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Nitrergic modulation of an oviposition digging rhythm in locusts

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Summary

In locusts, a central pattern generator underlies the rhythmic movements of the ovipositor valves that serve to drive the abdomen into damp soil in order to lay eggs. We have investigated the role of nitric oxide (NO) in the control of this oviposition digging rhythm. NO increases the frequency of the rhythm by acting *via* sGC to elevate cGMP, which in turn acts *via* PKG. Increasing exogenous NO levels using the NO donors SNAP and PAPANONOate increased the cycle frequency of the fictive digging rhythm, as did increasing endogenous NO by bath application of the substrate for NOS, L-arginine. On the other hand, application of the NO scavenger PTIO decreased the cycle frequency, indicating that NO must normally exert a continuous and dynamic role on the central pattern generator underlying the oviposition rhythm. Inhibiting the main molecular target of NO, soluble guanylate cyclase, with ODQ reduced the cycle frequency of the rhythm, suggesting that NO mediated its effects *via* sGC and cyclic GMP. Further evidence for this was produced by bath application of 8-Br-cGMP, which increased the frequency of the rhythm. Bath application of the generic protein kinase inhibitor and a selective PKG inhibitor, H-7 and KT-5823, respectively, reduced the frequency of the rhythm, suggesting that PKG acted as a target for cGMP. Thus, we conclude that NO plays a key role in regulating the frequency of the central pattern generator controlling rhythmic egg-laying movements in locusts by acting *via* sGC/cGMP–PKG.

Key words: oviposition, central pattern generator, nitric oxide, egg laying, locust, *Schistocerca gregaria*.

Introduction

The behaviour of animals needs to be shaped to suit the demands posed by their fluctuating internal and external environments. Previous studies, in different model organisms, have shown that central pattern generators (CPGs) are not fixed, hard-wired neural circuits but instead are flexible multifunctional systems capable of producing different motor outputs (Harris-Warrick and Marder, 1991). In insects, CPGs underlie a number of rhythmic movements including egg laying, or oviposition, in locusts (Woodrow, 1965), in which specialized structures located at the distal-most segment of the abdomen, called the ovipositor valves, function to dig into the substrate to lay eggs. Locusts lay their eggs in damp sand or soil (Popov, 1980) by producing cyclical opening and closing movements of the ovipositor valves that are accompanied by protractive and retractive movements of the abdomen, thereby driving it into the substrate (Vincent and Wood, 1972). These movements extend the abdomen by up to four times its original length (Vincent and Wood, 1972; Jorgensen and Rice, 1983; Rose et al., 2001) to lay eggs at depths that protect them from desiccation, parasitization and predation (Jorgensen and Rice, 1983). The movements are generated by a CPG within the genital ganglia that is normally under descending inhibition, and transecting the connectives, or isolating the abdomen, results in release of inhibition and generates a fictive digging rhythm (Thompson, 1986a; Thompson, 1986b). The rhythmic movements of the ovipositor valves are produced by direct muscular attachments to the ovipositor itself, or indirectly by rhythmic movements of the entire abdomen (Facciponte and Lange, 1996; Vilhelmsen et al., 2001). Both types of rhythm serve to manoeuvre the abdomen into position to lay eggs and both are variable and need to be altered to suit the changing demands of the animal and the substrate, such as the density of the substrate or its chemical composition.

In invertebrates, the most intensively studied examples of CPGs include those controlling locomotion in stick insects (Brunn, 1998), flight in locusts (Wilson, 1961), the feeding movements of molluscs (Elliott and Benjamin, 1985; Staras et al., 1998) and control of the stomatogastric rhythms of crustaceans (Simmers et al., 1995), and in many of these behaviours the ubiquitous free radical nitric oxide (NO) serves as a key modulator of the rhythm (e.g. Scholz et al., 2001). NO has been well established as a signalling molecule in the central nervous system (Garthwaite et al., 1988) and, at low concentrations, acts as a neuromodulator by diffusing from its site of synthesis in three dimensions (Wood and Garthwaite, 1994) to potential targets throughout an animal. NO is synthesized from its substrate L-arginine in a Ca^{2+}/calmodulin-dependent process by the enzyme nitric oxide synthase (NOS), in a reaction requiring oxygen and

nicotinamide adenine dinucleotide phosphate (NADPH) (Moncada et al., 1991). As a result of NO being identified as an important signalling molecule in the nervous system, many studies have focused on the molecular targets of NO. NO can act on a range of molecular targets to produce physiological effects including a direct action on cGMP-regulated phosphodiesterases (Takemoto et al., 1993), sodium and potassium ion channels (Hammarström and Gage, 1999; Hampl et al., 1995) and the enzyme soluble guanylate cyclase (sGC) (Bredt and Snyder, 1989). The subsequent targets of cGMP include cyclic nucleotide gated ion channels (Ahmad et al., 1994), cGMP-dependent protein kinases (Clementi et al., 1995) and cGMP-regulated cyclic nucleotide phosphodiesterases (Lincoln and Cornwell, 1993). The most common target of NO is the enzyme sGC, which results in the production of cGMP. cGMP may in turn act upon cGMP-dependent protein kinase (PKG), which is thought to phosphorylate downstream target proteins or affect the opening and closing of potassium channels that regulate neuronal responses (Bredt and Snyder, 1989).

NOS-containing neurones have been revealed throughout the central nervous system in various invertebrates by using NADPHd histochemistry. Neurones that contain NOS have been identified in the central nervous system of molluscs (Moroz et al., 1992), insects (Ott and Burrows, 1999; Ott et al., 2001) and crustaceans (Schuppe et al., 2001). In locusts, stained clusters of neurones containing NOS are localized in varying densities in all 11 ganglia located along the entire length of the ventral nerve cord (Ott et al., 2001). As a consequence, NO has been hypothesized to play a crucial role in the modulation of sensory input in locusts (Elphick et al., 1996; Ott et al., 2001) but may also play a key role in modifying rhythmic activity, such as that produced during egg laying.

Recent studies have implicated NO in modulating contact chemoreception and feeding behaviours (Schuppe et al., 2007). The neuronal networks that underlie the feeding movements in locusts are located in the sub-oesophageal ganglion (Schachtner and Bräunig, 1993). Bath application of the NO donor, sodium nitroprusside (SNP), to the sub-oesophageal ganglion initiates feeding movements (Rast, 2001), an effect that can be reversed by bath application of the sGC inhibitor, ODQ. These results suggest that NO acts directly on its target sGC to initiate feeding. Moreover, studies have also shown that NO can activate the CPG that underlies feeding in molluscs (Elphick et al., 1995). This raises the possibility that NO may have a more general role to play in regulating the activity of CPGs, and this study provides a detailed analysis of the role of NO in the modulation of the oviposition rhythm of locusts and reveals a pathway through which it mediates its effects.

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, aged from 9 to approximately 14 days after final moult, were used in all experiments. All adults were examined prior to use to ensure that the ovipositor valves and surrounding cuticle were intact and undamaged.

Preparation and physiological recording

Initiation of the digging rhythm was performed by releasing the CPG from descending inhibition by decapitating locusts with a twisting motion and sharply pulling the head to remove the gut. This procedure reliably produced rhythmic movements of the ovipositor valves similar to those seen in intact female locusts that have been defined as 'fictive digging movements' by Thompson (Thompson, 1986a).

The abdomen was then isolated from the thorax and pinned laterally on a Plasticine™ stage with the ovipositor valves overhanging the edge of the stage. This allowed chemicals to drain away from the valves and served to prevent constant chemosensory input. The anterior end of the abdomen was constantly perfused with fresh locust saline throughout an experiment.

Recordings were obtained from the dorsal and ventral opener muscles but particularly from the ventral opener muscles due to their large size and accessibility. Pairs of 63 μm copper wire, insulated except for their tips, were pushed through small holes in the cuticle into the muscles. The wires were then secured in place using cyanoacrylate glue. After each experiment, an animal was dissected and the locations of the wires visually confirmed. Signals from the electrodes were amplified with an AC pre-amplifier and displayed on a Tektronix TDS 210 oscilloscope (Texas, USA), digitised using a Cambridge Electronic Design 1401 interface (CED, Cambridge, UK) and displayed and analysed using Spike 2 v.4.0 software (CED).

Drug application

To apply drugs to the terminal abdominal ganglion, a small window of cuticle was removed from the ventral surface of the sub-genital plate. All pharmacological agents were obtained from Sigma Aldrich Chemical Co., Ltd (Poole, Dorset, UK) and Tocris Cookson, Ltd (Bristol, UK), including L-arginine (L-arg), *N*-nitro-L-arginine methyl ester (L-NAME), *N*-nitro-D-arginine methyl ester (D-NAME), *S*-nitroso-*N*-acetyl-penicillamine (SNAP), *N*-acetyl-penicillamine (NAP), 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANONOate), 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) and (9*S*, 10*R*, 12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9, 12-epoxy-1*H*-di indolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT-5823). 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).

With the exception of ODQ and KT-5823, each chemical was maintained in the dark and only dissolved in normal locust saline (Parker and Newland, 1995) to the required concentration immediately prior (3 min) to bath application, with the exception of de-gassed SNAP, which was maintained in the dark at room temperature for approximately 24 h prior to use. Both ODQ and KT-5823 were first dissolved in 100% ethanol and then serially diluted in locust saline to a final concentration

of 0.1% ethanol. The exposed terminal abdominal ganglion was constantly perfused with fresh locust saline using a 502S Watson-Marlow microtube pump (Falmouth, Cornwall, UK). Ganglia were exposed to a drug for 10 min and then washed with locust saline for 30 min. Each animal was tested only once per drug application.

Results

Activity patterns of the muscles involved in the digging rhythm

The locust oviposition digging rhythm has been shown to be tri-phasic (Fig. 1A) (Thompson, 1986a), during which the ovipositor valves protract (Fig. 1Ai,ii) and then open (Fig. 1Aiii,iv), followed by a simultaneous closing and retraction movement (Fig. 1Aiv–vi). The musculature that produces the movements of the valves consists of four groups of muscles – the openers, closers, protractors and retractors – that insert on both ventral and dorsal regions of the abdomen. All muscles are situated between the posterior-most midline of tergite 6 and sternite 6 and extend to tergite 11 and sternite 8, respectively, where they attach to various regions of their respective ovipositor valves (Albrecht, 1953). The activation patterns of these muscles during oviposition have been well described (Thompson, 1986a; Thompson, 1986b). During digging, the four dorsal and ventral opener muscles are all activated in phase (Fig. 1B). Recordings from the ventral closer and retractor muscles show that their activity occurs directly after opener muscle activity ceases (Thompson, 1986a), indicating that the closing and retraction phases of the movements of the ovipositor valves occur simultaneously (Fig. 1C,D).

Effects of increasing endogenous and exogenous NO levels on the digging rhythm

To determine if NO acts to modulate the motor output of the oviposition digging rhythm, the endogenous and exogenous NO levels were manipulated within the terminal abdominal ganglion by bath applying a variety of pharmacological agents. Their effects on the motor output of the CPG that underlies the oviposition digging movements were then analysed.

To elevate endogenous levels of NO, the substrate for its synthesis, L-arginine, was bath applied at a concentration of

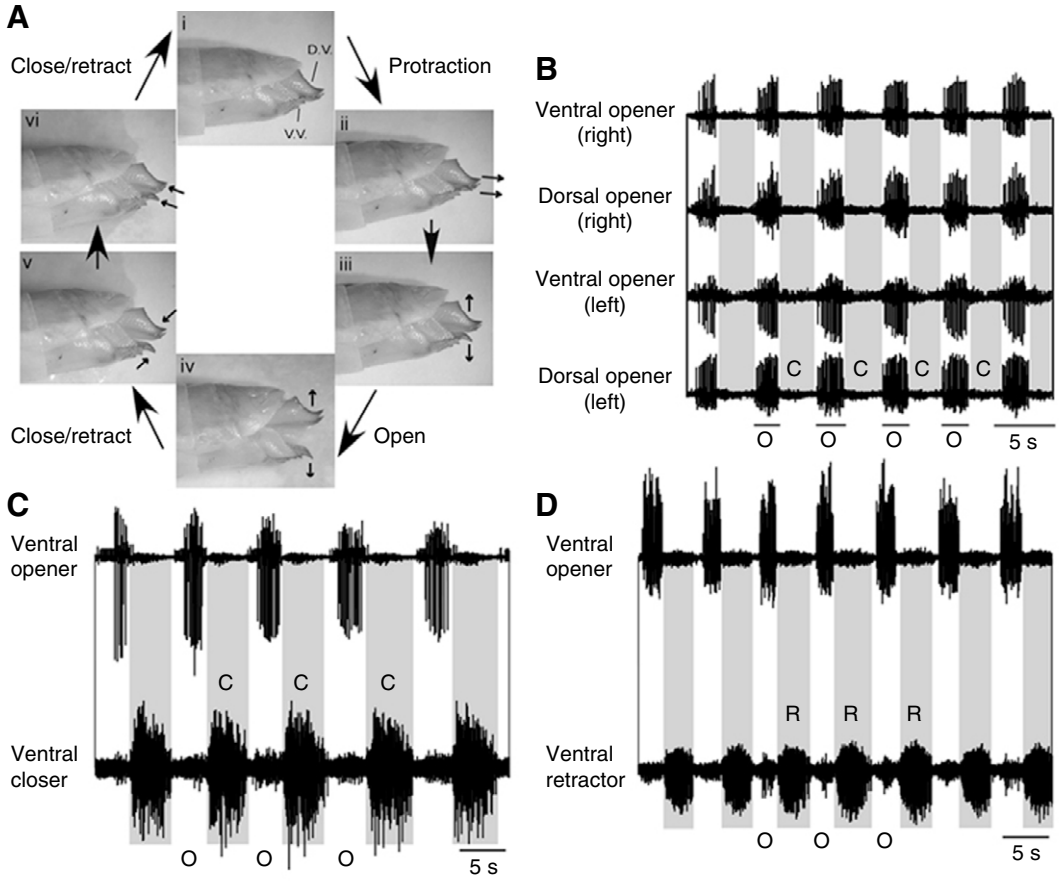


Fig. 1. The oviposition digging rhythm. (A) Photographs showing the cyclical movements of the locust ovipositor valves. (i) The dorsal valves (D.V.) and ventral valves (V.V.) in their closed positions. (ii) The protractive movements of the ovipositor push the valves out of the abdomen. (iii,iv) The opening movements of the ovipositor valves can be seen (iv=fully open). (v,vi) The simultaneous closing and retraction movements of the valves, returning the valves back to their original closed position. Small arrows indicate the direction of movement of the valves. (B) A recording from the left and right dorsal and ventral opener muscles. All four muscles were active and in phase during the opening (O) movement of the digging rhythm. 'C' represents the closed phase of the digging rhythm. (C) A recording from a ventral opener and ventral closer muscle. The ventral closer muscle bursts (C) directly after the ventral opener muscle (O) in order to pull the valves shut. (D) A recording from the ventral opener and ventral retractor muscle. The ventral retractor muscle bursts (R) directly after the ventral opener muscle.

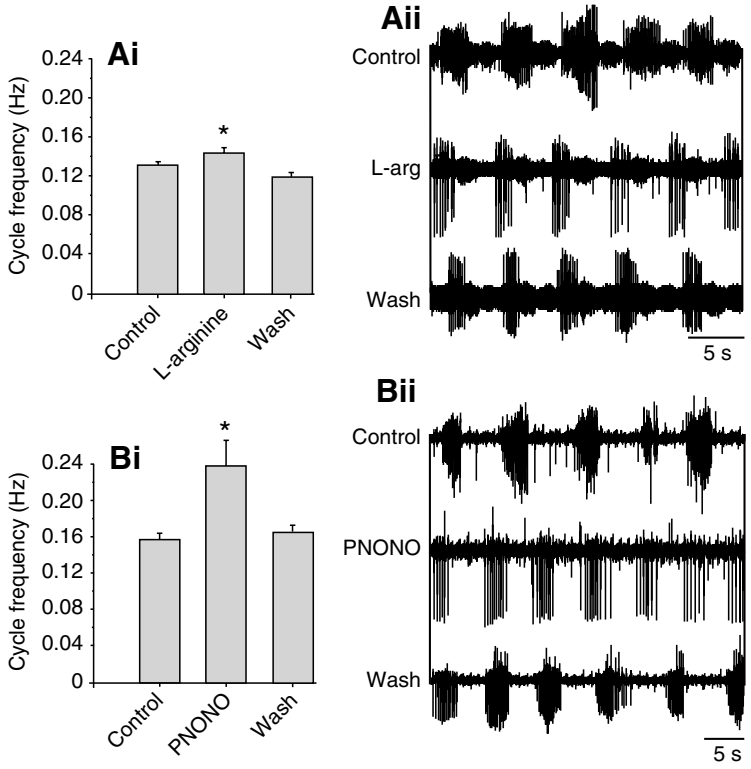
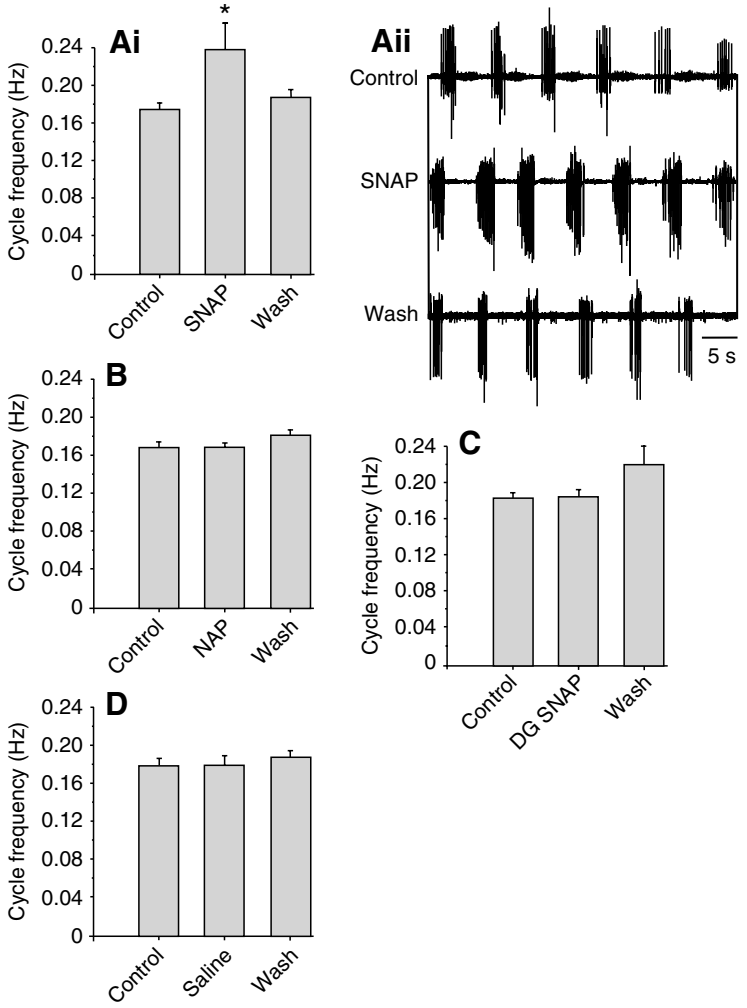


Fig. 2. The effect of NO on the cycle frequency of the digging rhythm. (Ai) The bath application of 20 mmol l^{-1} L-arginine caused a statistically significant increase in the oviposition cycle frequency (t=2.44, *P<0.05, mean ± s.e.m. from eight animals). (Aii) An example showing the effect of a 10 min bath application of 20 mmol l^{-1} L-arginine on the ovipositor valve opener muscles. The number of cycles of muscle activity increased from a control value of 5 to 6 after L-arginine application within the same time period. After a 30 min wash with locust saline, the number of cycles of opener muscle activity returned to the control value. (Bi) The effect of the NO donor PAPANONOate (PNONO) on the cycle frequency. A 10 min bath application of 0.2 mmol l^{-1} PAPANONOate caused a statistically significant increase in the cycle frequency (t=–2.82, P<0.01) (*=P<0.05). Mean ± s.e.m. of five animals. (Bii) An example showing a 10 min bath application of 0.2 mmol l^{-1} PAPANONOate resulted in a significant increase in the number of cycles of muscle activity within the same time period. A 30 min wash with locust saline resulted in the digging rhythm partially returning to its original cycle frequency.



20 mmol l^{-1} to the terminal abdominal ganglion (Fig. 2Ai,ii). This produced a significant increase in the cycle frequency of the oviposition rhythm, as determined from myogram recordings from the opener muscles, from 0.13±0.001 Hz to 0.14±0.001 Hz (mean ± s.e.m.; N=8 animals; Student's t-test, t=2.44, P<0.05). Following a 30 min wash in fresh saline, the cycle frequency returned to control levels (0.12±0.0004 Hz) and was not significantly different from initial control levels (Fig. 2Ai,ii).

Exogenous levels of NO were increased within the terminal abdominal ganglion by bath application of the NO donors PAPANONOate and SNAP, both at a

Fig. 3. The effects of the NO donor SNAP on oviposition digging. (Ai) A 10 min bath application of SNAP resulted in a statistically significant increase in the cycle frequency of the digging rhythm (t=2.5, *P<0.05, mean ± s.e.m. of five animals). (Aii) An example showing the effect of increased exogenous levels of NO on the digging rhythm. A 10 min bath application of 0.2 mmol l^{-1} SNAP resulted in an increase in the number of cycles of muscle activity while a 30 min wash with locust saline resulted in the number of cycles of muscle activity returning to control. (B) The effect of bath application of the inactive isomer of SNAP, NAP, on the cycle frequency. A 10 min bath application of 0.2 mmol l^{-1} NAP had no effect on the cycle frequency (t=0.14, P>0.05, N=4). (C) A 10 min bath application of 0.2 mmol l^{-1} de-gassed SNAP (DG SNAP) had no significant effect on cycle frequency (t=0.17, P>0.05, mean ± s.e.m. from four animals). (D) Bath application of normal locust saline similarly resulted in no significant effect on cycle frequency (t=–1.81, P>0.05, mean ± s.e.m. from four animals).

concentration of 0.2 mmol l^{-1}. An increase in NO by bath application of 0.2 mmol l^{-1} PAPANONOate (Fig. 2) resulted in a statistically significant increase in the cycle frequency of the rhythm, from a control value of 0.16±0.007 Hz to 0.24±0.03 Hz (N=5 animals; Student's t-test, t=–2.82, P<0.05) (Fig. 2Bi,ii). A 10 min wash with locust saline resulted in the cycle frequency of the rhythm returning close to its control value (0.17±0.007 Hz).

Similarly, an increase in the level of exogenous NO using the donor SNAP produced a significant increase in the cycle frequency of the digging rhythm, from a control value of 0.17±0.001 Hz to 0.21±0.012 Hz (N=5 animals; Student's t-test, t=2.5, P<0.05) (Fig. 3Ai,ii). A 30 min wash with normal locust saline again resulted in a recovery of the digging rhythm to a cycle frequency (0.19±0.001 Hz) that was not significantly different from the control. As additional controls, the inactive isomer of SNAP, NAP, was bath applied for 10 min and had no significant effect on the oviposition rhythm (N=4; Student's t-test, t=0.14, P>0.05) (Fig. 3B), nor did degassed SNAP (Fig. 3C) or saline (Fig. 3D).

Effects of reducing NO levels on the oviposition digging rhythm

To decrease the availability of endogenous NO, a NOS inhibitor, L-NAME, was bath applied (Fig. 4). 20 mmol l^{-1} L-NAME produced a significant decrease in the cycle frequency of opener muscle activity from 0.15±0.0006 Hz to 0.13±0.0006 Hz (N=8 animals; Student's t-test, t=2.08, P<0.05). Following a 30 min wash with normal locust saline, the digging rhythm showed a partial recovery to its original control value (0.15±0.012 Hz), which was not significantly different from the initial control (Fig. 4Ai,ii).

To confirm the specificity of the action of L-NAME, D-NAME was also applied. A 10 min bath application of the isomer D-NAME (20 mmol l^{-1}) had no effect upon the cycle frequency of the digging rhythm (control=0.15±0.0007 Hz, test= 0.15±0.0008 Hz; N=4 animals; Student's t-test, t=0.12, P>0.05) (Fig. 4Bi,ii).

Decreasing endogenous NO levels were also achieved by bath applying the NO scavenger PTIO at a concentration of 0.5 mmol l^{-1} (Fig. 5A,B). This resulted in a significant decrease in the cycle frequency of the digging rhythm from a control value of 0.17±0.001 Hz to 0.14±0.001 Hz (N=11 animals; Student's t-test, t=–2.04, P<0.05). A 30 min wash with normal locust saline resulted in a return of the cycle frequency to a level that was not significantly different from its original control value (0.18±0.001 Hz) (Fig. 5A,B).

The effect of NO on the digging rhythm is mediated via a sGC/cGMP signalling pathway

sGC acts as one molecular target of NO (Bredt and Snyder, 1989). To determine whether NO acts to modulate the oviposition digging rhythm *via* the sGC/cGMP signalling pathway, a specific inhibitor of sGC, ODQ, was bath applied to the terminal abdominal ganglion (Fig. 6Ai,ii). A 10 min bath application of 0.1 mmol l^{-1} ODQ resulted in a significant decrease in the cycle frequency of the digging rhythm from a control value of 0.17±0.001 Hz to a value of 0.14±0.001 Hz (N=5 animals; Student's t-test, t=2.85, P<0.05). A 30 min wash with normal locust saline resulted in a return of the digging rhythm to a frequency (0.17±0.013 Hz) that was not significantly different from the original control value (Fig. 6Ai,ii).

To demonstrate that the L-arginine-dependent effect on cycle frequency was due to increased NO synthesis acting *via* sGC, both L-arginine and ODQ were simultaneously bath applied to the terminal abdominal ganglion. This had the effect of elevating endogenous NO levels whilst simultaneously inhibiting sGC. Bath application of L-arginine alone significantly increased the frequency of the digging rhythm (see

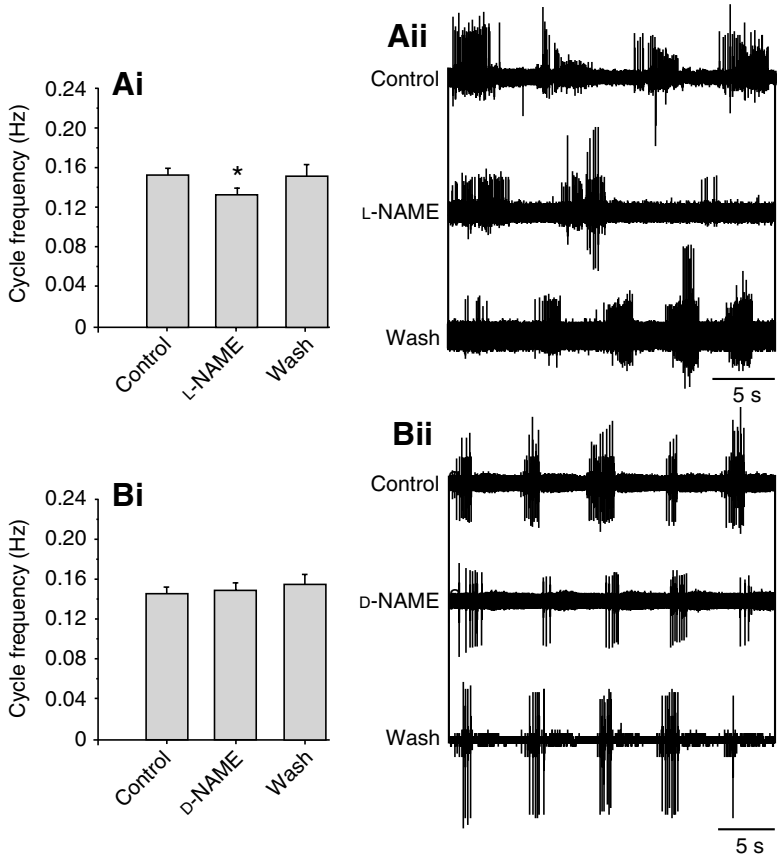


Fig. 4. The effect of inhibiting NO synthesis by bath application of the NOS inhibitor L-NAME. (Ai) A 10 min bath application of 20 mmol l^{-1} L-NAME caused a statistically significant decrease in the cycle frequency of the digging rhythm (t=2.08, *P<0.05, mean ± s.e.m. from eight animals). (Aii) The effects of L-NAME were reversed by a 30 min wash with locust saline. (Bi) A 10 min bath application of the inactive isomer 20 mmol l^{-1} D-NAME caused no statistically significant change in cycle frequency (t=0.12, P>0.05, mean ± s.e.m. from four animals). (Bii) An example showing that a 10 min bath application of 20 mmol l^{-1} D-NAME resulted in no significant decrease in the number of cycles between test and control values.

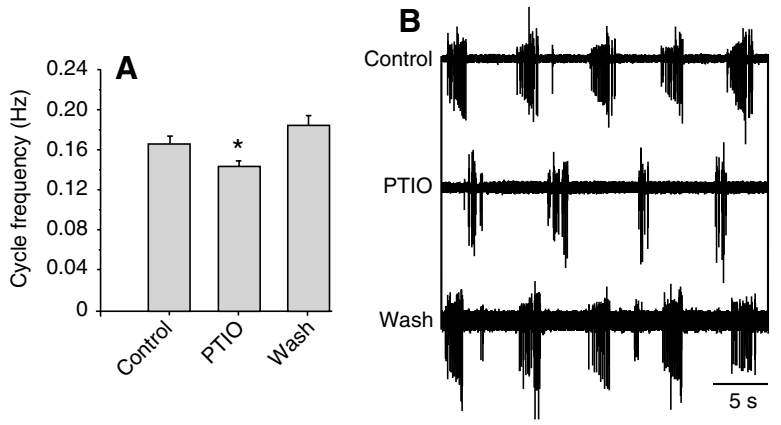


Fig. 5. The effect of removing endogenous NO by bath application of the NO scavenger PTIO. (Ai) A 10 min bath application of 0.5 mmol l^{-1} PTIO caused a statistically significant decrease in cycle frequency (t=2.04, *P<0.05, N=11). A 30 min wash with locust saline resulted in the digging rhythm returning to its original cycle frequency. (Aii) An example showing that a 10 min bath application of 0.5 mmol l^{-1} PTIO resulted in a decrease in the number of cycles within the same time period.

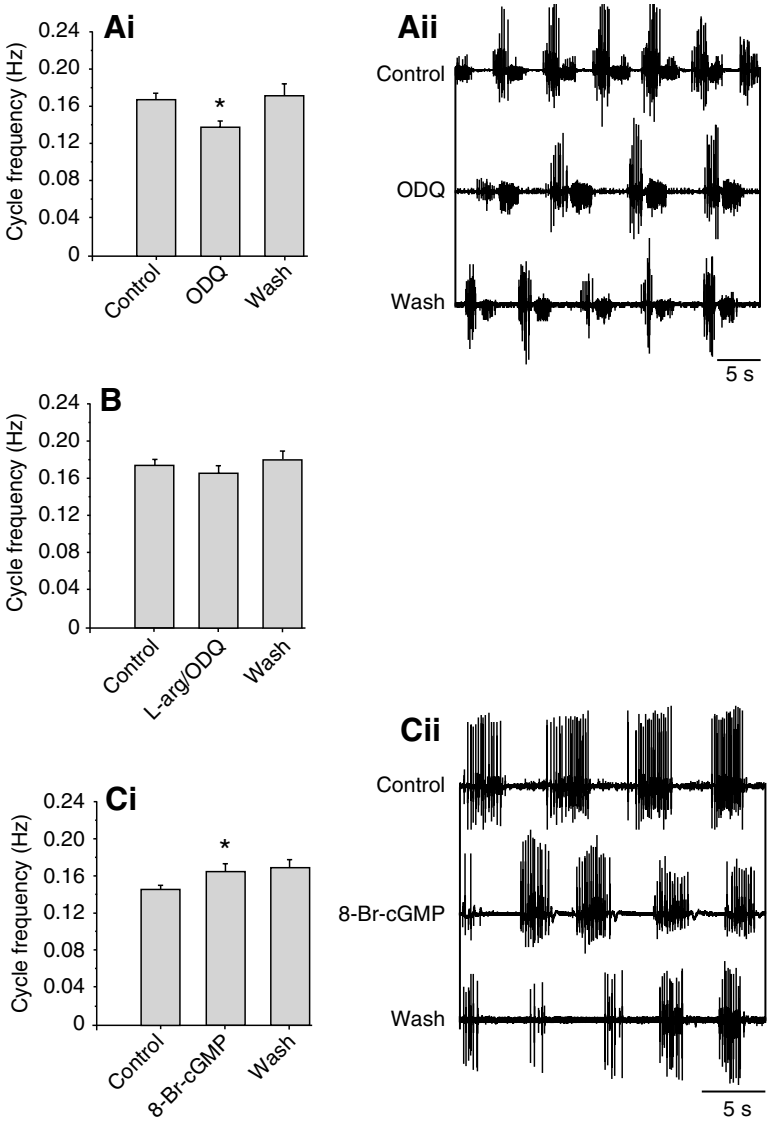


Fig. 6. The effects of the sGC inhibitor, ODQ, and the cGMP analogue, 8-Br-cGMP, on the digging rhythm. (Ai) A 10 min bath application of 0.1 mmol l^{-1} ODQ resulted in a significant decrease in cycle frequency (t=2.85, *P<0.05, mean ± s.e.m. from five animals). (Aii) An example showing that a 10 min bath application of ODQ resulted in a decrease in the cycle number that was reversed by a 30 min wash with locust saline. (B) The simultaneous bath application of L-arginine and ODQ resulted in no statistically significant difference between control and test values (t=–1.34, P>0.05, mean ± s.e.m. from five animals). (Ci) A 10 min bath application of 0.1 mmol l^{-1} 8-Br-cGMP resulted in a significant increase in cycle frequency of the digging rhythm (t=2.22, *P<0.05, mean ± s.e.m. from six animals). A 30 min wash with locust saline did not result in the cycle frequency returning back to its original value. (Cii) An example showing that bath application of 8-Br-cGMP resulted in an increase in the number of cycles of muscle activity of the digging rhythm, from a control value of four cycles to a test value of five within the same time period.

Fig. 2Ai). However, simultaneous bath application of L-arginine and ODQ had no significant effect on cycle frequency (N=5 animals; Student's t-test, t=–1.34, P>0.05) (Fig. 6B), suggesting that L-arginine acts to increase NO levels that in turn then act to increase cycle frequency by activating sGC.

Cyclic GMP levels were also elevated by bath applying a membrane-permeable analogue of cGMP, 8-Br-cGMP, at a concentration of 0.1 mmol l^{-1}. Increasing the level of cGMP resulted in a significant increase in the cycle frequency of the digging rhythm, from a control value of 0.15±0.0004 Hz to 0.17±0.001 Hz (N=6 animals; Student's t-test, t=2.22, P<0.05). A 30 min wash, however, failed to reverse the effects of 8-Br-cGMP within the wash time window (0.17±0.001 Hz) (Fig. 6Ci,ii).

Does cGMP act via a protein kinase signalling pathway?

To determine whether NO acts to modulate the digging rhythm by acting on protein kinases, the generic protein kinase inhibitor H-7 was bath applied to the terminal abdominal ganglion (Fig. 7Ai,ii). Bath application of 0.1 mmol l^{-1} H-7 resulted in a significant decrease in the cycle frequency of the valve opener muscles from a control value of 0.19±0.012 Hz to 0.09±0.014 Hz (N=5 animals; Student's t-test, t=4.93, P<0.05). A 30 min wash with locust saline resulted in a partial recovery of the cycle frequency to 0.16±0.014 Hz, which was not significantly different from the original control value (Fig. 7Ai,ii).

To determine *via* which protein kinase NO was mediating its effects, the selective PKG inhibitor KT-5823 was bath applied (Fig. 7B). Bath application of 10 μmol l^{-1} KT-5823 resulted in a significant decrease in the cycle frequency of the digging rhythm from a control value of 0.15±0.005 Hz to

0.12±0.01 Hz (N=5 animals; Student's t-test, t=2.8, P<0.01). A 30 min wash with locust saline resulted in a recovery of the digging rhythm that was not significantly different from the original control value (Fig. 7Bi,ii).

Discussion

Our results show that increasing the availability of NO in the terminal abdominal ganglion of the locust significantly increases the cycle frequency of the digging rhythm. Conversely, decreasing the availability of NO results in a significant decrease in the cycle frequency. Inhibiting the enzyme sGC resulted in a significant decrease in the cycle frequency of the digging rhythm. Inhibiting a known target of cGMP, PKG, in the terminal abdominal ganglion also resulted in a significant decrease in the cycle frequency of the oviposition digging rhythm. Thus, our study suggests that NO modulates the oviposition rhythm *via* a sGC/cGMP–PKG signalling pathway.

Regulation of the oviposition rhythm

Many factors may influence the oviposition rhythm in order that effective behaviour is produced. For example, the physical and chemical composition of the substrate is highly variable and locusts must adapt the oviposition rhythm to suit these needs (Popov, 1980; Woodrow, 1965). Belanger and Orchard suggested that the peptide proctolin was necessary for normal oviposition digging (Belanger and Orchard, 1993), where it is thought to play a role in muscle tension and in maintaining internal pressure (Rose et al., 2001). Clearly, in more compact substrates, the motor pattern would have to be modified in order to allow animals to excavate successfully, and such modulation by proctolin can aid in this. The biogenic amines serotonin and octopamine are known to have effects on the related movements of the oviduct, where serotonin increases the frequency and amplitude of the contractions required to expel eggs, and in maintaining the basal tension of the oviduct (Lange, 2004). Kalogianni and Theophilidis showed that the activation of octopaminergic dorsal unpaired median neurones in the seventh abdominal ganglion of two orthopteran species reduced the firing rate of the oviduct motor neurones (Kalogianni and Theophilidis, 1993). It is not surprising that, given the presence of NOS in the central nervous system of locusts (Ott and Burrows, 1999; Ott et al., 2001), and its known effects on other rhythms (Rast, 2001), it also appears to have a continuous and dynamic control over the digging rhythm.

The digging rhythm is also regulated peripherally by mechano- and chemosensory input from receptors on the ovipositor valves (Kalogianni, 1996; Tousson and Hustert, 2000). Schuppe et al. have recently shown that NO also plays a key role in regulating the chemosensory responses of locusts (Schuppe et al., 2007), raising the possibility that NO may have an influence at the peripheral level in the afferent input onto the oviposition CPG; however, this remains to be investigated in the future.

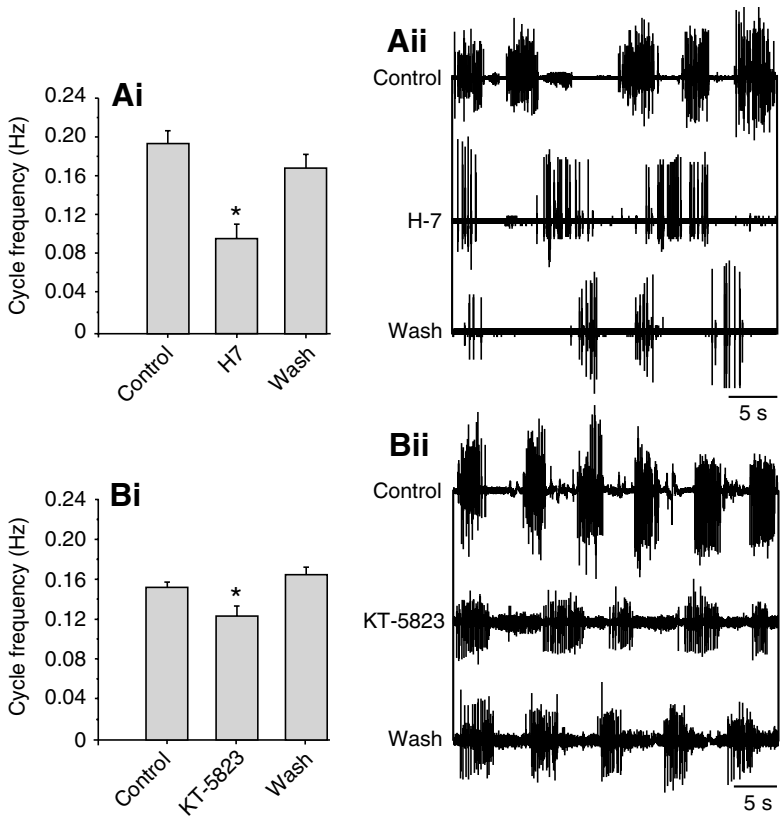


Fig. 7. The effect of the generic protein kinase inhibitor H-7 and the PKG inhibitor KT-5823 on the digging rhythm. (Ai) A 10 min bath application of 0.1 mmol l^{-1} H-7 resulted in a significant decrease in cycle frequency (t=4.93, *P<0.05, mean ± s.e.m. from five animals). A 30 min wash with locust saline resulted in a partial recovery of the digging rhythm to control levels. (Aii) A 10 min bath application of H-7 resulted in a decrease in the number of cycles (three cycles) compared with control (five cycles). A 30 min wash with locust saline resulted in a partial recovery of the digging rhythm to control levels. (Bi) A 10 min bath application of 10 μmol l^{-1} KT-5823 resulted in a significant decrease in the cycle frequency (t=2.8, *P<0.05, mean ± s.e.m. from five animals). (Bii) A 10 min bath application of KT-5823 resulted in a decrease in the number of cycles (five cycles) from a control value of six. This particular recording shows that a 30 min wash with locust saline did not result in the number of cycles returning to its control value.

Sources of NO in the abdominal ganglia

A number of studies have focused on the sources of NO in the central nervous system of locusts, which provides some insights to the potential sources of NO that may result in the modulation of the digging rhythm. In particular, Müller and Bicker showed that Ca^{2+}/calmodulin-activated NOS was responsible for the fixation-insensitive NADPH diaphorase activity of cells in the brain and specific thoracic ganglia (Müller and Bicker, 1994). More recently, using both NOS immunostaining and NADPH diaphorase histochemistry, Bullerjahn and Pflüger revealed the distribution of over 30 bilaterally symmetrical pairs of stained neurones within the terminal abdominal ganglion (Bullerjahn and Pflüger, 2003). Some of these neurones have since been shown to be efferent peptidergic

neurosecretory cells (Bullerjahn et al., 2006). Whether any of the neurones that contain NOS are actually part of the CPG networks that produce the digging rhythm has yet to be demonstrated; however, the presence of such neurones within the terminal ganglion at the very least indicates that neurones that are part of the oviposition CPG are within NO diffusion distance of NOS-containing neurones (Philippides et al., 2000).

The role of NO in modulating CPGs

Rast showed that NO activated the CPG that underlies the rhythmic feeding movements of the mouthparts of locusts and that the effect was reversed (feeding inhibited) by bath application of L-NAME (Rast, 2001). Moreover, blocking sGC with ODQ resulted in an inhibition of the feeding motor pattern, suggesting that NO was acting *via* sGC. Rast did not reveal, however, the targets of cGMP (Rast, 2001). Our study goes one step further and suggests that the CPG that underlies the oviposition digging rhythm is modulated *via* an NO/cGMP signalling pathway that acts *via* PKG.

NO also has a behavioural role in the chemosensory activation of feeding in the pond snail, *Lymnaea stagnalis* (Elphick et al., 1995). In freely behaving *L. stagnalis*, stimulating the lips with sucrose initiates a feeding response consisting of a rhythmic tri-phasic protraction, rasp and swallowing movement of the mouthparts (Straub et al., 2002). The rhythmic feeding movements are produced by a CPG located in the buccal ganglion, and sucrose application to the mouthparts activates a fictive feeding rhythm in isolated lip and buccal ganglion preparations. Bath applying the NOS inhibitor L-NAME whilst stimulating the mouthparts with sucrose, however, resulted in an inhibition of the rhythm. NO application in the absence of sucrose re-initiated the rhythm, indicating that, as with the locust sub-oesophageal CPG (Rast, 2001), NO can act to initiate CPG activity (Elphick et al., 1995). Elphick et al. identified potential sources of NO within the buccal ganglion using NADPH diaphorase staining, which revealed staining in regions of the neuropil in which the median and superior lip nerves project (Elphick et al., 1995).

In *L. stagnalis*, feeding initiation depends on NO levels within the buccal ganglia that contain the feeding CPG (Elphick et al., 1995; Benjamin and Rose, 1979). Recent studies have indicated that the generation of NO in the buccal ganglion following feeding (Sadamoto et al., 1998) serves an inhibitory role, contradicting the findings of Elphick et al. (Elphick et al., 1995). Preventing NO synthesis, and its subsequent effects on the feeding network, by lesioning a neurone that is part of the rhythmic oesophagus network (B2) leads to an increase in the frequency of the rhythmic feeding movements (Kobayashi et al., 2000). This result suggests that NO can have an inhibitory effect on the fictive feeding rhythm. This was subsequently confirmed by bath application of the exogenous NO scavenger PTIO and the NOS inhibitor L-NAME to the buccal ganglion, both of which resulted in a significant increase in the frequency of the fictive feeding rhythm. This has led to the hypothesis that NO modulates the feeding rhythm in *L. stagnalis* by inhibiting CPG activity in preparation for the next cycle of feeding movements (Kobayashi et al., 2000).

NO has also been shown to be involved in the modulation of vertebrate CPG networks. In the tadpole of the frog *Xenopus laevis*, for example, increases in NO levels result in a significant decrease in the frequency of swimming movements (McLean and Sillar, 2004). By contrast, our study shows that an increase in NO increases the cycle frequency of the CPG underlying oviposition, but why it should have this effect remains to be examined in the future. One possibility is that NO acts at the level of the synapse and modulates the release of neurotransmitter (Wildemann and Bicker, 1999). At *Drosophila* neuromuscular junctions, increasing NO levels significantly increase the number of synaptic vesicles releasing neurotransmitter (Wildemann and Bicker, 1999).

PKG signalling

We have also shown that the application of the generic protein kinase inhibitor H-7 and the PKG inhibitor KT-5823 results in a modulation of the motor pattern by significantly decreasing the cycle frequency of the rhythm. The effect of KT-5823 therefore demonstrates that the oviposition rhythm is modulated *via* a sGC/cGMP–PKG signalling pathway. Although it has been established that NO can act *via* a cGMP/PKG signalling cascade, a common target of PKG is potassium channels (Hirsch and Schlatter, 1995). In the rat, the principal cell of the basolateral membrane of the cortical collecting duct of the kidney is potassium (K^+) conductive. Two K^+ channels have been described in the principal cell: a small conductance K^+ and an intermediate conductance K^+ channel (Costa and Assreuy, 2005). Activation of small conductance K^+ channels is blocked in the presence of the PKG inhibitor KT-5823, indicating that PKG can act on small conductance K^+ channels. This suggests that neuronal events such as re-polarization may be mediated by the presence of PKG *via* the phosphorylation of K^+ channels (Reddy, 2006). Inhibition of PKG using a specific inhibitor such as KT-5823 could therefore prevent, or decrease, the rate of K^+ channel phosphorylation. This in turn could decrease the activity of K^+ channels and decrease the rate of re-polarisation of individual neuronal components of a CPG network and thus could decrease the frequency of the oviposition digging rhythm. While this remains to be tested, it is clear that, as with many other rhythmic movements, NO appears to play a key role in exerting a continuous and dynamic control over the behaviour to match it to the demands of the environment.

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