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Nitric oxide induces centrally generated motor patterns in the locust suboesophageal ganglion

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Summary

The stimulatory effects of nitric oxide (NO) on central motor pattern generation in isolated locust suboesophageal ganglia (SOGs) were studied using extracellular recordings from motor nerves. Different NO donor molecules and a specific inhibitor of soluble guanylyl cyclases were used to confirm that the observed motor pattern occurred in response to activation of the NO/cyclic GMP signalling pathway. Experiments with muscarinic agonists and antagonists showed that the NO-induced motor pattern is generated independently from the motor pattern induced by muscarinic agonists described previously. Staining for NADPH-diaphorase and an antiserum directed against cyclic GMP were used to identify neurones representing potential sources of NO and their target cells within the SOG. Using intracellular dye injection and backfilling of peripheral nerves in combination with anti-cGMP immunohistochemistry, it was shown that identified efferent neurones involved in the mandibular motor pattern are potential target cells of NO.

Key words: nitric oxide, soluble guanylyl cyclase, pilocarpine, migratory locust, *Locusta migratoria*.

Introduction

In various model systems, motor patterns can be induced using externally applied neurotransmitters or modulators as stimulants inducing the nervous system to reach and to maintain an oscillatory state, e.g. serotonin in leech swimming (Willard, 1981) or octopamine in insect motor patterns (Kinnamon et al., 1984; Sombati and Hoyle, 1984; Stevenson and Kutsch, 1988). The most common stimulatory agents are muscarinic agonists in arthropods (Chrachri and Clarac, 1990; Elson and Selverston, 1992; Ryckebusch and Laurent, 1993; Büschges et al., 1995; Braun and Mulloney, 1995; Johnston and Levine, 1996; Heinrich et al., 1997; Rast and Bräunig, 1997) and glutamatergic agonists in vertebrate preparations (Cohen and Wallen, 1980; Soffe, 1996; Beato et al., 1997). As these motor patterns remain stable for considerable periods in deafferented preparations and many of their features resemble those of naturally occurring motor patterns, it is concluded that such central oscillating networks do not depend on timing cues provided by sensory feedback to generate these patterns. However, a large number of neuromodulators have been shown to influence centrally generated motor patterns, e.g. serotonin and neurotensin modulate the frequency of oscillation in the lamprey spinal cord (Harris-Warrick and Cohen, 1985; Barthe and Grillner, 1995). Other well-studied systems in this context are serotonergic modulation of swimming in *Clione limacina* (for a review, see Satterlie and Norekian, 1996) and modulation of the stomatogastric motor patterns by various modulators in crustaceans (for a review, see Simmers et al., 1995). Recently, the neurotransmitter and modulator nitric oxide (NO) was also found to influence centrally generated motor patterns (Hedrick et al., 1998; McLean and Sillar, 2000). In the pond snail *Lymnaea stagnalis*, NO induces a motor pattern mimicking chemosensory input (Elphick et al., 1995).

In insects, NO, acting on the soluble guanylyl cyclase (sGC) and thus stimulating the production of cyclic guanylyl monophosphate (cGMP), has been extensively demonstrated (Green and O'Shea, 1993; Müller and Buchner, 1993; Müller, 1997; Bicker, 1998; Zayas et al., 2000). NO signalling has been shown to be involved in diverse physiological contexts such as developmental processes (Truman et al., 1996; Ball and Truman, 1998; Schachtner at al., 1998; Schachtner et al., 1999; Wildemann and Bicker, 1999a), olfaction (Müller, 1994; Elphick et al., 1995; Bicker et al., 1996; Seidel and Bicker, 1997; Nighorn et al., 1998), vision (Elphick et al., 1996; Bicker and Schmachtenberg, 1997; Schmachtenberg and Bicker, 1999), memory (Müller, 1996) and motor systems (Shibanaka et al., 1994; Wildemann and Bicker, 1999b; Qazi and Trimmer, 1999).

The presence of NO/cGMP signalling in locust motor systems was proposed by Ott and Burrows (Ott and Burrows, 1998; Ott and Burrows, 1999; Ott et al., 1999), and the aim of the present study was to investigate the role of this signalling pathway in central motor pattern generation in the locust. As

a model system, the central control of the mouthparts was chosen, this being a system for which muscarinic pattern generation has previously been described (Rast and Bräunig, 1997). The interrelationship between these two signalling systems was of particular interest as interactions have previously been shown in the nervous system of the moth *Manduca sexta* (Qazi and Trimmer, 1999). For this purpose, specific antagonists for each of the signalling pathways were tested for their potency in inhibiting pattern generation induced by NO or muscarinic agonists. If NO/cGMP signalling played a role in the pattern induced by muscarinic agonists, it would be expected that a specific inhibitor of sGC would impair the muscarinic pattern. Similarly, if muscarinic signalling played a role in nitrergic pattern generation, specific inhibitors of muscarinic receptors should also affect the NO-induced pattern.

Histochemical staining for NADPH-diaphorase activity and for the presence of cGMP upon induction with NO were used to identify structures serving as potential natural sources and targets of NO. The localisation of these structures will assist future identification and characterisation of elements of the central-pattern-generating system.

Materials and methods

Insects and preparation

Experiments were performed on adult African migratory locusts (*Locusta migratoria migratorioides* R. & F.) taken from a crowded laboratory culture. Prior to dissection, locusts were immobilised by chilling to 4 °C. Subsequently, they were decapitated, and the suboesophageal ganglion (SOG) was exposed after the removal of the frons, clypeus, labrum and the remainder of the foregut. The peripheral nerves of the SOG were cut at suitable lengths. After severing the circumoesophageal connectives and the tentorium, the SOG was excised from the head capsule, leaving the main branches of the tracheal system intact, and was pinned into a Petri dish coated with Sylgard (Dow Corning Corp., Midland, Michigan, USA). It was superfused with saline (Clements and May, 1974). The stumps of the tracheae were opened at the surface of the saline. Anatomical terms are taken from Snodgrass (Snodgrass, 1928); for an anatomical description of the mandibular motor system, see Bräunig (Bräunig, 1990).

Electrophysiology

Extracellular potentials were measured using monopolar, tightly fitting suction electrodes feeding into Grass P15 bipolar preamplifiers whose negative input was grounded. Gain was set to 100, and the half-amplitude frequency of the high-pass filter was turned down to 0.1 Hz. Data were digitised using a personal computer equipped with the CED 1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). Off-line analysis was performed using software written by the author in C running on a personal computer under the LINUX operating system. Mean bursting frequency, duty cycle, intra-burst frequency and the number of units recruited were evaluated from 50 s stretches of activity. Bursts were defined as intervals in which no inter-spike gap was longer than 200 ms. The mean bursting frequency was calculated as the number of bursts per 50 s; the mean duty cycle was defined as the mean proportion of a burst compared with the whole cycle period; intra-burst frequencies were determined as the number of spikes per burst and subsequently averaged over the 50 s recordings. The number of units recruited was estimated from the number of spike sizes and shapes that could be distinguished in the extracellular recordings by visual inspection. Statistical significance of differences was tested using the sign test for paired data. An error probability of $P<0.05$ was accepted as significant.

Stock solutions of drugs were prepared as follows: 100 mmol l^{-1} sodium nitroprusside (SNP; Sigma Aldrich Chemie GmbH, Steinheim, Germany) in distilled water; 1 mol l^{-1} hydroxylamine (Merck AG, Darmstadt, Germany) in distilled water; 0.1 mol l^{-1} 1H-(1,2,4)oxa-diazolo(4,3a)-quinoxalin-1-one (ODQ; ICN Biomedicals Inc., Aurora, Ohio, USA) in dimethyl sulphoxide (DMSO); 10 mmol l^{-1} pilocarpine (Sigma) in saline; 10 mmol l^{-1} oxotremorine (Sigma) in saline; 10 mmol l^{-1} atropine (Sigma) in distilled water; 10 mmol l^{-1} scopolamine (Sigma) in saline; 4.5 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX; Sigma) in saline. The dosages of pilocarpine and IBMX were designed to give a maximal response (Rast and Bräunig, 2001); all other drugs were given at the lowest dose reliably yielding a response, as tested in preliminary experiments (data not shown). Stock solutions of drugs were added to a fixed bath volume using an Eppendorf pipette and dispersed by gentle agitation of the bath solution using the pipette after recordings had been established.

Histology

Ganglia destined for anti-cGMP immunohistochemistry were incubated in 5 mmol l^{-1} SNP and 0.45 mmol l^{-1} IBMX for 20 min at room temperature and with 100 W halogen illumination before fixation to promote the decomposition of SNP (see also Ball and Truman, 1998). Preparations were fixed in 4 % formaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature. Subsequently, they were infiltrated with 30 % sucrose for 10–18 h. After embedding in Tissue-Tek mounting medium (TED Pella, Inc., Redding, California, USA), cryosections were taken at 35–50 μm and immediately mounted on chrome alum/gelatine-coated slides. Sections were washed overnight at 4 °C in PBS containing 0.25 % Triton X-100 (PBS-TX) and subsequently blocked for 1 h in a mixture of 5 % normal donkey serum and 0.5 % bovine serum albumin (both Sigma) in PBS-TX 0.25 % at room temperature. Sheep anti-cGMP serum (kind gift of J. De Vente to P. Bräunig) was diluted 1:600 000 when using a horseradish peroxidase (HRP)-conjugated secondary antibody or 1:100 000 when using a (CY3)-conjugated secondary antibody in the blocking medium, and sections were incubated overnight at 4 °C. After repeated rinsing in 0.25 % PBS-TX, sections were incubated in

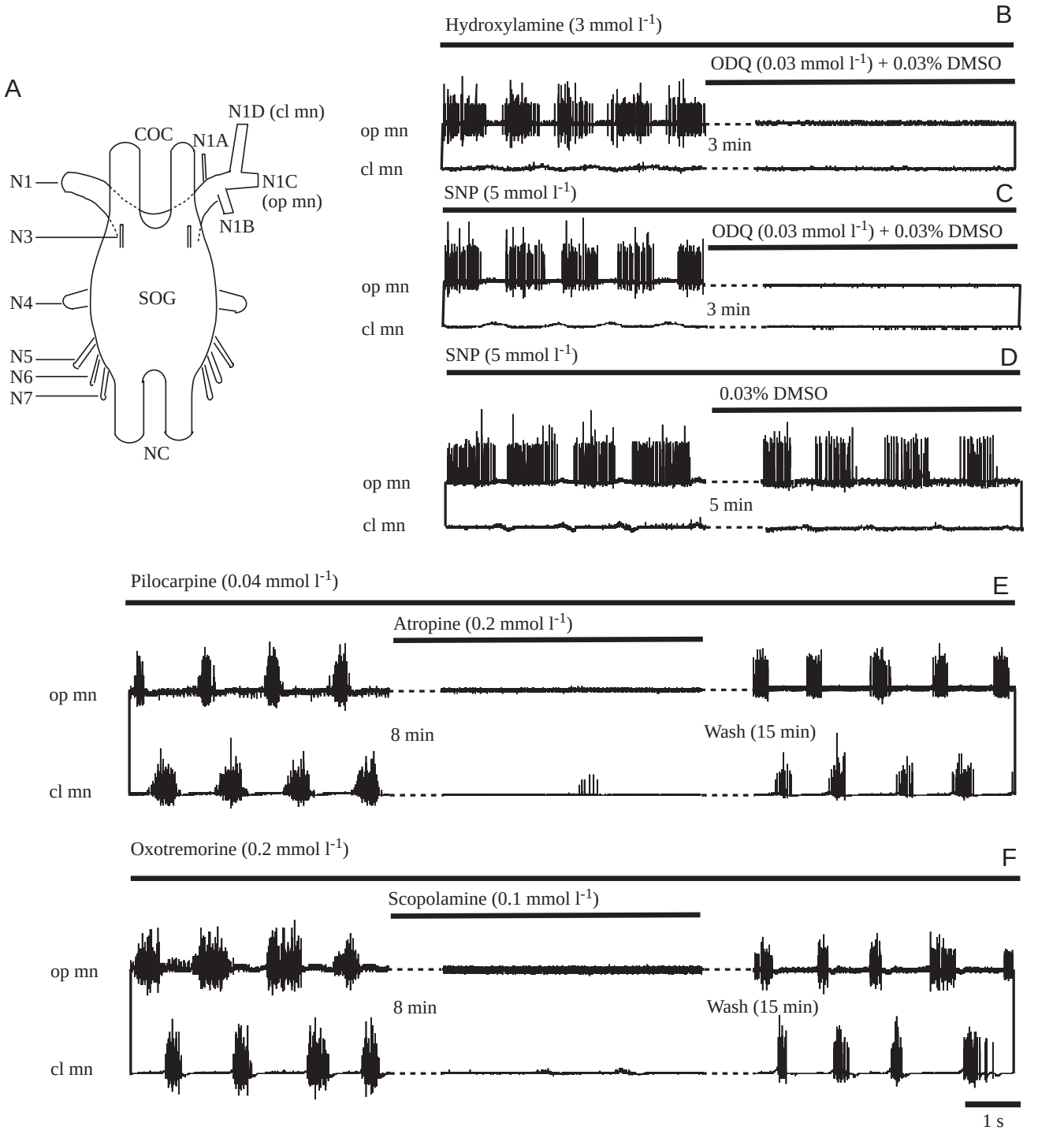

Fig. 1. Nitrergic and muscarinic pattern generation in the locust suboesophageal ganglion (SOG). (A) Diagrammatic dorsal view of the SOG showing nerves 1C (N1C) and 1D (N1D), from which mandibular opener and closer motor patterns were recorded. Nerves are numbered consecutively (see Altman and Kien, 1979). (B) Motor pattern induced by the NO donor hydroxylamine before (left) and after (right) application of the soluble guanylyl cyclase (sGC) inhibitor 1H-(1,2,4)oxa-diazolo(4,3a)-quinoxalin-1-one (ODQ). Upper trace, mandibular opener motor nerve; lower trace, mandibular closer motor nerve. (C) Motor pattern induced by the NO donor sodium nitroprusside (SNP) before (left) and after (right) application of the sGC inhibitor ODQ. (D) The motor pattern induced by SNP is not affected by the amount of dimethyl sulphoxide (DMSO) required to dissolve ODQ. (E) Motor pattern induced by the muscarinic agonist pilocarpine (left). After application of the muscarinic antagonist atropine, the pattern is abolished but reappears after washing and reapplication pilocarpine (right). (F) Motor pattern induced by the muscarinic agonist oxotremorine (left). After application of the muscarinic antagonist scopolamine, the pattern is abolished but reappears after washing and reapplication of oxotremorine (right). COC, circumoesophageal connectives; NC, neck connectives; N, nerve; cl mn, closer motor nerve; op mn, opener motor nerve.

a donkey anti-sheep serum conjugated with HRP or with CY3 (both from dianova GmbH, Hamburg, Germany) overnight at 4 °C. Preparations were then rinsed thoroughly in PBS-TX 0.25 % and, in the case of HRP-conjugated secondary antibodies, the reaction product was developed using 30 % diaminobenzidine (DAB; Sigma) with 0.015 % H_2O_2 and 0.03 % NiCl in PBS for 10–30 min in the dark. Preparations were dehydrated in an alcohol series and mounted in DePeX (Serva, Heidelberg, Germany; DAB reaction) or Fluoromount (Serva; CY3 fluorescence).

To identify mandibular closer motoneurones and salivary neurones in double-labelled sections, motoneurones were dye-injected with hyperpolarising current pulses (–5 nA, 1 Hz, 500 ms for 20 min) using microelectrodes filled with 5 % Lucifer Yellow (Molecular Probes, Eugene, Oregon, USA) in 1 mol l^{-1} LiCl in the tip and 1 mol l^{-1} LiCl in the shaft (resistance 40–80 MΩ), while the salivary nerve (N7B; Altman and Kien, 1979) was backfilled with Lucifer-Yellow-labelled dextranamine (Molecular Probes). The subsequent treatment of these preparations was as described above.

For diaphorase histochemistry, ganglia were fixed in 4 % formaldehyde in PBS for 1 h at room temperature without any pretreatment. Cryosections were prepared as described above and soaked overnight at 4 °C in PBS-TX 3 %. They were then preincubated in 100 mmol l^{-1} Nitroblue Tetrazolium (NBT, Sigma) in PBS-TX 0.1 % for 1 h at room temperature in the dark. Staining was performed with 100 mmol l^{-1} NBT and 100 mmol l^{-1} β-nicotinamide adenine dinucleotide phosphate in its reduced form (NADPH; Sigma) in PBS-TX 0.1 % for 1–6 h in the dark at room temperature or overnight at 4 °C. After staining, sections were rinsed in PBS, dehydrated in an alcohol series and embedded in DePeX (Serva).

Sections were documented with an Axiophot microscope (Carl Zeiss GmbH, Jena, Germany) and a CoolPix 950 digital camera (Nikon, Tokyo, Japan). Photographs were corrected for brightness, and their contrast was enhanced using the GNU Image Manipulation Program (GIMP).

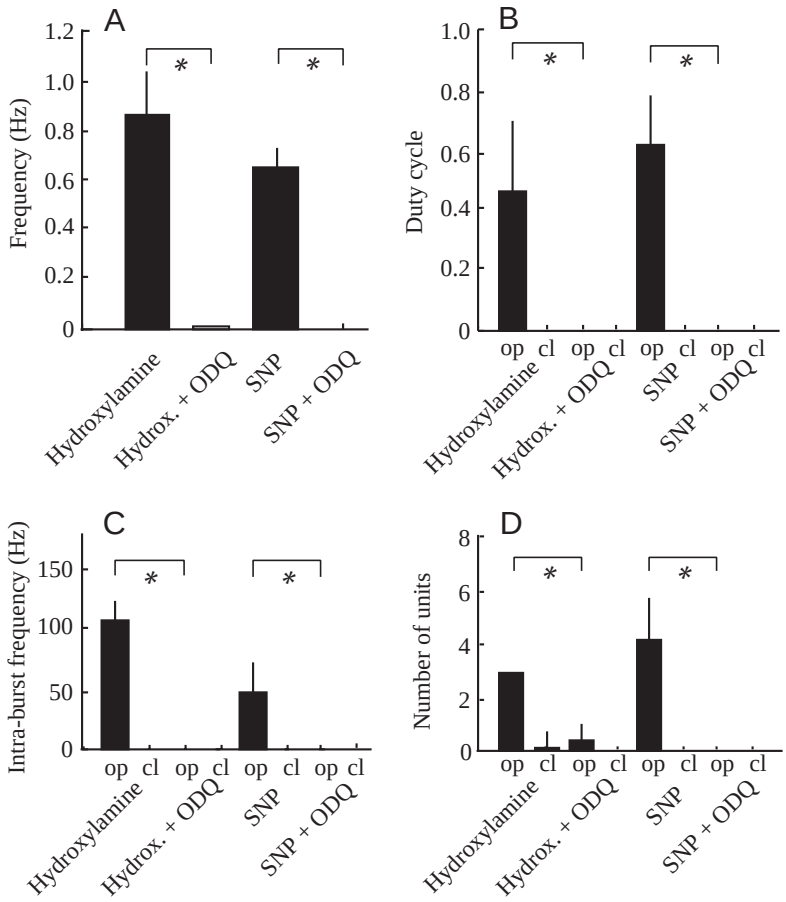

Fig. 2. Comparison of the characteristics of the nitrergic motor patterns determined from 50 s stretches of activity. Depending on the mean bursting frequency, this observation period covered 26–57 cycles. Asterisks mark significant differences (sign-test: $P<0.05$; $N=5$). (A) Frequency of bursting. (B) Mean duty cycle (burst duration divided by cycle period). (C) Intra-burst frequency. (D) Number of units distinguishable in the extracellular recordings by visual inspection. cl, closer; op, opener; Hydrox., hydroxylamine; ODQ, 1H-(1,2,4)oxa-diazolo(4,3a)-quinoxalin-1-one; SNP, sodium nitroprusside. Values are mean + S.D.

Results

Nitric oxide donors and muscarinic agonists induce a mandibular motor pattern

Before application of drugs, no rhythmic motor activity occurs in isolated locust suboesophageal ganglia (SOG) (Rast and Bräunig, 1997; Rast and Bräunig, 2001). Patterns of motor activity were induced by nitric oxide (NO) donors: 80 % of preparations exposed to hydroxylamine and 73.3 % of preparations treated with sodium nitroprusside (SNP) showed motor patterns ($N=15$ each). These lasted for 20–30 min and occurred with a latency of 0.5–2 min after application of the respective drug. The pattern is best seen in the mandibular opener motor nerve (N1C; Fig. 1A,B–D, upper trace). Mandibular closer motoneurones usually do not spike; however, when recording from the mandibular closer motor nerve (N1D; Fig. 1A), oscillations of the baseline phase-locked to the mandibular opener pattern are visible (Fig. 1B–D, lower trace). While it is not clear yet whether these baseline deflections are always exclusively due to subthreshold activity in the motoneurones, intracellular recordings from up to three motoneurones in parallel consistently indicated phase-locked subthreshold patterning that matched the extracellularly recorded signal (data not shown).

Soluble guanylyl cyclase (sGC), a common receptor for NO, is probably involved in pattern generation since 1H-(1,2,4)oxa-diazolo(4,3a)-quinoxalin-1-one (ODQ), a specific inhibitor of

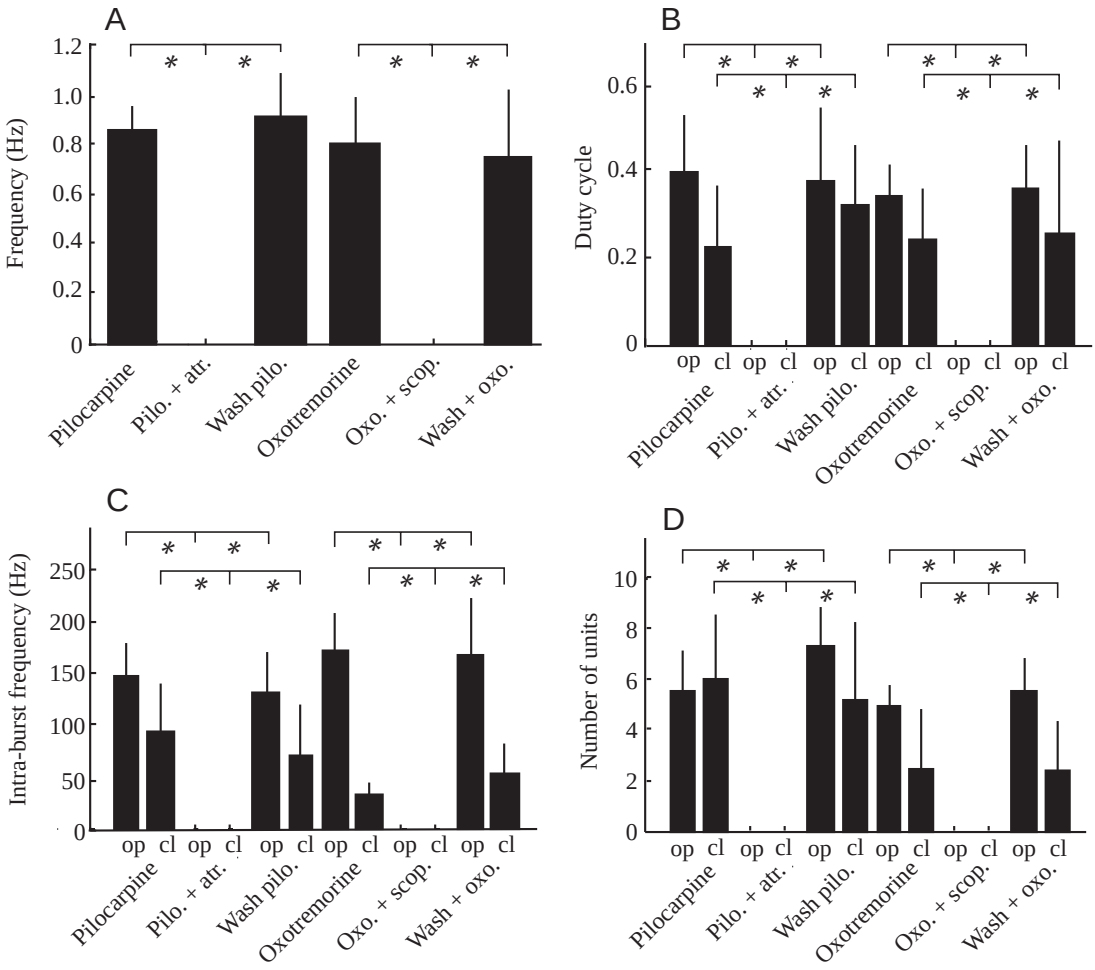

Fig. 3. Comparison of characteristics of the muscarinic motor patterns determined from 50 s stretches of activity. Depending on the mean bursting frequency, this observation period covered 26–57 cycles. Asterisks mark significant differences (sign-test: $P<0.05$; $N=5$). (A) Frequency of bursting. (B) Mean duty cycle (burst duration divided by cycle period). (C) Intra-burst frequency. (D) Number of units distinguishable in the extracellular recordings by visual inspection. cl, closer; op, opener; atr., atropine; oxo., oxotremorine; pilo., pilocarpine; scop., scopolamine. Values are mean + S.D.

sGC, blocks both the motor pattern in the mandibular opener motor nerve and the subthreshold oscillations in the mandibular closer motor nerve after a latency of 1–3 min (Fig. 1B,C; $N=5$). A control for the amount of dimethyl sulphoxide (DMSO) used to dissolve ODQ is shown in Fig. 1D ($N=3$). For all preparations, bursting frequency, duty cycle (burst duration/cycle period), intra-burst frequency and the number of units detectable in extracellular recordings were evaluated (see Materials and methods). Application of ODQ abolished the mandibular opener motor pattern except for occasional single spikes; this is reflected by a decline in the value of all variables to near zero, which represents a significant change compared with the situation prior to ODQ application (Fig. 2; sign-test: $P<0.05$, $N=5$ each). As the above evaluation is based on the occurrence of spikes, no comparisons are available for subthreshold mandibular closer activity.

The muscarinic agonists pilocarpine and oxotremorine induced motor patterns in 100 % of preparations ($N=15$ each); these lasted for 1.5–3 h and occurred with a latency of 5–10 min after application of the drug (Fig. 1E,F). The muscarinic antagonists atropine and scopolamine blocked the motor pattern after a latency of 5–10 min, but subsequent thorough washing restored it (Fig. 1E,F; $N=5$). This is reflected in the statistically significant changes in all evaluated variables for both mandibular opener and closer motor patterns (Fig. 3; sign-test: $P<0.05$, $N=5$ each).

Nitrergic pattern generation is independent of the muscarinic pathway

Both NO and muscarinic agonists are sufficient to induce a mandibular motor pattern; however, neither muscarinic receptors nor sGC are necessary for the induction of the pattern. Fig. 4A,B shows motor patterns induced by the NO donors hydroxylamine and SNP before and after the ganglia had been treated with the muscarinic antagonists atropine and scopolamine ($N=5$). The motor patterns induced by the muscarinic agonists pilocarpine and oxotremorine before and

after application of the sGC inhibitor ODQ are shown in Fig. 4C,D (N=5). The inhibitors lack any effect (Fig. 4) at doses that were shown to block any reaction to the respective agonist (Fig. 1, Fig. 2, Fig. 3). For all preparations, bursting frequency, duty cycle, intra-burst frequency and the number of units detectable in extracellular recordings were evaluated before and after application of the respective agonist. Statistical significance for differences between the pattern before and after application of the respective antagonist could not be established (Fig. 5, Fig. 6; sign-test: P>0.05; N=5 each). No differences from the above results were found when the respective antagonist was applied before pattern induction by a muscarinic agonist or an NO donor instead of application of the antagonist during an ongoing motor pattern (data not shown, N=5 each).

Structures potentially involved in nitrergic pattern generation

NADPH-diaphorase staining

Neuronal somata, fibres and neuropilar structures show NADPH-diaphorase activity that is insensitive to mild formaldehyde fixation (Fig. 7D–H). The examples shown in Fig. 7 come from a set of 50 preparations (for orientation purposes, schematic views of the SOG with the structures and planes of section discussed below are shown in Fig. 7A–C). As control experiments to examine the specificity of diaphorase staining preparations were overfixed (>1 h up to 4 h at room temperature), this resulted in a rapid decrease in staining intensity, suggesting that no diaphorase activity existed that was more resistant to formaldehyde fixation than nitric oxide synthase (NOS) was present. Preparations were also incubated for up to 12 h in Nitroblue Tetrazolium without NADPH. This resulted in only a faint background staining, which showed that Nitroblue Tetrazolium is reduced in an NADPH-dependent manner for the specific stainings shown in Fig. 7D–H.

The most prominently stained structures are a pair of anterior cells projecting ipsilaterally into the circumoesophageal connectives, usually accompanied by 1–3 smaller cells (Fig. 7E), and a pair of posterior cells projecting contralaterally into the neck connectives (Fig. 7F). The projection pattern was verified both in serial sections and in wholemount preparations. A group of 3–4 cells (15–20 μm in diameter), whose relative position varies, is regularly found dorsally between the circumoesophageal connectives (Fig. 7G). In addition, groups of 5–11 very small ventral cells (5–15 μm in diameter) are found just behind the insertion of nerve 2 (Fig. 7D, dashed circles), and these are always separated from up to 36 loosely arranged larger cell bodies located posteriorly.

An interesting feature of the NADPH-diaphorase-positive neuropilar regions is the Ω-shaped structure

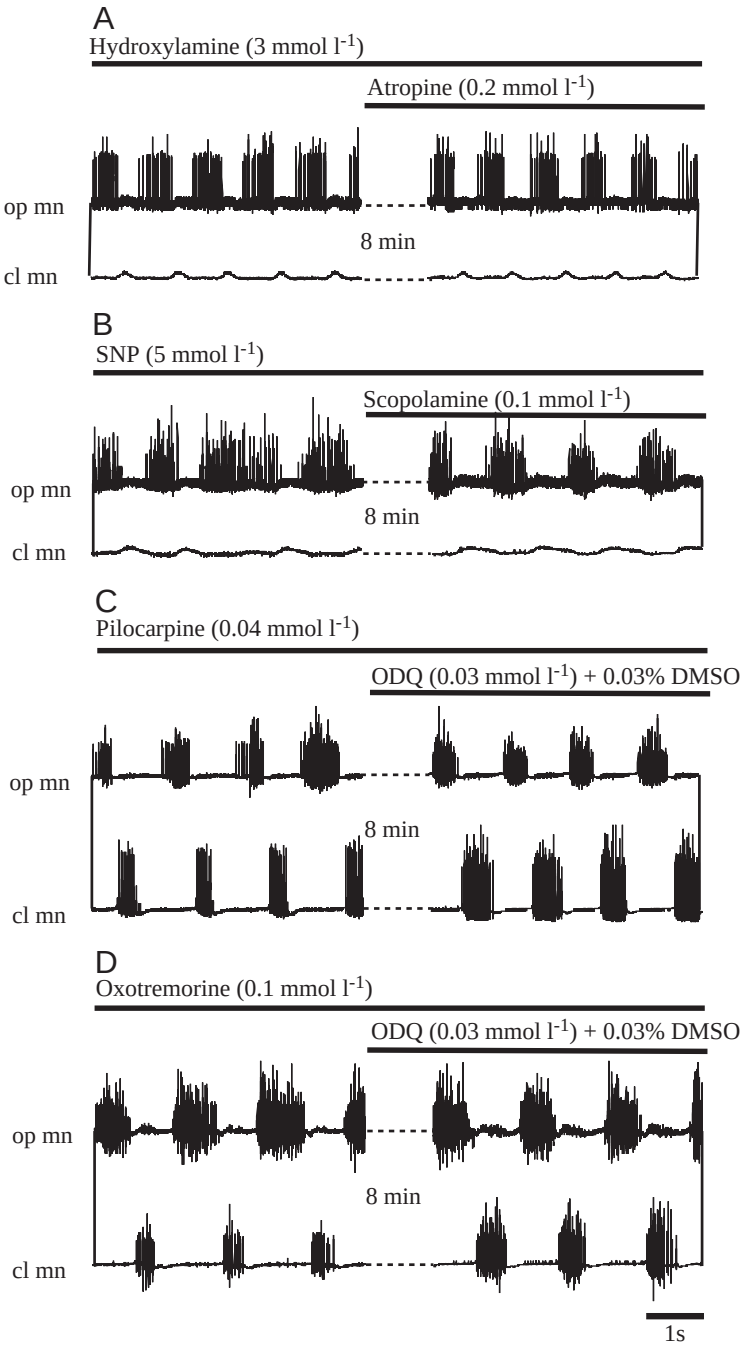

Fig. 4. The motor patterns induced by muscarinic agonists and by nitric oxide are independent. (A) The generation of the motor pattern induced by the NO donor hydroxylamine (left) does not involve muscarinic signalling since it is not blocked by the muscarinic antagonist atropine. (B) The generation of the motor pattern induced by the NO donor sodium nitroprusside (SNP) does not involve muscarinic signalling, since it is not blocked by the muscarinic antagonist scopolamine. (C) The generation of the motor pattern induced by the muscarinic agonist pilocarpine does not involve NO signalling, since it is not blocked by the soluble guanylyl cyclase (sGC) inhibitor 1H-(1,2,4)oxa-diazolo(4,3a)-quinoxalin-1-one (ODQ). (D) The generation of the motor pattern induced by the muscarinic agonist oxotremorine does not involve NO signalling, since it is not blocked by the sGC inhibitor ODQ. cl mn, closer motor nerve; op mn, opener motor nerve; DMSO, dimethylsulphoxide.

with dense fine processes and boutons in the ventral posterior SOG (Fig. 7H). This region differs from other stained neuropilar structures (e.g mandibular neuropile, Fig. 7H, dashed outline) in the density of the profiles but not in the intensity of the staining. While NADPH-diaphorase-positive fibres are found in the longitudinal tracts and the connectives (e.g. Fig. 7G), almost no NADPH-diaphorase staining is found in the peripheral nerves of the SOG.

Anti-cGMP immunohistochemistry

Immunohistochemistry directed against cGMP reveals cell bodies, neuropilar processes and fibres (Fig. 8, representative examples of 45 preparations). Control experiments without pretreatment with SNP and IBMX or with IBMX alone failed to show any staining. Preparations pretreated with 0.03 mmol l^{-1} ODQ before application of SNP and IBMX were, similarly, unlabelled. This shows that all the stained structures described below accumulated cGMP directly or indirectly upon induction with NO. The most prominent stained structure is a pair of a large (50–60 μm in diameter) ventral posterior cells (Fig. 8D). As in NADPH-diaphorase-stained preparations, a group of 1–4 cells (30–40 μm in diameter) with varying relative positions is found dorsally between the circumoesophageal connectives (Fig. 8F). Large (50–70 μm in diameter) but less intensely stained cell bodies are located laterally and towards the anterior end of the ganglion (Fig. 8E; see also double labellings below). Stained fibres occur in the circumoesophageal and neck connectives (data not shown). In contrast to NADPH-diaphorase staining, anti-cGMP immunoreactivity was also found widely in peripheral nerves, e.g. the labial nerve (N5) in Fig. 8D and the mandibular nerve (N1) in Fig. 8G. The number of fibres seen in the mandibular nerve (Fig. 8G) exceeds the number of efferent neurones known to project into this nerve and some of these stained for cGMP (see below), suggesting that afferent profiles show anti-cGMP immunoreactivity. Prominent anti-cGMP-immunoreactive neuropilar regions occur as very fine anterior ventral arborizations at the anterior edge of the mandibular neuropil and in a bilateral neuropilar structure that lies ventrally and posteriorly close to the midline (Fig. 8H).

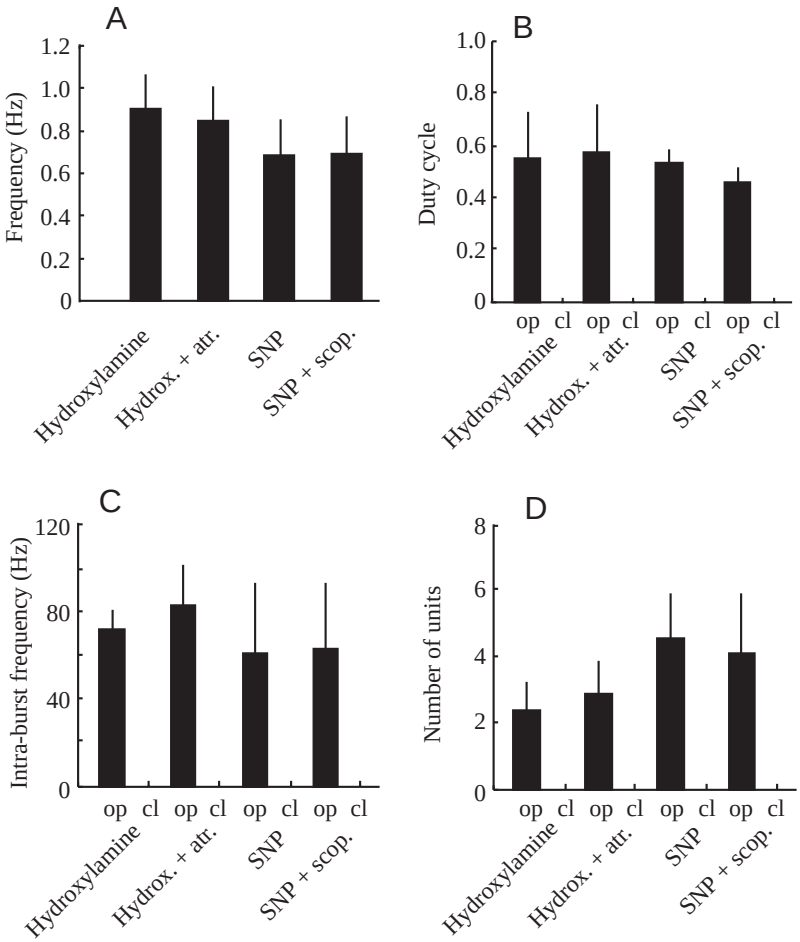

Fig. 5. Comparison of characteristics of the nitrergic motor patterns determined from 50 s stretches of activity in five preparations before and after application of muscarinic antagonists. Depending on the mean bursting frequency, the observation period covered 31–55 cycles. (A) Frequency of bursting. (B) Mean duty cycle (burst duration divided by cycle period). (C) Intra-burst frequency. (D) Number of units distinguishable in the extracellular recordings by visual inspection. cl, closer; op, opener; atr., atropine; hydrox., hydroxylamine; scop., scopolamine; SNP, sodium nitroprusside. Values are mean + S.D.

Double labellings

To identify some of the cGMP-immunoreactive cell bodies, double-labelling experiments were performed. Intracellular injection of Lucifer Yellow and subsequent anti-cGMP immunohistochemistry showed that a subpopulation of the mandibular closer motoneurones shows anti-cGMP immunoreactivity upon induction with NO (Fig. 9A,B). Backfilling of the salivary nerve (N7B) with dextran-amine-conjugated Lucifer Yellow revealed that salivary neurone 1 (SN1), but not salivary neurone 2 (SN2), shows anti-cGMP immunoreactivity (Fig. 9C–F). In SN1, only the cell body was immunoreactive, but this labelling did not extend to the neurite or the dendritic arborizations.

Discussion

The present study shows that NO induces a centrally generated motor pattern in the locust SOG that is distinct from the motor pattern induced by muscarinic agonists (Fig. 1, Fig. 4; Rast and Bräunig, 1997). Staining for NADPH-diaphorase and anti-cGMP immunohistochemistry revealed structures that are potential physiological sources and targets of NO (Fig. 7, Fig. 8, Fig. 9).

Nitrergic pattern generation

A motor pattern is induced by NO donors and does not seem to be influenced by side-effects of the drugs or their degradation products since similar motor patterns can be induced by chemically distinct NO donors. The action of a specific inhibitor of sGC, ODQ (Hobbs, 1997), corroborates the conclusion that the induced pattern is the result of stimulating the NO/cGMP signalling pathway (Fig. 1B,C). The action of ODQ on sGC is probably irreversible (Hobbs, 1997), which accords with the observation that NO-induced pattern generation may not be restored after thorough washing (data not shown). The reasons for the short lifetime (20–30 min) of preparations treated with NO donors are not known, but this was always long enough to allow a sequential application of drugs.

Nitric oxide has modulatory effects on central pattern generation in some model systems. For example, it increases the cycle period of breathing in the bullfrog (Hedrick et al., 1998) and of spinal swimming in *Xenopus laevi*s tadpoles (McLean and Sillar, 2000). In the pond snail, NO donors trigger the feeding motor pattern by mimicking the activity of nitrergic chemosensory neurones (Elphick et al., 1995). These examples show that NO may have either an inhibitory or an excitatory effect on pattern generation. In insects, NO also plays a role in the chemosensory system (Müller, 1994; Elphick et al., 1995; Bicker et al., 1996; Seidel and Bicker, 1997; Nighorn et al., 1998) and, since both NADPH-diaphorase-positive and cGMP-immunoreactive fibres occur in the circumoesophageal connectives, the important question arises of whether chemosensory information from the brain contributes to the generation or modulation of feeding motor patterns *via* the NO/cGMP signalling pathway.

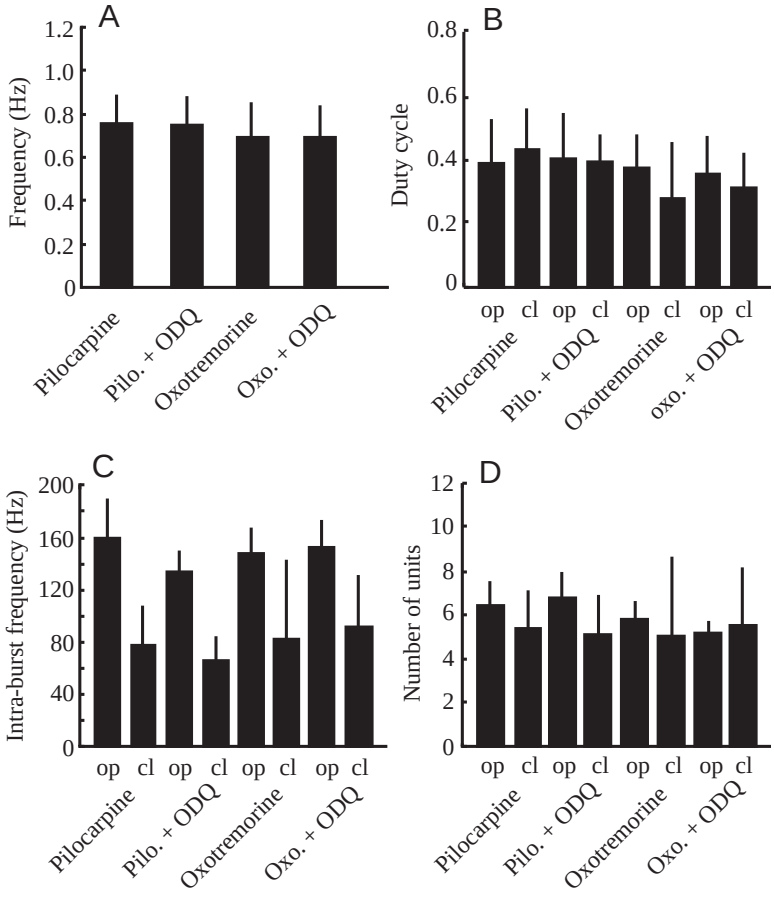

Fig. 6. Comparison of characteristics of the muscarinic motor patterns determined from 50 s stretches of activity in five preparations before and after application of the soluble guanylyl cyclase inhibitor 1H-(1,2,4)oxadiazolo(4,3a)-quinoxalin-1-one (ODQ). Depending on the mean bursting frequency, the observation period covered 31–55 cycles. (A) Frequency of bursting. (B) Mean duty cycle (burst duration divided by cycle period). (C) Intra-burst frequency. (D) Number of units distinguishable in the extracellular recordings by visual inspection. cl, closer; op, opener; oxo., oxotremorine; pilo., pilocarpine. Values are mean + S.D.

Muscarinic pattern generation

Muscarinic induction of motor patterns is common in arthropods (see Introduction). In the locust mandibular system, the action of a muscarinic agonist, pilocarpine, has been described (Rast and Bräunig, 1997) and, to support this study on a broader basis of evidence, experiments were performed with the muscarinic agonists pilocarpine and oxotremorine in parallel (Fig. 1, Fig. 4). For the same reason, different muscarinic antagonists (atropine and scopolamine) were used as specific blockers of the muscarinic motor pattern. The similarity of action of the different agents makes it unlikely that the observed effects are due to side-effects of the drugs. The results obtained with pilocarpine and atropine are in good agreement with similar experiments on locusts, stick insects and the tobacco hornworm (Ryckebusch and Laurent, 1993; Büschges et al., 1995; Johnston and Levine, 1996).

Independence of the motor patterns

The induction of mandibular motor patterns by NO and muscarinic agonists is independent, i.e neither of the signalling pathways is essential for the induction of pattern generation, but both pathways are sufficient (Fig. 4). The fact that the results of the experiments shown in Fig. 4 do not change even if the respective antagonist is applied before pattern induction by the agonist confirms that the signalling pathway being blocked is relevant neither for the induction nor for the maintenance of a motor pattern. From the above data, it is concluded that the NO/cGMP pathway and the muscarinic pathway work in parallel. This does not, however, preclude the possibility that these pathways may converge on a third pathway involved in central pattern generation further downstream, e.g. a network of premotor interneurones.

In the central nervous system of *Manduca sexta* larvae, cGMP production in response to application of muscarinic

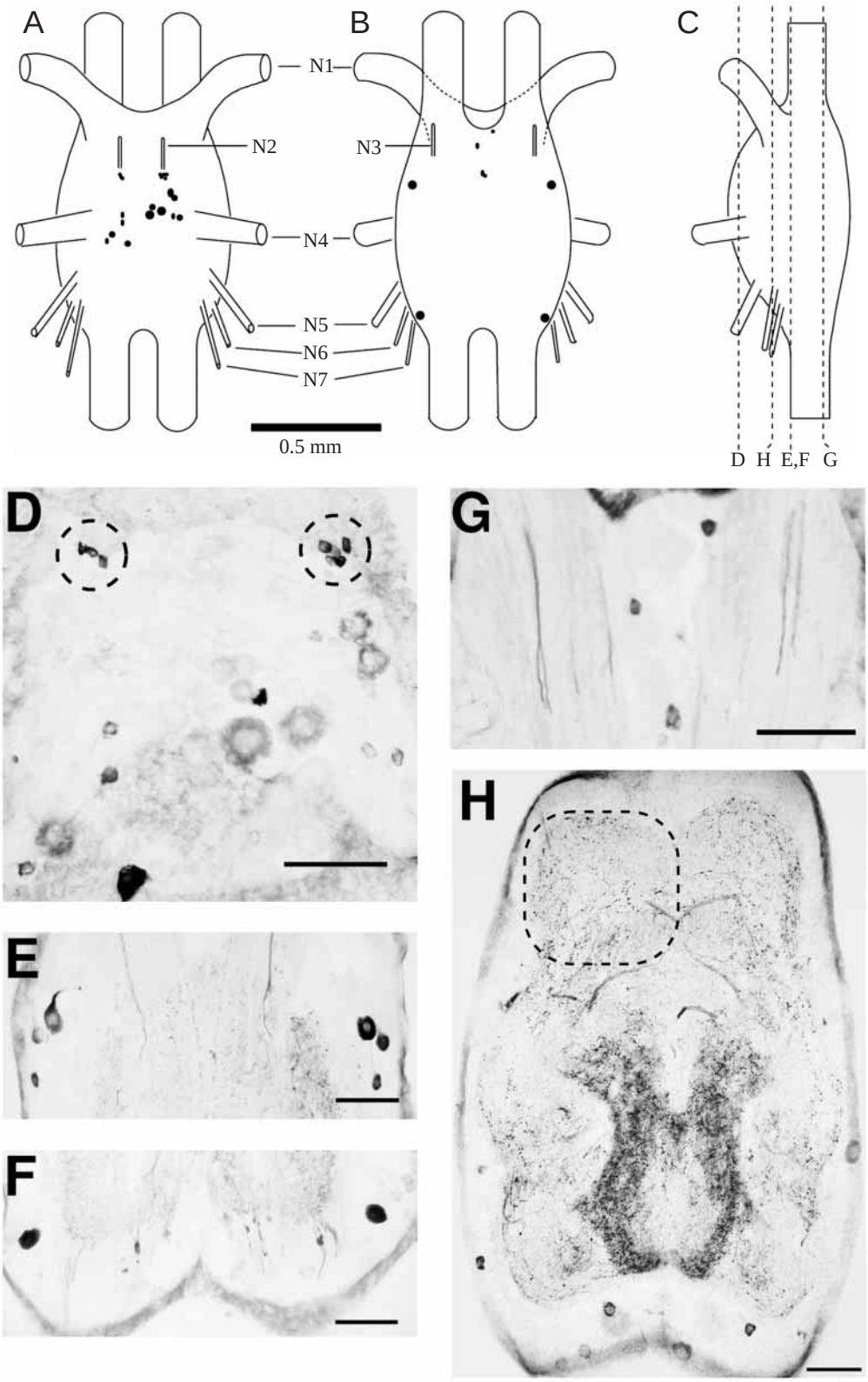

Fig. 7. NADPH-diaphorase staining in horizontal sections of the suboesophageal ganglion (SOG). (A–C) Schematic drawings of the SOG in a ventral (A), dorsal (B) and lateral (C) view. For orientation, the cells shown in D–H are indicated by dots in the drawings (D in A, E–H in B). In C, the planes of sectioning for D–H are indicated. Anterior is to the top in all panels. (D) Ventral section with anterior groups of small cells (dashed circles). (E) Lateral anterior cells. (F) Lateral posterior cells. (G) Dorsal anterior medial cells. (H) Prominent neuropilar structures in a mid-ventral plane with the dense posterior Ω-shaped neuropilar region showing fine processes and boutons and the anterior ventral mandibular neuropil (dashed line). N1–N7, nerves 1–7. Scale bars, 100 μm.

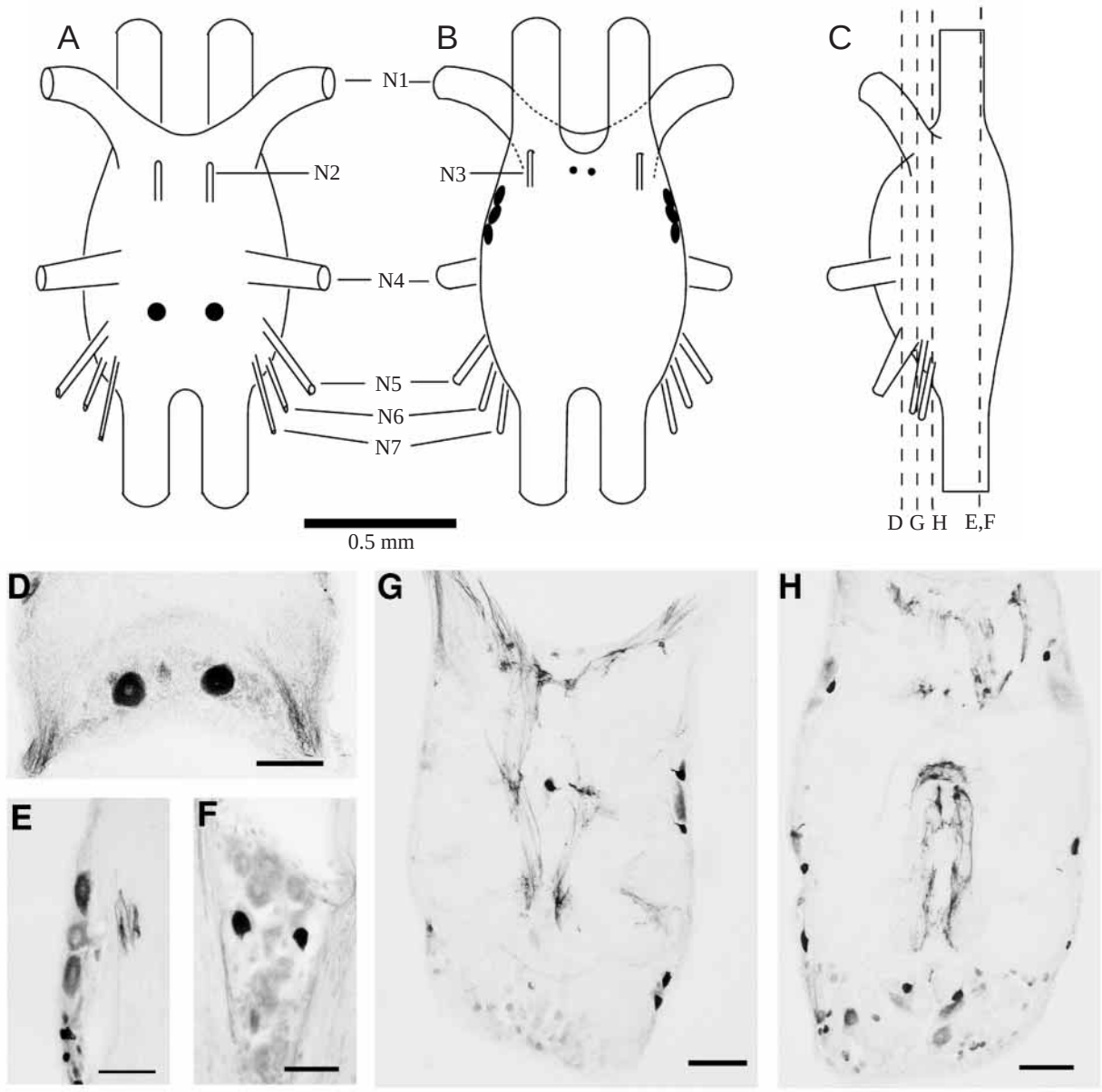

Fig. 8. Cyclic-GMP-like immunoreactivity in the suboesophageal ganglion (SOG). (A–C) Schematic drawings of the SOG in a ventral (A), dorsal (B) and lateral (C) view. For orientation, the cells shown in D–H are indicated by dots in the drawings (D in A, E–H in B). In C, the planes of sectioning are indicated. Anterior is to the top in all panels. (D) Ventral section showing two very prominent cGMP-immunoreactive cells located between the roots of nerve 5. Note that this nerve also contains immunoreactive fibres. (E) Large anterior and lateral cGMP-immunoreactive neurones. (F) Dorsal anterior medial cells. (G) Ventral section showing immunoreactive fibres in the mandibular nerve (N1) and stained neuropilar regions along the midline. (H) Stained structures in the mandibular neuropil (anterior) and a posterior medial neuropilar area. N1–N7, nerves 1–7. Scale bars, 100 μm.

agonists can be blocked by NOS inhibitors. This suggests a NO-dependent and sGC-mediated rise in cGMP concentration in response to muscarinic agonists (Qazi and Trimmer, 1999). However, Qazi and Trimmer (Qazi and Trimmer, 1999) have also shown that this effect is not responsible for the increase in proleg motoneurone spike activity in response to either muscarinic agonists or NO donors. This means that, in larval *Manduca sexta*, both coupling between muscarinic and nitrergic pathways and, as in the proleg motoneurones, an independent parallel action of the two pathways upon a single target occur. Similarly, the possibility that, in the locust SOG, coupling of nitrergic and muscarinic pathways occurs cannot be ruled out, although this does not seem to apply for mandibular pattern generation.

Potential sources and targets of the NO/cGMP pathway

NADPH-diaphorase staining

NADPH-diaphorase staining is an indicator of the presence of NOS and has been widely used in insect nervous tissue (e.g. Dawson et al., 1991; Hope et al., 1991; Müller and Bicker,

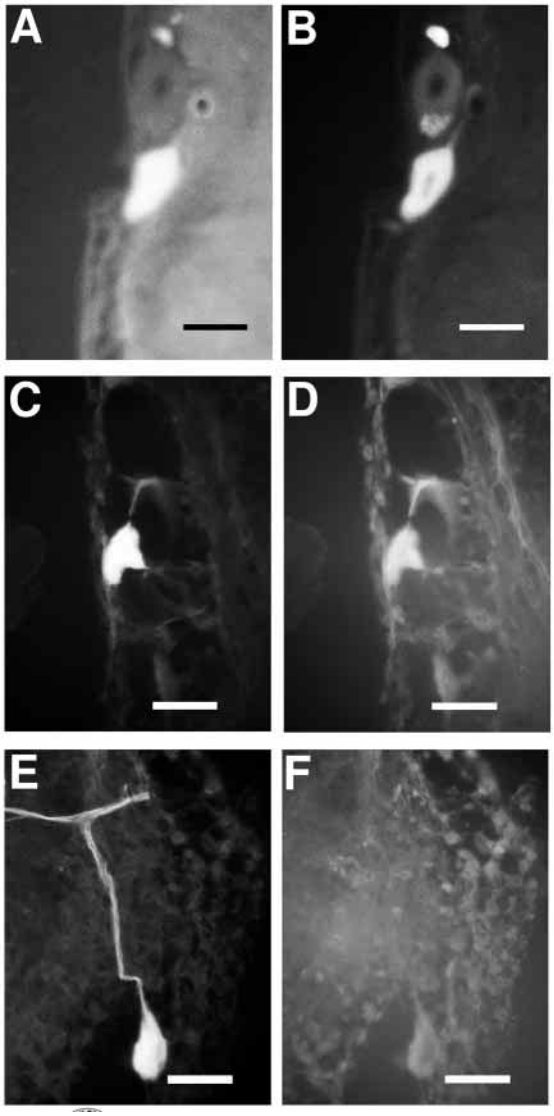

Fig. 9. Identified suboesophageal ganglion (SOG) neurones showing anti-cGMP immunoreactivity. (A) Mandibular closer motoneurone injected with Lucifer Yellow and photographed with an appropriate filter set. (B) The same neurone shows anti-cGMP immunostaining (CY3-labelled secondary antibody photographed with an appropriate filter set). (C) Salivary neurone 1 (SN1) labelled by backfilling of nerve 7B with dextran-amine-conjugated Lucifer Yellow. (D) SN1 is anti-cGMP-immunoreactive. (E) Salivary neurone 2 (SN2) labelled by backfilling of nerve 7B with dextran-amine-conjugated Lucifer Yellow. (F) SN2 does not show anti-cGMP immunoreactivity. Scale bars, 100 μm.

1994; Müller, 1994; Ott and Burrows, 1998). However, it is possible that the NADPH-diaphorase method stains cells that contain a NADPH-diaphorase that is insensitive to mild formaldehyde fixation and is different from NOS. It is also possible that some NOS may fail to be revealed even under mild fixation conditions (Truman et al., 1996; Ott and Burrows, 1999). The control experiments described, however, suggest that the staining obtained in the locust SOG is likely to be due to NOS activity.

In the locust SOG, a number of structures appear to express NOS (Fig. 7), and this supports the hypothesis that, in the intact animal, NO from local physiological sources may influence pattern generation. A comparison of the neural structures stained in the SOG with data from thoracic ganglia (Ott and Burrows, 1998; Ott and Burrows, 1999) is not possible at present. The structural organisation of the three fused neuromeres of the SOG is highly derived so that any correlation between homologous parts remains unsatisfactory. However, both in thoracic ganglia (Ott and Burrows, 1998) and in the SOG, the staining is confined mainly to local and intersegmental interneurones and, in both cases, large neuropilar areas show strong NADPH-diaphorase staining. The modulatory role of NO in information processing suggested by Ott and Burrows (Ott and Burrows, 1998) may, therefore, also hold for the SOG.

Anti-cGMP immunohistochemistry

Specificity controls for the anti-cGMP serum used are described by De Vente et al. (De Vente et al., 1987; De Vente et al., 1996). The high specificity and the extraordinarily high dilution used in the present study (1:600 000) make nonspecific cross reactions extremely unlikely, and this suggests that the cGMP-like immunoreactivity observed is the result of cGMP accumulation in the labelled cells. Since the treatment with SNP induces considerable neural activity in the ganglia, it is possible that some cGMP detected in response to SNP treatment may also be produced by membrane-bound guanylyl cyclases activated by chemical messengers released during neural activity induced by NO. For example, certain neurohormones are known to induce a NO-independent rise in cGMP levels (Ewer et al., 1994; Morton and Simpson, 1995; Baker et al., 1999).

There is cGMP-like immunoreactivity in both efferent neurones and sensory fibres (Fig. 8). Double-labelling experiments show that both mandibular closer motoneurones and salivary neurone 1 (SN1) are among the large anterior lateral neurones exhibiting anti-cGMP immunoreactivity (Fig. 9). Salivary neurone 2 (SN2) does not accumulate cGMP upon induction with NO (Fig. 9). The difference between the salivary neurones with regard to sGC expression emphasizes the potential functional difference between these neurones, which is also suggested by the different transmitters found in them (for a review, see Ali, 1997). The physiological role of cGMP production in these identified neurones is not yet clear, but cGMP might modulate their membrane properties to fine-tune their response to synaptic input provided by the central-pattern-generating network.

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