor pulse. A positive test resulted in observed feeding movements of the proboscis including full extension, uncoiling and pumping of the 'knee' (see supplementary material Movies 1–3). Animals were scored based on each odor presentation; for example, 'moth A showed proboscis extension one out of three times to the CS+'. Animals were also tested with a blank syringe to test the effect of airflow (blank PER%=23%). To examine the effect of L-NAME in different memory stages, the moths were injected with L-NAME prior to conditioning with hibiscus and tested at 5 min, 1 h, 4 h and 24 h post-conditioning.

Odor detection

To test whether the L-NAME impairment was caused by a learning or memory deficit or a disruption in odor detection, L-NAME injections were performed after conditioning. Injections were performed 15–30 min prior to testing, and testing commenced 1 h post-conditioning.

Discrimination between dissimilar odorants

Some neuromodulators, such as serotonin, have been suggested to enhance contrast resolution between different molecular classes of odorants (Dacks et al., 2008). NO was tested in this capacity by determining the animal's ability to discriminate between two commonly encountered odorants in plant headspaces: linalool (a monoterpenoid structure) and methyl salicylate (an aromatic structure). Animals were conditioned to associate one odorant with a sucrose reward. Odor–sucrose conditioning was performed before L-NAME injection to rule out association impairments from lack of NO. Odor–sucrose conditioning consisted of the presentation of linalool (monoterpenoid) and methyl salicylate (aromatic) to each animal six times spaced 4 min apart. One odorant was alternatively assigned per experiment day to be the CS+ and paired with sucrose. The CS+ was always presented first. The other odorant was presented without sucrose (CS–). The animals were injected and then tested 1 h later after conditioning. Evaluation of odor discrimination consisted of the CS+ and CS– presented alternatively (CS+ 2×; CS– 2× per animal) and evaluated on the proboscis extension criteria described above.

Statistical analysis

All statistical analyses were performed using JMP 9.0.1 (SAS Institute, Cary, NC, USA). NO concentrations between scotophase and photophase were evaluated for statistical significance using a two-tailed Student's *t*-test. In all learning and memory experiments, responses were recorded with a 1 or 0 to employ parametric tests. A one-way ANOVA was employed with a Tukey–Kramer HSD *post hoc* test to evaluate means among groups. In all tests, α=0.05 and a 95% confidence level was used. Data are expressed as means ± s.e.m. unless otherwise noted.

RESULTS

NO levels are higher during scotophase in the AL and optic lobes

NO concentrations in the ALs, the optic lobes and the remainder of the brain were measured at a singular time point (3 h post-induction of the light cycle) in scotophase and photophase. NO levels are substantially higher in the ALs and optic lobes during scotophase, when the moths are most active (Fig. 2). In the ALs, the mean NO concentration during scotophase [115.70±19.75 nmol l^{-1} (s.d.), N=11 from eight moths] is significantly higher than the mean concentration during photophase [47.86±15.59 nmol l^{-1} (s.d.), N=8 from five moths; t_{17}=8.04, P=<0.0001]. Similarly, in the optic lobes, the mean NO concentration during scotophase [131.68±36.72 nmol l^{-1} (s.d.), N=8 from five moths] is significantly higher than during photophase [42.72±23.24 nmol l^{-1} (s.d.), N=8 from six moths; t_{14}=5.78, P=<0.0001]. The remainder of the brain, encompassing the protocerebrum, the tritocerebrum and the sub-esophageal ganglion, does not show a significant change in NO levels with light phase

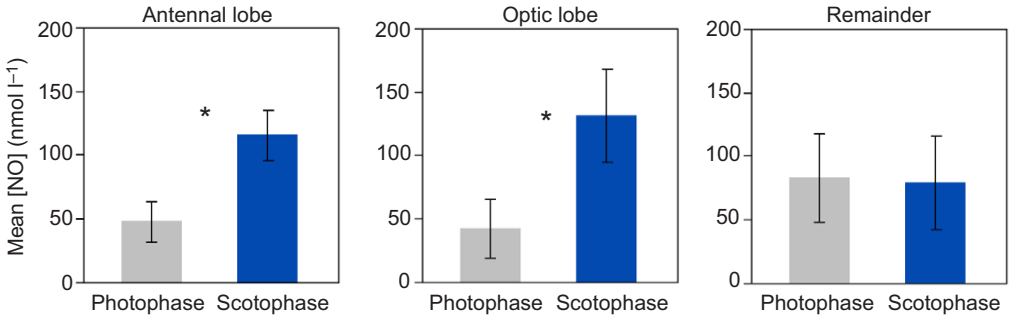

Fig. 2. Basal nitric oxide (NO) levels fluctuate with light cycle in the *Manduca sexta* brain. ALs, optic lobes and the remainder of the brain (protocerebrum, tritocerebrum and the sub-esophageal ganglion) were measured for NO concentration during scotophase and photophase. Mean NO concentration is significantly higher during scotophase than photophase in the ALs (Student's *t*-test, t_{17}=8.04, *P*=<0.0001, *N*=11 scotophase, *N*=8 photophase) and optic lobes (t_{14}=5.78, *P*=<0.0001, *N*=8, 8) but not in the remainder of the brain (t_9=0.17, *P*=0.87, *N*=5, 6). Error bars denote ±s.d.

[scotophase: 79.22±36.99 nmol l^{-1}, *N*=5; photophase: μ 82.87± 4.55 nmol l^{-1}, *N*=6; t_9=0.17, *P*=0.87]. These results suggest that NO concentrations are subject to light cycle and are likely indicative of roles in nocturnal activity.

NOS inhibition impairs odor associations and does not affect odor detection

The effect of NO in olfactory learning was examined using associative-odor learning assays paired with NOS inhibition before and after conditioning (Fig. 3A). This experiment was first performed using *D. wrightii*, the preferred host plant of *M. sexta*, as the conditioned odor. Conditioning was performed 2.5 h into scotophase to mimic the approximate time of day *M. sexta* forage in the field (Gregory, 1963; Raguso and Willis, 2005). When NOS is inhibited before conditioning, there is a significant reduction in the number of proboscis extensions 1 h later as compared with vehicle controls ($F_{1,64}$=11.18, *P*=0.001, *N*=11 L-NAME injected, *N*=11 saline injected). To test whether this impairment is the result of learning or odor detection, NOS was inhibited after conditioning (Fig. 3A). In contrast, we found no significant impairment of proboscis extension 1 h later ($F_{1,28}$=0.35, *P*=0.59, *N*=5, 5). These results suggest that NO does not interfere with odor detection or retrieval, but does affect learning or memory to the conditioned odor. To further investigate NO and the odor-associative effects, responses to a novel odor were examined. The same sets of experiments were performed using hibiscus (Fig. 3A). Similar to the results with *D. wrightii*, when NOS inhibition is performed before conditioning, there is significant reduction in the number of proboscis extensions ($F_{1,40}$=15.92, *P*=0.0003, *N*=7, 7). When NOS is inhibited after conditioning, there is no significant difference compared with vehicle controls ($F_{1,43}$=2.87, *P*=0.097, *N*=8, 7). NO appears to be a necessary component during the conditioning process to recognize an odor as rewarding. Taken together, there is a significant deficit imposed by NOS inhibition prior to conditioning ($F_{3,104}$=9.12, *P*=<0.0001) without regard to the conditioned odor (*P*=0.59, *post hoc* Tukey–Kramer HSD).

NOS inhibition does not affect odorant discrimination between dissimilar odorants

To test whether NO affects odorant discrimination between chemically dissimilar odorants, moths were tasked with associating linalool (monoterpenoid) or methyl salicylate (aromatic) with a sucrose reward (CS+) (Fig. 3B). The moths were tested by presenting the CS+ alternatively with the unrewarded odorant (CS–), and proboscis extension was monitored. NOS inhibition was performed after conditioning to rule out learning impairments caused by lack of NO. NOS inhibition was performed after conditioning to rule out memory impairments due to the lack of NO. Both groups, saline treated and L-NAME treated, showed successful discrimination between the CS+ and CS– [saline treated ($F_{1,38}$=7.33, *P*=0.01, *N*=10); L-NAME ($F_{1,38}$=7.6, *P*=0.009, *N*=10)]. NOS inhibition does not affect successful discrimination between dissimilar odorants linalool and methyl salicylate.

NOS inhibition affects short-term memory trace(s)

To examine whether NOS inhibition affects learning acquisition or memory, moths were tested at multiple time points over 24 h (Fig. 4A,B). If moths show continued impairment throughout the time points, this would suggest that learning acquisition is affected by NO. L-NAME-injected moths show a significant impairment at the 1 h time point compared with saline controls ($F_{1,79}$=23.55, *P*=0.0001, *N*=18 L-NAME injected, *N*=12 saline injected), but unexpectedly show significant improvement 24 h later ($F_{2,159}$=4.48, *P*=0.01, *post hoc* Tukey–Kramer HSD, *N*=18; Fig. 4A). These results suggest that NO does not affect learning acquisition, but does affect a memory trace appearing 1 h after conditioning.

In comparison with memory traces found in *Drosophila*, this time window borders short-term and intermediate-term memory. A short-term memory trace appears immediately after conditioning in the *Drosophila* ALs and disappears after 7 min (Yu et al., 2004). To test the effects of NO more conclusively in the short-term memory window, we also tested moths at 5 min post-conditioning in addition to the 1, 4 and 24 h time periods (Fig. 4B). At 5 min, L-NAME-injected moths show significant reductions in PER compared with saline controls ($F_{1,49}$=4.09, *P*=0.048, *N*=10 L-NAME injected, *N*=7 saline injected) and confirm our previous findings of a significant PER reduction at 1 h ($F_{7,196}$=6.08, *P*=0.0003, *post hoc* Tukey–Kramer HSD). These moths also do not show significant reductions in PER at 4 and 24 h. As a result, there is significant improvement in the PER from short-term time points (5 min and 1 h) to longer-term time points (4 and 24 h) ($F_{3,116}$=7.347, *P*=<0.0001, *N*=10 L-NAME injected, *N*=7 saline injected). These results suggest that NO affects either one memory trace that spans from at least 5 min to 1 h, or that NO affects two short-term memory traces. Taken altogether, these studies (Fig. 4A,B) reveal that L-NAME-injected moths fall into three main categories when observed over time: (1) those that are inhibited in the short-term and improve (57%), (2) those that remain consistently impaired (18%), and (3) those that do not show short-term impairments (21%).

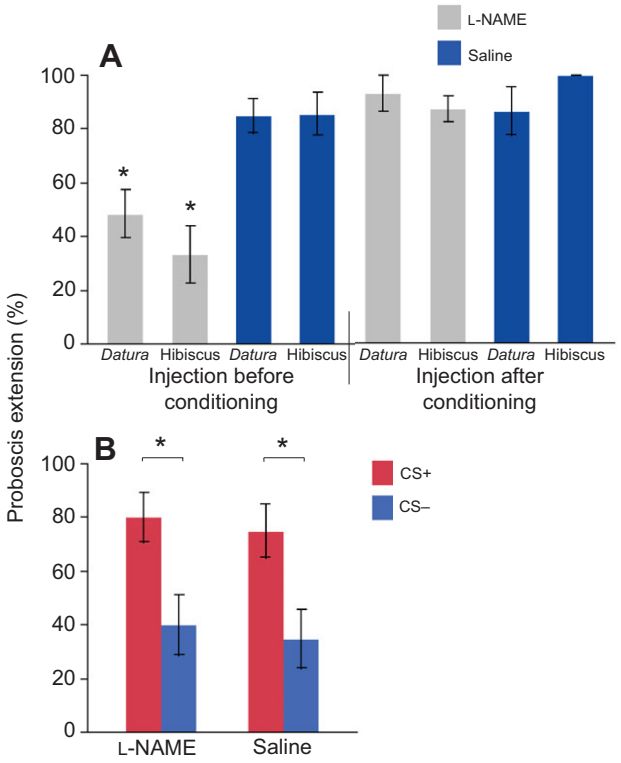

Fig. 3. Nitric oxide synthase (NOS) inhibition diminishes the proboscis extension reflex (PER) before odor conditioning, but not after. (A) NOS inhibition by L-NAME reduces PER when injected into the ALs before conditioning but not after conditioning to both *Datura wrightii* and hibiscus odors. The effect of L-NAME on the PER was measured *versus* a saline control using a one-way ANOVA. Before conditioning, L-NAME significantly reduced PER using *D. wrightii* ($F_{1,64}$=11.18, P=0.001, N=11 L-NAME injected, N=11 saline injected) and hibiscus ($F_{1,40}$=15.92, P=0.0003, N=7 L-NAME injected, N=7 saline injected). Asterisks denote significant differences between L-NAME and saline groups conditioned with the same odor. (B) L-NAME injection after conditioning does not affect successful discrimination between the chemically dissimilar odorants linalool and methyl salicylate ($F_{1,38}$=7.6, P=0.009, N=10). Linalool and methyl salicylate were alternatively presented as the CS+ and CS– throughout testing. CS+ denotes sucrose-rewarded odor and CS– denotes unrewarded odor.

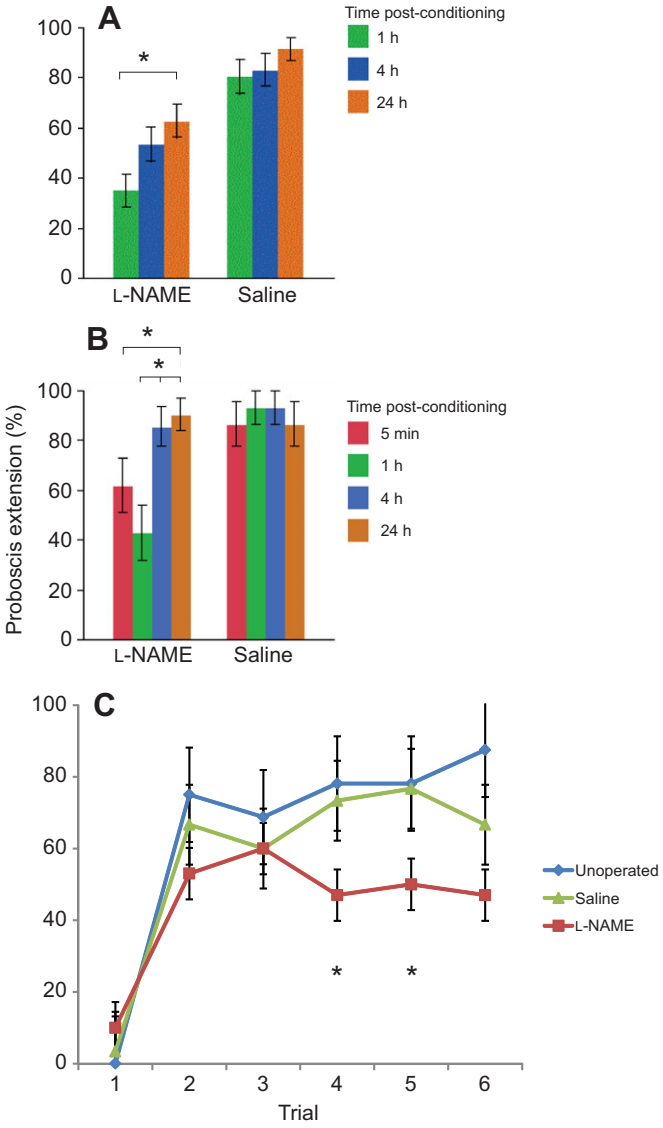

Fig. 4. NOS inhibition affects the PER differently over time. (A) *Manduca sexta* were tested at 1, 4 and 24 h post-conditioning. L-NAME-treated moths show significant improvement in PER from 1 to 24 h ($F_{2,159}$=4.48, *P=0.01, *post hoc* Tukey–Kramer HSD, N=18). (B) L-NAME also impairs the PER at 5 min post-conditioning and again at 1 h post-conditioning. At 4 and 24 h post-conditioning, moths significantly improve PER to the CS+ ($F_{3,116}$=7.347, *P=<0.0001, *post hoc* Tukey–Kramer HSD, N=10 L-NAME injected, N=7 saline injected). (C) L-NAME reduces PER during the later trials of conditioning with hibiscus as the CS+. L-NAME-injected moths significantly drop in PER during trials four (one-way ANOVA, $F_{1,58}$=4.64, *P=0.035, N=30, 30) and five ($F_{1,58}$=4.81, *P=0.032, N=30, 30) when compared with saline controls of the same trial.

The anticipatory PER responses observed during conditioning also suggest that NO affects short-term memory. The results of the conditioning trials (collected across experiments with hibiscus as the CS+) reveal that memory deficits by L-NAME appear as early as the fourth trial (Fig. 4C). Moths were conditioned to the CS+ during six trials spaced 4 min apart. During the first trial, before the CS+ is paired with sucrose, there are minimal proboscis extensions to the CS+ odor. By the second and third trial, all treatment groups extend their proboscis in anticipation more than 50% of the time. The responses of the control groups, both un-operated and saline-injected moths, continue to increase with additional trials. However, by the fourth trial, L-NAME-injected moths significantly drop in the number of proboscis extensions compared with saline controls of the same trial ($F_{1,58}$=4.64, P=0.035, N=30 L-NAME injected, N=30 saline injected) and remain significantly impaired through Trial 5 ($F_{1,58}$=4.81, P=0.032, N=30 L-NAME injected, N=30 saline injected). In Trial 6, L-NAME moths show reduced PER at 47% (Trials 4 and 5: PER 47 and 50%, respectively) but this is not significantly different from the Trial 6 saline control. These findings further implicate NO as an important signaling component in the creation of short-term memory traces.

DISCUSSION

NO signaling is likely common to all olfactory systems. Previous studies have shown that NO exists at tonic low levels (Wilson et al., 2007) that dramatically increase in response to odorants (Collmann et al., 2004; Lowe et al., 2008). We also know that NO modifies whole-cell current in AL neurons (Higgins et al., 2012).

While this evidence strongly implicates a role for NO, the functional significance of this modulation is not known. In this study, we have used chemical detection of NO and the PER odor-conditioning assay to understand whether NO basal levels fluctuate and the involvement in basic olfactory tasks.

We have discovered that NO levels are significantly higher in the optic lobes and ALs during the nocturnal active period (Fig. 2). These findings suggest a dynamic temporal role for NO that may contribute to the circadian time of olfactory-dependent activity. In *M. sexta*, *period* gene products are found in several cell types, including the compound eye photoreceptors, neurons in the optic lobes, and glia surrounding the glomeruli in the ALs (Wise et al., 2002). In addition, *period* immunoreactivity identified putative circadian pacemaker cells in the antennae that include olfactory receptor neurons and antennal nerve glia (Schuckel et al., 2007). In *Drosophila*, antennae pacemaker cells are found to be necessary and sufficient for olfactory rhythms, therefore suggesting that the components of the olfactory signal transduction cascade could be targets of circadian regulation (Tanoue et al., 2004). NO could very likely be an important modulator in this process, especially given the expression of NOS in the olfactory receptor neurons in *M. sexta*. NO could affect pacemaker cells in the antennae, AL and optic lobes directly, similar to the basal retinal neurons in the mollusk (Bullmann and Stevenson, 2008), or be a downstream result. Given the dramatic physiological effects of NO in AL neurons, NO could act as a 'priming' agent that adjusts olfactory and optical circuitry to enable nocturnal behaviors. It would be interesting to note whether the NO peak fluctuation is reversed in diurnal animals, and whether multiple measurements of NO over the light cycle reveal a light-entrainable circadian pattern.

Heightened NO release during the active period may also indicate specific roles in modulating olfactory-guided behaviors. NO can affect cells in several ways (e.g. by activating protein kinases, phosphodiesterases and cyclic nucleotide-gated channels) and therefore could mediate many different aspects of olfactory processing. In our studies using the PER assay, we found that NO does not affect odor detection (Fig. 3A) or odor discrimination between dissimilar odorants once the CS+ has been learned (Fig. 3B). NO may play more subtle roles at the cellular level, but these are undetectable using the PER assay. Interestingly, NO does mediate aspects of appetitive-associative conditioning. NOS inhibition revealed a strong impairment to the conditioned odor when tested 1 h later (Fig. 3A). Given these initial results, we tested whether NO affects the acquisition of learning – by affecting those biochemical processes that enable learning to occur – or underlies a memory trace present at the time the animals were tested. Testing at additional time intervals suggests the latter (Fig. 4) and specifically implicates NO in short-term memory.

In *Drosophila*, researchers have identified six olfactory memory traces occurring in the ALs and the mushroom bodies (for a review, see Davis, 2011). These memory traces are likely formed by specific molecular substrates activated through odor conditioning and appear at distinct time lengths after the conditioning period. In our studies, the greatest NOS inhibition impairment was observed at 1 h post-conditioning. In comparison with *Drosophila*, this memory trace window falls between short-term and intermediate-term memory and does not appear to distinctly correspond with an identified trace. One short-term *Drosophila* memory trace, however, recruits AL projection neurons into the CS+ representation (Yu et al., 2004). This trace appears and disappears 7 min after conditioning. We tested moths at 5 min and found a significant NOS inhibition impairment, although not as robust as at 1 h (Fig. 4B). This finding suggests that NO may affect two memory traces, or that these time points could be representative of one trace. The trial data collected during conditioning further implicate NO in short-term memory. During the six conditioning trials, NOS inhibition impaired later trial responses, starting with the fourth trial or 12 min into conditioning (Fig. 4C). Taken altogether, these results suggest that instead of deficits in learning acquisition, NO affects specific molecular substrates underlying short-term memory trace(s), while leaving intermediate-term (4 h post) and long-term traces (24 h post) intact.

The time course of these NO-mediated memory traces is strikingly similar to the nocturnal habits of flowering and feeding between *Datura* (Solanaecea) and hawkmoths (Sphingidae). In Southern Arizona, *M. sexta* feed from trumpet-shaped *D. wrightii* flowers in a relationship that has co-evolved over time (Riffell et al., 2008a; Riffell et al., 2008b; Raguso et al., 2003). *Datura wrightii* flowers open at dusk and wilt during the morning hours of the next day (Grant, 1983; Raguso and Willis, 2005). Nectar production is slight when flowers first open, but flows at peak abundance 1–2 h later (Grant, 1983) and significantly decreases 3.3 h after opening (Guerenstein et al., 2004). *Manduca sexta* and other hawkmoths forage at this peak nectar time for 1–2 h and sometimes beyond, but never at the levels observed during the first hour (Gregory, 1963; Raguso and Willis, 2005). It appears that this co-evolved relationship depends on a narrow, 1–2 h time range, with an emphasis on the first hour. The importance of this feeding window unexpectedly corresponds to our observation that NOS inhibitory effects are strongest at 1 h. These observations suggest that a 1-h memory trace between volatiles and nectar may be biologically significant and part of the co-evolution between *M. sexta* and *D. wrightii*. It is interesting that *D. wrightii*, known to cause innate responses in *M. sexta*, would still illicit strong memory impairments after NOS inhibition. This suggests that NO may contribute to the physiology underlying the tightly coupled timing of foraging and nectar production. Moreover, when *D. wrightii* are not locally abundant, it becomes necessary for *M. sexta* to learn to feed from other species such as *Agave* spp. (Riffell et al., 2008a; Riffell et al., 2008b), thus demonstrating that learning and memory is important at this time of day.

In conclusion, our observations reveal functional roles of NO in the olfactory system. NO production is higher during the nocturnal active period and is necessary for short-term memory. This increased level of NO coincides with robust learning and memory responses in the laboratory and prior observations in the field. The precise timing of foraging and nectar production between *M. sexta* and *D. wrightii* suggests that NO may be important for the timing of olfactory-guided behaviors. It is, therefore, a plausible hypothesis for future studies that NO may mediate the coordination of physiological processes that enable animals to anticipate regular stimuli in the environment.

LIST OF ABBREVIATIONS

AL	antennal lobe
cGMP	cyclic guanosine monophosphate
CS–	conditioned stimulus, unrewarded
CS+	conditioned stimulus, rewarded
L-NAME	*N*-nitro-L-arginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
PER	proboscis extension reflex
sGC	soluble guanylyl cyclase

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AUTHOR CONTRIBUTIONS

S.L.G., K.C.D. and A.N. designed the experiments. S.L.G. performed and analyzed the experiments. S.L.G., K.C.D. and A.N. wrote the manuscript.

COMPETING INTERESTS

No competing interests declared.

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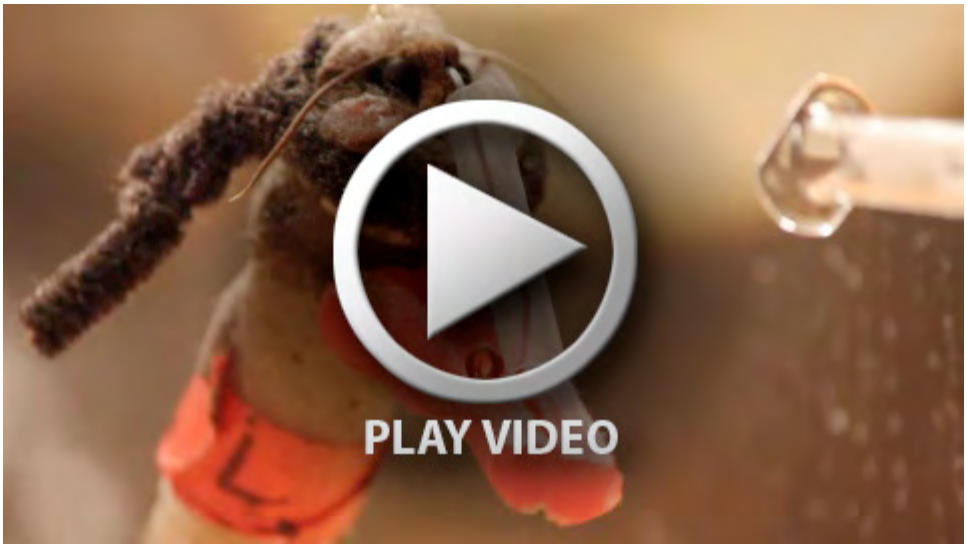
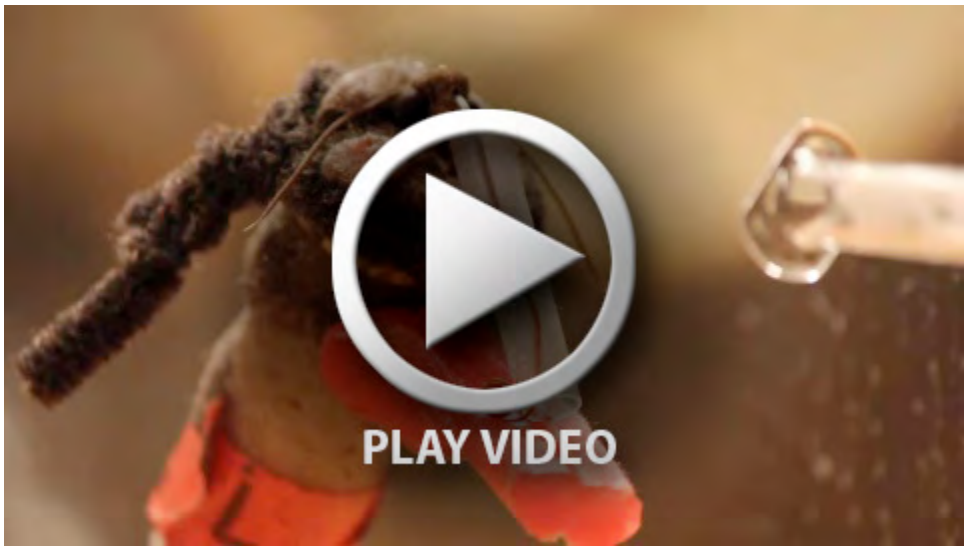


Movie 1. Proboscis extension. Example of proboscis extension to the conditioned odor. Odor puff delivered at 00:00:02.



Movie 2. Proboscis extension: example of uncoil. Example of proboscis extension to the conditioned odor. Odor puff delivered on click at 00:00:01.

Movie 3. Proboscis extension: example of pump. Example of proboscis extension to the conditioned odor. Odor puff delivered on click at 00:00:01.

Table S1. *Manduca* diet recipe (1.8 l)

Ingredient	Amount
Agar	35.5 g
Wheat germ	360 g
Casein	75 g
Salt mix	24 g
Sucrose	36 g
Cholesterol	5 g
Ascorbic acid	12 g
Sorbic acid	6 g
Methyl paraben	3 g
Vitamin mix (30 ml)	
Nicotinic acid	1800 mg
Riboflavin	900 mg
Thiamine	420 mg
Pyridoxine	420 mg
Folic acid	420 mg
Biotin	36 mg
Water	1800 ml
Formalin (60 ml)	
Formaldehyde (37%)	50 ml
Water	450 ml
Linseed oil	10 ml
Water	1800 ml