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Nitric Oxide Modulates Salt and Sugar Responses via Different Signaling Pathways

Philip L. Newland and Paul Yates

School of Biological Sciences, Biomedical Science Building, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Correspondence to be sent to: Philip L. Newland, School of Biological Sciences, Biomedical Science Building, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, United Kingdom. email: pln@soton.ac.uk

Abstract

Locusts lay their eggs by digging into a substrate using rhythmic opening and closing movements of ovipositor valves at the end of the abdomen. The digging rhythm is inhibited by chemosensory stimulation of chemoreceptors on the valves. Nitric oxide (NO) modulated the effects of chemosensory stimulation on the rhythm. Stimulation with either sucrose or sodium chloride (NaCl) stopped the digging rhythm, whereas simultaneous bath application of the NO inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), increased the duration for which the digging rhythm stopped. Increasing NO levels caused a significant reduction in the cessation of the rhythm in response to the same 2 chemicals. Bath applying cyclic guanosine monophosphate (cGMP), the soluble guanylate inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and the generic protein kinase inhibitor H-7 had no effect on the duration for which the rhythm stopped in response to NaCl stimulation. Conversely, bath application of cGMP and ODQ resulted in a significant decrease and increase, respectively, in the duration for which the digging rhythm stopped when stimulated with sucrose. Moreover, bath application of the selective protein kinase G (PKG) inhibitor KT-5823 also resulted in a significant increase in the duration of cessation of the rhythm when stimulated with sucrose. Results suggest that NO modulates the behavioral responses to NaCl via a cGMP/PKG-independent pathway while modulating the responses to sucrose via a NO-cGMP/PKG-dependent pathway.

Key words: cGMP, chemoreception, grasshopper, gustation, nitric oxide synthase, oviposition

Introduction

An animal's behavioral response to sensory input needs to be modified in the context of its current internal physiological state (Elliott and Susswein 2002). Although a range of neuromodulators have been shown to affect behavior (see e.g., Harris-Warrick and Marder 1991), nitric oxide (NO) has been shown to modulate sensory input and behavioral responses to all of the known sensory modalities, including mechanosensation (Schuppe and Newland 2004), vision (Bicker and Schmachtenberg 1997), audition (Zdanski et al. 1998; Hanson et al. 2003), olfaction (Gelperin et al. 1996; Bicker 1998; Nighorn et al. 1998; Schmachtenberg and Bacigalupo 1999; Collmann et al. 2004), and chemoreception (Elphick, Kemenes, et al. 1995; Morley et al. 1997; Kretz et al. 1998; Krizhanovsky et al. 2000; Murata et al. 2004) in both vertebrates and invertebrates.

NO has been known as an important regulator in olfaction in vertebrates and invertebrates for a number of years. Nitric oxide synthase (NOS), for example, is present in areas of the central and peripheral nervous system that are involved in the processing of olfactory input (Dellacorte et al. 1995; Elphick, Rayne, et al. 1995) and recently has been shown to be involved in olfactory processing by interneurons in the olfactory lobe of the moth, *Manduca sexta* (Wilson et al. 2007) and in olfactory learning (Menzel et al. 1996; Müller 1996).

A growing number of studies have implicated NO in modulating responses to contact chemosensory stimuli or taste (Huque and Brand 1994). In vertebrates, NOS is present in the taste organs of the channel catfish, *Ictalarus punctatus* (Huque and Brand 1994), and in the taste buds (vallate and foliate papilla) of rats (Kretz et al. 1998; Krizhanovsky et al. 2000). In invertebrates, NO plays a modulatory role in feeding and is found in the central neuronal networks that underlie the feeding movements of molluscs (Straub et al. 2002). In the pond snail *Lymnaea stagnalis*, stimulating the mouthparts with a sucrose solution results in the generation of NO around the buccal ganglia (Kobayashi et al. 2000).

Ott et al. (2000) showed that one of the key molecular targets of NO, soluble guanylate cyclase (sGC), is present in the

terminals of sensory neurons within the metathoracic ganglion of locusts and also in peripheral contact chemosensors, the basiconic sensilla, indicating a crucial link between NO and taste. More recently, Murata et al. (2004) and Nakamura et al. (2005) suggested that the NO/cyclic guanosine monophosphate (cGMP)-signaling cascade was involved in the chemosensory transduction of sucrose stimuli, although direct pharmacological evidence for the involvement of the NO/cGMP pathway was lacking, as was a peripheral source of NO. Recently, however, we showed that a peripheral source of NO is present within glandular cells of the epithelium of locusts using both immunohistochemistry and fluorescence imaging (Schuppe et al. 2007) and that NO was involved in regulating responses to NaCl and sucrose in the periphery.

Contact chemoreception also plays a vital role in the selection of egg-laying sites (Tousson and Hustert 2000). During egg laying any chemical can be aversive and prevent oviposition digging if present at sufficiently high concentrations (Newland and Yates 2007). The concentration at which a chemical becomes aversive, as with leg avoidance (Rogers and Newland 2002), depends on whether the chemical is a normal component of the diet. Newland and Yates (2008) have also shown that NO regulates the frequency of the digging rhythm via a NO/sGC-cGMP signaling pathway. Given these recent observations and the potential for NO to act via different signaling cascades (Murata et al. 2004; Schuppe et al. 2007) we have utilized this digging motor pattern to reveal the mechanisms through which NO modulates 2 different tastes, NaCl and sucrose.

Materials and methods

Female desert locusts, *Schistocerca gregaria* (Forskål) were taken from a colony maintained at the University of Southampton, reared under crowded conditions and fed daily on seedling wheat and oats. The colony was, on occasion, supplemented with female locusts obtained from Blades Biological Supplies (Edenbridge, Kent, UK). Adult locusts were used in all experiments, aged from 14 to approximately 21 days postmoult. All adults were examined prior to use to ensure that the ovipositor valves and surrounding cuticle were intact and undamaged.

Physiological methods

The digging rhythm was initiated by decapitating a locust, removing its gut, and isolating the abdomen (Figure 1A,B). This reliably caused rhythmic movements of the ovipositor valves similar to those defined as "fictive digging movements" (Thompson 1986a, 1986b) (Figure 1C). The abdomen was pinned laterally to a plasticine stage with the ovipositor valves overhanging the edge of the stage to allow chemicals to drain away and prevent constant chemosensory input. The anterior end of the abdomen was constantly perfused with fresh locust saline throughout an experiment.

Muscle recordings (Figure 1D) were obtained primarily from the ventral opener muscles due to their large size and accessibility, using pairs of 63 μm copper wire, insulated except for their tips, pushed through small holes in the cuticle into the opener muscles, and secured in place using cyanoacrylate glue.

The cycle frequency of the digging rhythm was determined from the first spike of each muscle burst to the first spike of the next burst. After each experiment, an animal was dissected and the locations of the myogram wires visually confirmed. Signals from the electrodes were amplified with an AC preamplifier and displayed on a Tektronix TDS 210 oscilloscope, digitized using a Cambridge Electronic Design 1401 interface and displayed and analyzed using Spike 2 v4.0 software.

Chemical stimulation

Individual droplets of 2 chemicals were applied to the ventral surface of the ovipositor valves (Figure 1B) at 3 min intervals using a Pasteur pipette as this area of the ovipositor valves has a high density of basiconic sensilla (Tousson and Hustert 2000). This procedure provided a baseline with which to compare preparations in which the NO levels around the terminal abdominal ganglia were pharmacologically manipulated while repeatedly applying chemical stimuli to the valves. A total of 13 chemical applications were made to the ovipositor valves per experiment. The first 3 chemical stimuli represent the control period, the following 3 stimuli, when drug application occurs, represent the test period, and the last 3 stimuli, when the terminal ganglion is perfused with fresh saline, represent the wash period. The chemicals used for stimulation of the ovipositor valves were 250 mM NaCl and 1 M sucrose, concentrations that have previously been shown to have an effect on the oviposition rhythm (Newland and Yates 2007).

Drug application

A small window was cut in the cuticle at the caudal end of the abdomen, and the terminal and last abdominal ganglion 7 exposed (where the central pattern generator [CPG] network that underlies the digging rhythm is located [Thompson 1986a, 1986b]). These ganglia were then perfused with control saline or saline containing drugs that were applied after the third presentation of a chemical stimulus and lasted for a duration of 10 min. Preliminary experiments were performed to establish the duration taken for the optimal effects of the drugs to be exhibited, these were maximal after stimulus 8 (1440 s), and these were compared with control (stimulus 3, 180 s) and wash (stimulation 13, 2340 s). The effects of the NOS inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), and the NO donor 3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANONOate), on the cessation of the digging rhythm were performed for both chemicals (NaCl and sucrose).

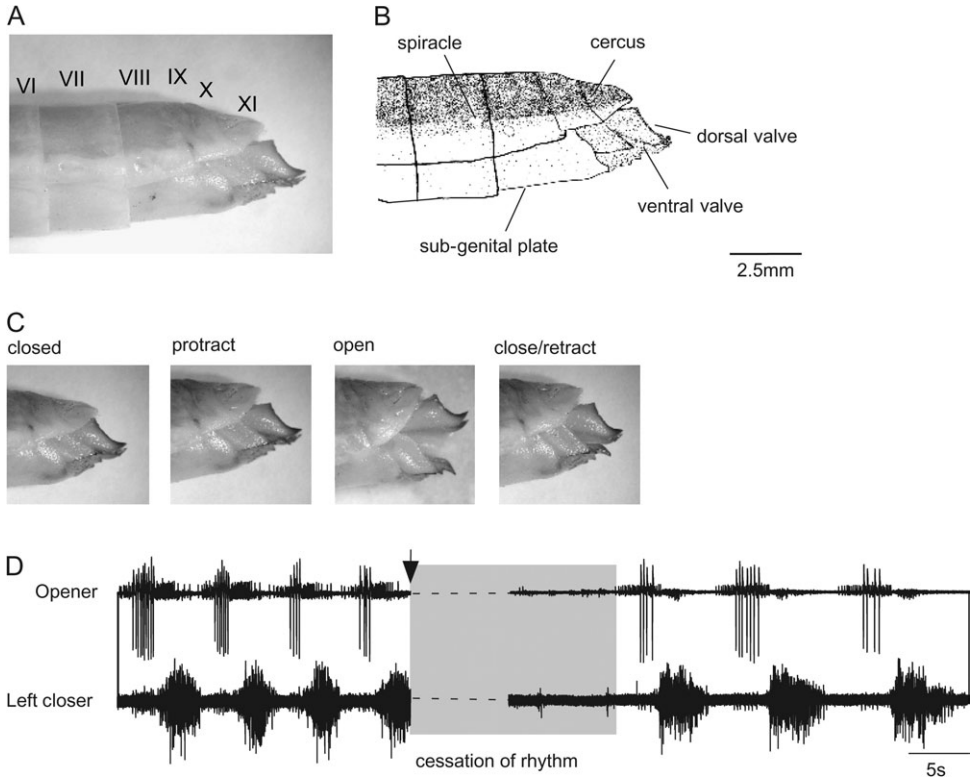

Figure 1 The locust abdomen and the digging rhythm. **(A)** Detailed anatomy of the distal end of the female abdomen. A lateral view of the abdomen and the ovipositor valves. The muscles that produce the digging movements of the valves are contained in the abdomen from tergites VI–XI (dorsal) and sternites VI–VIII (ventral) to the tip of the abdomen. **(B)** Two pairs of valves are involved in the digging of suitable substrates, the dorsal and ventral ovipositor valves. The dorsal valves project from tergite XI and the ventral valves project distally from subgenital plate (sternite 8). A mechanosensitive cercus and a spiracle are also labeled. At least 1 spiracle is found on each tergite of the abdomen (Albrecht 1953). **(C)** From a fully closed and retracted position, the oviposition digging rhythm consists of a protraction of the valves followed by an opening movement of the dorsal and ventral valves before the valves are retracted and closed simultaneously. **(D)** Myogram recordings from the ventral opener and closer muscles that move the valves showing reciprocal activation. Droplets of test chemicals were applied when the valves were closed and the period for which they remain closed (cessation of the rhythm) determined (gray box).

The pathways by which NO affects the behavioral responses to NaCl and sucrose were also investigated. For both chemicals, the effect of the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and the membrane-permeable analogue of cGMP, 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP) on the oviposition rhythm were investigated. An investigation of potential downstream molecular targets of cGMP was also carried out for both NaCl and sucrose responses. For this purpose, the generic protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) was bath applied to the terminal abdominal ganglion. The protein kinase G (PKG) blocker (9S, 10R, 12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-di indolo[1,2,3-fg:3′,2′,1′,-kl]pyrrolo [3,4-I][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT-5823) was also used for sucrose stimulation experiments. The concentrations of drugs used were based on preliminary experiments and on the results of previous studies (Aonuma and Newland 2001; Schuppe et al. 2007). An animal was used only once for one drug application.

Statistical methods

The statistical significance of the effects of drug application on the period for which the rhythm stopped after chemical stimulation (test periods compared with control periods) were tested using Student's *t*-tests. Where data sets were non-parametric, Mann–Whitney *U*-tests were used. To test the differences between the effects of control (without drug application to the terminal abdominal ganglion), L-NAME and PAPANONOate on the rhythm for control, test, and wash times, a Kruskal–Wallis test was appropriate because of the large variations in the data set. Where data sets were parametric, a 1-way analysis of variance was used. The association between preceding cycle frequency and the subsequent duration a rhythm stopped was tested using a Pearson product–moment correlation analysis.

Results

Previous studies have shown that the detection of chemicals by chemoreceptors on the ovipositor valves leads to an

inhibition, or cessation, of the oviposition digging rhythm (Newland and Yates 2007). In addition, Newland and Yates (2008) have shown that the frequency of the digging rhythm is modulated by NO. Since chemoreception plays such a fundamental role in egg laying, these previous studies raise the question as to whether NO may also modulate the responses to chemical stimulation, as it does on the leg (Schuppe et al. 2007). Thus, the effect of NO on the motor output representing the digging rhythm was analyzed in response to chemical stimulation of the ovipositor valves with sucrose and NaCl and the signaling pathways through which NO mediates its effects determined.

NO modulates the behavioral response to NaCl

The ovipositor valves were repeatedly stimulated with 250 mM NaCl at 3 min intervals and the effect on the cessation of the digging rhythm analyzed. The duration for which the digging rhythm stopped was not statistically different between control (23.3 ± 6.6 s) and test (14.6 ± 4.1 s) time periods (mean ± standard error of the mean [SEM], n = 10 animals; $P > 0.05$, Mann–Whitney U-test) (Figure 2Ai). A recording from a ventral opener muscle showed that the duration between NaCl stimulation and the return of the digging rhythm steadily decrease between control (C), test (T), and wash (W) time periods (Figure 2Aii).

Bathing the terminal abdominal ganglion for 10 min with 20 mM L-NAME (a NO synthase inhibitor) while repeatedly stimulating the ovipositor valves with 250 mM NaCl resulted in a significant increase in the duration for which the digging rhythm stopped, from a control value of 10.2 ± 3.1 s to a test value of 32.6 ± 5.9 s (mean ± SEM, n = 5; Student's t-test, t = −3.39, $P < 0.05$) (Figure 2Bi). A recording from a ventral opener muscle during 10 min L-NAME bath application significantly increased the duration for which the digging rhythm stopped (Figure 2Bii). After washing, the duration of cessation of the digging rhythm began to decrease and approach its control value (Figure 2Bi,Bii).

Increasing the levels of NO by bath application of the donor PAPANONOate (0.2 mM) to the terminal abdominal ganglion resulted in a statistically significant decrease in the duration for which the rhythm stopped, from a control value of 17.9 ± 6.2 s to a test value of 3.01 ± 0.83 s (mean ± SEM, n = 6; Student's t-test, t = 2.38, $P < 0.05$) (Figure 2Ci). A recording of ventral opener muscle activity showed that the digging rhythm stopped during the control period when stimulated with 250 mM NaCl but not when the donor was simultaneously applied (Figure 2Cii).

NO modulates the behavioral response to sucrose

The ovipositor valves were also repeatedly stimulated with 1 M sucrose at 3 min intervals and the effect on the cessation of the digging rhythm analyzed. There were no statistically significant differences between control (11.4 ± 2.9 s) and test durations (9.5 ± 2.5 s) (mean ± SEM, n = 5; Student's t-test,

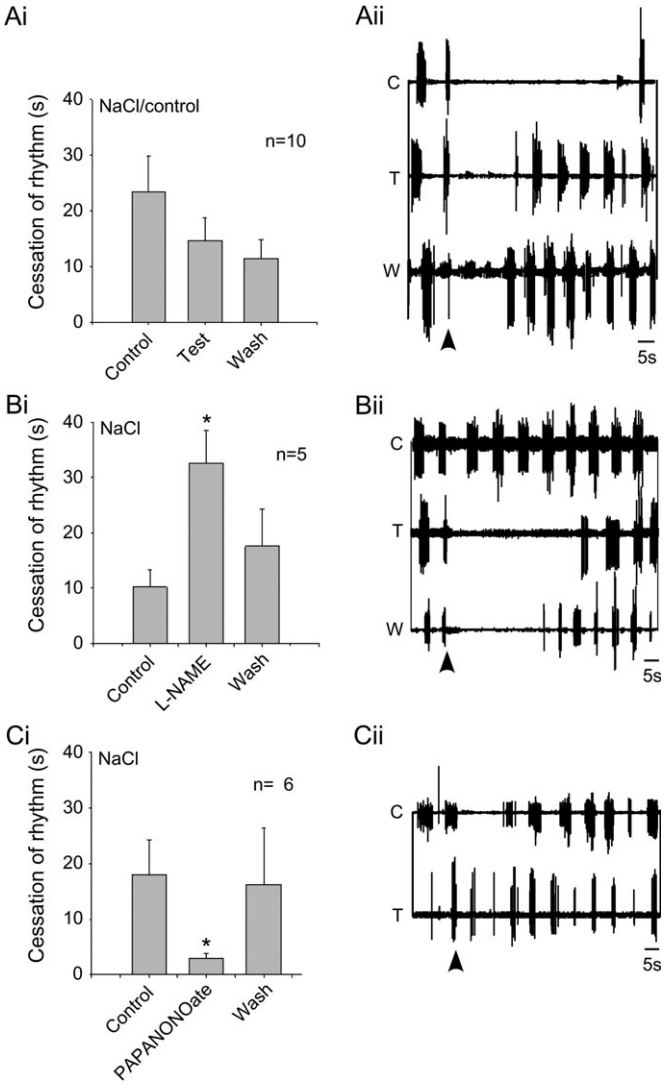

Figure 2 NO modulates the response of the digging rhythm to NaCl. **(Ai)** Repetitive stimulation of the ovipositor valves with 250 mM NaCl while simultaneously bathing the terminal abdominal ganglion in locust saline showed no statistically significant difference in cessation of the digging rhythm, between control (23.3 ± 6.5 s) and test (14. ± 4.1 s) periods (mean ± SEM $P >$ 0.05, Mann–Whitney U-test). **(Aii)** A recording of opener muscle activity showing control (C), test (T), and wash (W) periods of chemical stimulation. The duration between chemical stimulation and the return of muscle activity gradually decreased on repeated chemical stimulation. **(Bi)** Repetitive stimulation of the ovipositor valves with 250 mM NaCl while simultaneously bathing the terminal abdominal ganglion with 20 mM L-NAME resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from a mean duration of 10.2 ± 3.1 s to 32.6 ± 5.9 s (mean ± SEM, Student's t-test, t = −3.39, $P < 0.05$). The arrowhead indicates the time of onset of the chemical stimulus. **(Bii)** After a 10 min application of L-NAME, opener muscle activity stopped for longer durations when chemically stimulated compared with control. **(Ci)** Bathing the terminal abdominal ganglion with the NO donor PAPANONOate while repeatedly stimulating the valves with 250 mM NaCl significantly decreased the duration for which the digging rhythm stopped, from a control of 17.9 ± 6.2 s to 3.0 ± 0.8 s (mean ± SEM, Student's t-test, t = 2.38, $P < 0.05$). **(Cii)** Stimulating the ovipositor with 250 mM NaCl when the terminal abdominal ganglion has been bathed for 10 min with 0.2 mM PAPANONOate caused a significant decrease in the duration of the cessation of the digging rhythm compared with control.

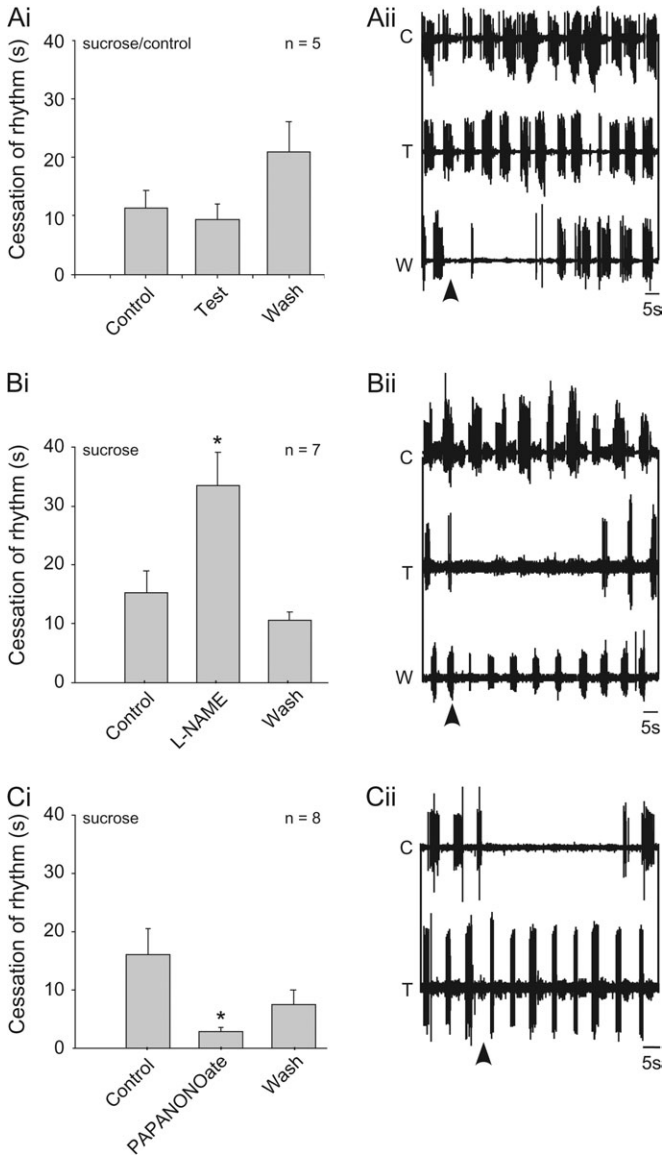

Figure 3 NO modulates the response of the digging rhythm to sucrose stimulation. **(Ai)** The ovipositor valves were repeatedly stimulated with 1 M sucrose while bathing the terminal abdominal ganglion with locust saline. No statistically significant difference between control (11.4 ± 2.9 s) and test (9.5 ± 2.6 s) values was observed for the cessation of the digging rhythm (mean ± SEM, Student's *t*-test; $t = 0.5$, $P > 0.05$). **(Aii)** A recording of opener muscle activity showed no difference between control and test values for the cessation of the digging rhythm. The wash period (20.9 ± 5.2 s), however, showed an increase in the cessation of the digging rhythm compared with the control and test period, although this difference was not statistically significant (Student's *t*-test; $t = -2.03$, $P > 0.05$). S, chemical stimulus. **(Bi)** Repetitive stimulation of the ovipositor valves with 1 M sucrose while simultaneously bathing the terminal abdominal ganglion with 20 mM L-NAME, resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from 15.2 ± 3.8 s to 33.6 ± 7 s (mean ± SEM, Student's *t*-test, $t = -2.77$, $P < 0.05$). **(Bii)** A 10 min application of L-NAME to the terminal abdominal ganglion resulted in an increase in the cessation of opener muscle activity when chemically stimulated compared with control. The arrowhead indicates the time of onset of the chemical stimulus. **(Ci)** Bathing the terminal abdominal ganglion with 0.2 mM PAPANONOate while repeatedly stimulating the ovipositor valves with 1 M sucrose significantly decreased the cessation of the digging rhythm from 16.1 ± 4.5 s to 2.8 ± 0.7 s (mean ± SEM, $P < 0.01$, Mann–Whitney *U*-test). **(Cii)** A recording of opener muscle activity showed a significant decrease in the duration of cessation of the digging rhythm when the ovipositor valves were stimulated with 1 M sucrose while the terminal abdominal ganglion had been bathed with 0.2 mM PAPANONOate compared with control.

$t = 0.5$, $P > 0.05$) (Figure 3Ai). An example of a recording from a ventral opener muscle showed no statistically significant difference in the cessation of the rhythm between control and test values. The wash period, however, showed an increase in the duration for which the rhythm stopped, although this difference was not statistically significant (Student's *t*-test, $t = -2.03$, $P > 0.05$) (Figure 3Aii).

NO levels were decreased by bath applying the NOS inhibitor L-NAME (20 mM) to the terminal abdominal ganglion, while repeatedly stimulating the ovipositor valves with 1 M sucrose. This resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from 15.2 ± 3.8 s to 33.6 ± 5.7 s (mean ± SEM, $n = 7$; Student's *t*-test, $t = -2.77$, $P < 0.05$) (Figure 3Bi). An example of a recording of ventral opener muscle activity showed that 20 mM L-NAME increased the duration for which the rhythm ceased (Figure 3Bii).

Increasing NO levels by bath application of 0.2 mM PAPANONOate to the terminal abdominal ganglion resulted in a statistically significant decrease in the duration for which the digging rhythm stopped when the ovipositor valves were repeatedly stimulated with 1 M sucrose (Figure 3Ci). The duration of cessation of the digging rhythm decreased from a control of 16.1 ± 4.5 s to 2.8 ± 0.7 s (mean ± SEM, $n = 8$; $P < 0.05$, Mann–Whitney *U*-test). An example of a recording from the ventral opener muscle showed that sucrose stimulation during PAPANONOate application had almost no effect on the rhythm (Figure 3Cii).

How are the effects of NO on behavioral responses to NaCl and sucrose mediated?

One of the main molecular targets of NO is the enzyme sGC that brings about the synthesis of cGMP which in turn can have an affect on a number of downstream targets (Bredt and Snyder 1989). The application of the membrane-permeable analogue of cGMP, 0.1 mM 8-Br-cGMP, to the terminal abdominal ganglion, while repeatedly stimulating the ovipositor valves with 250 mM NaCl, had no statistically significant effect on the duration of the cessation of the digging rhythm in response to NaCl (control, 13.4 ± 6.4 s; test, 17.4 ± 6.6 s) (mean ± SEM, $n = 5$; Student's *t*-test, $t = -0.43$, $P > 0.05$) (Figure 4Ai). An example of a recording of the ventral opener muscle shows no difference in the cessation of the digging rhythm between control and test (Figure 4Aii).

By contrast, bath application of 0.1 mM 8-Br-cGMP while simultaneously stimulating the valves with 1 M sucrose resulted in a statistically significant decrease in the duration

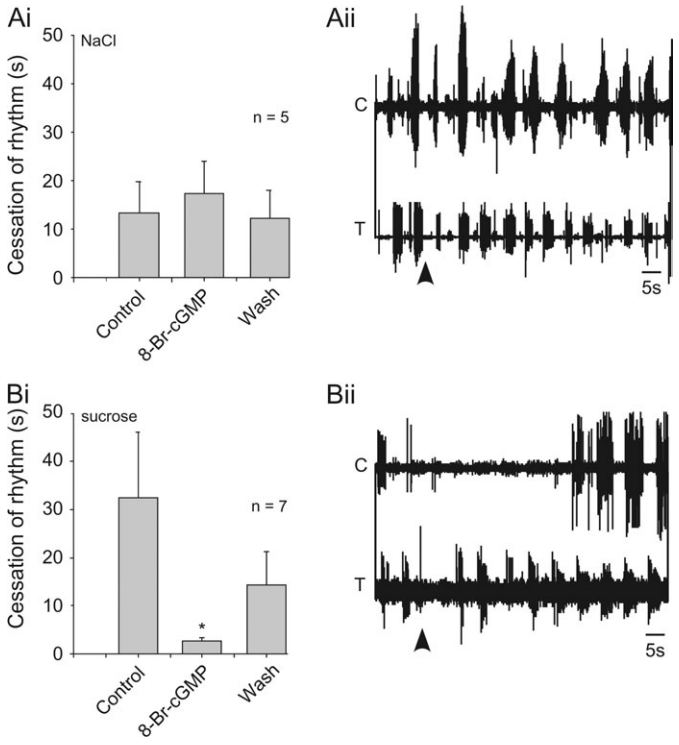

Figure 4 The effect of the cGMP analogue, 8-Br-cGMP, on the motor output of the digging rhythm. **(Ai)** Bath application of 0.1 mM 8-Br-cGMP while repeatedly stimulating the ovipositor valves with 250 mM NaCl resulted in no statistically significant change in the cessation of the digging rhythm over time, between control (C) (13.4 ± 6.4 s) and test (T) (17.4 ± 6.6 s, mean ± SEM, Student's *t*-test, $t = -0.43$, $P > 0.05$) time periods. **(Aii)** A recording of opener muscle activity showed no difference in the cessation of the digging rhythm when the ovipositor valves were stimulated with NaCl. The arrowhead indicates the time of onset of the chemical stimulus. **(Bi)** Bath application of 0.1 mM 8-Br-cGMP while repeatedly stimulating the ovipositor valves with 1 M sucrose resulted in a statistically significant decrease in the duration for which the digging rhythm stopped, from 32.5 ± 13.5 s to 2.7 ± 0.6 s (mean ± SEM, $P < 0.05$, Mann–Whitney *U*-test). **(Bii)** A recording of opener muscle activity showed a decrease in the cessation of the digging rhythm when the ovipositor valves were stimulated with sucrose in the presence of 8-Br-cGMP. The arrowhead indicates the time of onset of the chemical stimulus.

of the cessation of the digging rhythm compared with control (mean ± SEM, $n = 7$, control, 32.5 ± 13.5 s; test, 2.7 ± 0.6 s; $P < 0.05$, Mann–Whitney *U*-test) (Figure 4Bi). Similarly, a recording of opener muscle activity showed a decrease in the duration of cessation of the digging rhythm during chemical stimulation with 1 M sucrose when cGMP was applied to the terminal abdominal ganglion (Figure 4Bii).

Activation of sGC by NO is known to result in a sustained increase in intracellular levels of cGMP (Bredt and Snyder 1989). An inhibitor of sGC, 0.1 mM ODQ, was bath applied to the terminal abdominal ganglion while the ovipositor valves were repeatedly stimulated with either NaCl or sucrose. A small increase in the cessation of the digging rhythm was observed for NaCl after a 10 min bath application of 0.1 mM ODQ, from a control of 17.9 ± 3.3 s to 24.9 ± 9.6 s, although this increase was not statistically significant (mean ± SEM, $n = 5$; $P > 0.05$, Mann–Whitney *U*-test) (Figure 5A). Bath application of the terminal abdominal ganglion with 0.1 mM ODQ, however, resulted in a statistically significant increase in the cessation of the digging rhythm (control, 14.14 ± 4.15 s; test, 60.8 ± 9.3 s (mean ± SEM, $n = 5$; Student's *t*-test, $t = -4.59$, $P < 0.05$), when the ovipositor valves were simultaneously stimulated with sucrose (Figure 5B).

These results suggest that the effects of NO on the chemosensory responses to NaCl and sucrose input are mediated by different molecular mechanisms with NaCl being regulated via a cGMP-independent mechanism, whereas sucrose responses were mediated via a cGMP-dependent pathway.

To determine whether the NO effects on NaCl input could be dependent on protein kinases, the generic protein kinase inhibitor H-7 was bath applied. A 10 min bath application of 0.1 mM H-7 resulted in no significant differences in cessation of the digging rhythm, between control (16.5 ± 5.9 s) and test time periods (10.5 ± 4.7 s; mean ± SEM, $n = 5$; $P > 0.05$, Mann–Whitney *U*-test) for repeated NaCl stimulation (Figure 5C).

The generic protein kinase inhibitor H-7 was also bath applied to the terminal abdominal ganglion while the ovipositor valves were repeatedly stimulated with 1 M sucrose. Bath application of 0.1 mM H-7 resulted in a statistically significant increase in the duration of cessation of the digging rhythm to sucrose stimulation, between control (11.5 ± 2.9 s) and test (54.1 ± 10.4 s) time periods (mean ± SEM, $n = 5$; Student's *t*-test, $t = -3.93$, $P < 0.05$) (Figure 5D).

Are behavioral responses to sucrose stimulation modulated by PKG?

To establish if the responses of the digging rhythm to sucrose stimulation are modulated via a NO/cGMP-PKG signaling pathway, the PKG inhibitor KT-5823 was bath applied to the terminal abdominal ganglion and the valves repeatedly stimulated with 1 M sucrose. A 10 min bath application with 10 μM KT-5823 resulted in a statistically significant increase in the duration of cessation of the digging rhythm, from a control value of 15.1 ± 6.2 s to a test value of 66.1 ± 10.5 s (mean ± SEM, $n = 5$ animals; $P < 0.05$, Mann–Whitney *U*-test) (Figure 5Ei). A recording of the ventral opener muscle showed an increase in the cessation of the digging rhythm for test stimulations of the ovipositor valves with 1 M sucrose compared with control (Figure 5Eii).

In summary, NO was found to be involved in the modulation of responses to both chemicals used in the analysis. Increases and decreases of NO levels within the terminal abdominal ganglion both significantly influenced the cessation of the digging rhythm in response to these chemicals. Moreover, our results suggest that NO modulates the digging rhythm response to sucrose input via a NO/cGMP-PKG signaling pathway, whereas NO modulates the digging rhythm response to NaCl via a NO/cGMP-independent signaling pathway.

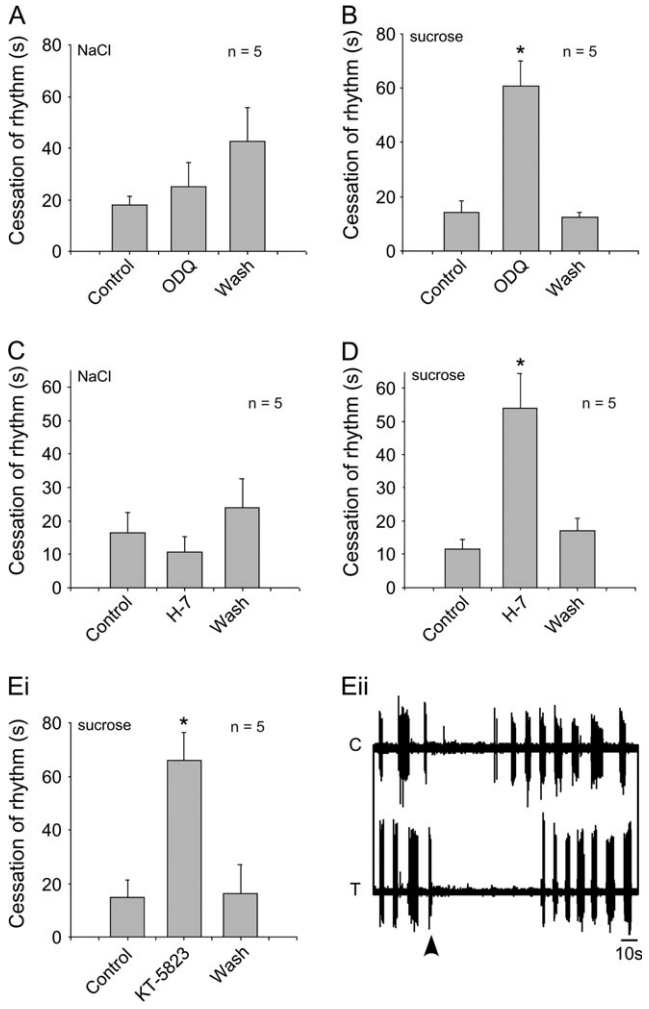

Figure 5 The effect of blocking sGC and PKG activity on the digging rhythm. **(A)** Bath application of the sGC blocker, 0.1 mM ODQ while repeatedly stimulating the ovipositor valves with 250 mM NaCl, resulted in no statistically significant difference in cessation of the digging rhythm over time (control, 17.9 ± 3.3 s, test, 24.9 ± 9.7 s; mean ± SEM, $P > 0.05$, Mann–Whitney *U*-test). **(B)** Repetitive stimulation of the ovipositor valves with 1 M sucrose while bath applying the terminal abdominal ganglion with 0.1 mM ODQ resulted in a statistically significant increase in the cessation of the digging rhythm, from a control value of 14.1 ± 4.2 s to a test value of 60.8 ± 9.3 s (mean ± SEM, Student's *t*-test, $t = -4.59$, $P < 0.01$). **(C)** A 10 min bath application of the terminal abdominal ganglion with 0.1 mM H-7, while repeatedly stimulating the ovipositor valves with 250 mM NaCl, had no statistically significant effect on the cessation of the digging rhythm (control, 16.5 ± 5.9 s; test, 10.6 ± 4.7 s; mean ± SEM, $P > 0.05$, Mann–Whitney *U*-test). **(D)** A 10 min bath application of 0.1 mM H-7 resulted in a statistically significant increase in the duration of cessation of the digging rhythm, from a control (C) value of 11.5 ± 2.9 s to a test (T) value of 54.1 ± 10.4 s (mean ± SEM, Student's *t*-test, $t = -3.93$, $P < 0.01$) during sucrose stimulation. **(Ei)** Bathing the terminal abdominal ganglion with 10 μM KT-5823 while chemically stimulating the ovipositor valves with 1 M sucrose significantly increased the cessation of the digging rhythm, from a control of 15.1 ± 6.2 s to 66.1 ± 10.5 s (mean ± SEM, $P < 0.05$ Mann–Whitney *U*-test). **(Eii)** A recording of opener muscle activity showing the effect of bath application of 10 μM KT-5823 while stimulating the ovipositor valves with 1 M sucrose. A 10 min bath application with 10 μM KT-5823 resulted in an increase in the cessation of opener muscle activity (C, control and T, test). The arrowhead indicates the time of onset of the chemical stimulus.

Does the cycle frequency of the digging rhythm preceding chemical stimulation influence the duration of inhibition of the rhythm?

Increasing and decreasing NO levels in the central nervous system regulates the oviposition digging rhythm (Newland and Yates 2007b). This creates the possibility that the presence of NO and its known effects on the rhythmic motor pattern (in this case, preceding chemical stimulation) has an effect on the cessation of the digging rhythm in response to chemical stimulation. This association was tested for both NaCl and sucrose effects on the digging rhythm during L-NAME and PAPANONOate application.

No correlation was found between cycle frequency preceding chemical stimulation and the duration of cessation of the digging rhythm for NaCl control (correlation coefficient, $r = -0.07$), 20 mM L-NAME-treated ($r = 0.11$), and 0.2 mM PAPANONOate-treated ($r = -0.27$) experiments.

Similarly, no correlation between cycle frequency preceding chemical stimulation and the cessation of the digging rhythm was found for sucrose control ($r = -0.25$), L-NAME-treated ($r = 0.12$), and PAPANONOate-treated ($r = 0.071$) experiments. These results show that the cessation of the digging rhythm was due to chemical stimulation and dependent on the presence or absence of NO and not correlated with the cycle frequency preceding chemical stimulation.

Discussion

From a number of studies it is clear that NO is emerging as a key modulator of contact chemosensory-related behaviors in invertebrates (Kemenes et al. 1986; Kobayashi et al. 2000; Murata et al. 2004; Schuppe et al. 2007), and vertebrates (Rabin 1996; Prendergast et al. 1997; Roth and Rowland 1998). Here, we show that manipulating the levels of NO around the terminal abdominal ganglion of locusts modulates the motor output of the digging rhythm when the ovipositor valves are stimulated with different chemicals. We show that the nitrergic modulation of chemosensory responses to sucrose and salt tastes were mediated via 2 separate pathways: cGMP-dependent for sucrose and cGMP-independent pathways for NaCl. The effect of this modulation for each chemical was, however, similar with NO acting to decrease their effects on the oviposition rhythm. A number of studies have analyzed the effects of NO on sensory inputs, and although opposing differential effects have been well demonstrated, a common effect via independent pathways is not. For example, Aonuma and Newland (2001) showed that NO had differential effects on different subsets of ascending interneurons in the terminal abdominal ganglion of a freshwater crayfish that received proprioceptive inputs from a chordotonal organ in the tail fan (Aonuma et al. 1999). Schuppe et al. (2007) showed that at the chemosensory neuron level NO differentially modulates the sensory responses to NaCl and sucrose. More recently, in the other chemical sense, olfaction, Wilson et al. (2007) showed that

NO also plays a role in molding the responses of olfactory interneurons in the moth, *M. sexta*, and moreover the authors believe this to occur via both sGC-dependent and independent pathways, although what those pathways are was not determined. Taken together these results suggest that NO plays a crucial role in the chemical senses, that it can have very specific effects at the level of the sensory neurons and interneurons, and that it can act via very different signaling cascades.

Our results also show that different taste modalities can be modulated via NO to achieve the same effect, implying considerable independence in the responses to NaCl and sucrose. How this extends to other qualities of taste is not clear although preliminary studies by Yates (2006) suggest that NO is also involved in regulating responses to amino acids and bitter tastants. Recent nutritional studies highlight that protein intake is regulated independently from carbohydrate (Simpson and Raubenheimer 2005), and our results suggest that the specificity of NO and the multiple signaling cascades it acts through means that elevated NO levels caused by changes in the hemolymph (Schuppe et al. 2007) could have a number of very specific and independent effects on the responses to different taste qualities depending on the taste-related behavior.

Molecular targets of NO during the modulation of contact chemosensory input

Studies on the blowfly, *Phormia regina* have shown the involvement of NO in the modulation of the receptor cell response to sucrose (Murata et al. 2004; Nakamura et al. 2005). Although Nakamura et al. (2005) suggest that NO acts via a sGC/cGMP signaling pathway to modulate the taste receptor response to sucrose stimulation, they provide no experimental evidence to support this. The results of our study show, for the first time, that the responses to sucrose stimulation in insects are clearly modulated by a NO/cGMP signaling pathway and in the case of locusts, at least, via the action of PKG. There is also evidence to suggest a potential role of cGMP in the detection of sucrose in the oral cavity of vertebrates. In the circumvallate taste bud cells of rats, a rapid and transient increase in intracellular levels of cGMP is observed in response to sucrose stimulation (Krizhanovsky et al. 2000).

We found that bathing the terminal abdominal ganglion of the locust with the membrane-permeable analogue of cGMP, 8-Br-cGMP, although stimulating the ovipositor valves with sucrose decreased the duration for which the digging rhythm stopped. This suggests that increases in NO (and hence cGMP) levels in the terminal abdominal ganglion may decrease the aversive chemosensory input onto the rhythm (decrease duration of cessation of the digging rhythm) and function to maintain oviposition digging, for example, when a suitable oviposition substrate is encountered. Indeed, Schuppe et al. (2007) have shown that the presence of NO around the sensory neurons of basiconic sensilla of locusts markedly affects the sensory neurons response to NaCl. When sensory neurons were bathed with PAPANONOate, the frequency of action potentials evoked when stimulated with NaCl significantly decreased. As the frequency of action potentials increased with increasing concentration of NaCl and, therefore, the aversiveness of NaCl increased (Newland 1998; Rogers and Newland 2002), this suggests that the presence of NO in either the periphery or the terminal abdominal ganglion decreases the aversive input of a chemical stimulus to the central nervous system. Moreover, Schuppe et al. (2007) also showed that increased levels of cGMP in the sensory neurons had no effect on the frequency of action potentials when the sensory neurons were stimulated with NaCl, providing further support for behavioral responses to NaCl being modulated via an NO/cGMP-independent pathway as we have demonstrated here.

As behavioral responses to NaCl rely on an initial depolarization by Na^+ ions in the dendrites of sensory neurons located within the taste receptors, a potential target for NO in the modulation of behavioral responses to NaCl contact chemosensory input is sodium channels (Hammarström and Gage 1999). For example, in rat hippocampal neurons, the presence of exogenous NO affects the opening of sodium channels. This suggests that in contrast to the modulation of the digging rhythm by sucrose stimulation, NO may modulate the digging rhythm response to NaCl stimulation by having a direct effect on sodium channels of individual neurons at the level of the terminal abdominal ganglion, and hence modulating the motor output of the CPG.

It is thus clear that the effects of NO on responses to chemical stimulation may be quite different depending on the stimulus site and the behavioral response produced by stimulation of receptors on that site. Studies on the crayfish (Ott et al. 2007) have shown that there is considerable variability in NADPH staining in different segmental ganglia, arguing against a generic role for NO in modulating sensory inputs and instead suggesting a high degree of specificity for NO. Indeed, in locusts, this is supported by the finding that NO has a similar effect on responses to both NaCl and sucrose, albeit via very different signaling pathways, yet can have differential effects on NaCl and sucrose inputs underpinning leg avoidance behavior (Murata et al. 2004; Schuppe et al. 2007).

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