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OPPOSING ACTIONS OF NITRIC OXIDE ON SYNAPTIC INPUTS OF IDENTIFIED INTERNEURONES IN THE CENTRAL NERVOUS SYSTEM OF THE CRAYFISH

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Summary

Little is known of the action of nitric oxide (NO) at the synaptic level on identified interneurones in local circuits that process mechanosensory signals. Here, we examine the action of NO in the terminal abdominal ganglion of the crayfish *Pacifastacus leniusculus*, where it has modulatory effects on the synaptic inputs of 17 identified ascending interneurones mediated by electrical stimulation of a sensory nerve. To analyse the role of NO in the processing of sensory signals, we bath-applied the NO donor SNAP, the NO scavenger PTIO, the nitric oxide synthase (NOS) inhibitor L-NAME, the NOS substrate L-arginine, a cyclic GMP (cGMP) analogue, 8-Br-cGMP, and the soluble guanylate cyclase (sGC) inhibitor ODQ. The effects of these chemicals on the synaptic inputs of the interneurones could be divided into two distinct classes. The NO donor SNAP enhanced the inputs to one class of interneurone (class 1) and depressed those to another (class 2). Neither the inactive isomer NAP nor degassed SNAP had any effect on the inputs to these same classes of interneurone. The NO scavenger PTIO caused the opposite effects to those of the NO donor SNAP, indicating that endogenous NO may have an action in local circuits. Preventing the synthesis of NO using L-NAME had the opposite effect to that of SNAP on each response class of interneurone. Increasing the synthesis of endogenous NO by applying L-arginine led to effects on both response classes of interneurone similar to those of SNAP. Taken together, these results suggested that NO was the active component in mediating the changes in amplitude of the excitatory postsynaptic potentials. Finally, the effects of 8-Br-cGMP were similar to those of the NO donor, indicating the possible involvement of a NO-sensitive guanylate cyclase. This was confirmed by preventing the synthesis of cGMP by sGC using ODQ, which caused the opposite effects to those of 8-Br-cGMP on the two response classes of interneurone. The results indicate that a NO–cGMP signal transduction pathway, in which NO regulates transmitter release from mechanosensory afferents onto intersegmental ascending interneurones, is probably present in the local circuits of the crayfish.

Key words: NO–cGMP signalling pathway, neuromodulation, ascending interneurone, sensory neurone, local circuit, crayfish, *Pacifastacus leniusculus.*

Introduction

Detailed knowledge about the types of neurones comprising local circuits, their individual coding properties and their patterns of connectivity is often insufficient to allow us to predict the precise output of a circuit for any given input. This is because various modulatory mechanisms exist that can modify the neural signal at any level in the network so that it can be matched to appropriate patterns of behaviour or conditions. For example, depolarising synaptic potentials in the central terminals of sensory neurones (Kennedy et al., 1974; Rudomin, 1990a; Rudomin, 1990b; Schmidt, 1971) reduce the amplitude of presynaptic spikes and, hence, the effectiveness of synaptic transmission. Equally important is the role of neuromodulation in altering the properties of individual neurones (Elson and Selverston, 1992; Katz, 1999; Kiehn and Harris-Warrick, 1992) and their synaptic and network interactions (Dickinson et al., 1990; Harris-Warrick and Marder, 1991).

In recent years, much interest has been generated by the free radical nitric oxide (NO), and its role as a neuronal messenger has been well established. NO diffuses through the membranes of target cells, where it can activate soluble guanylate cyclase (sGC) and increase the level of the second messenger cyclic GMP (cGMP) (Bredt and Snyder, 1989). NO is generated by NO synthase (NOS) from L-arginine (Moncada et al., 1991; Stuehr and Griffith, 1992), and histochemical studies have established the distribution of NOS in the central nervous systems of both vertebrates (Bredt and Snyder, 1992; Vincent and Kimura, 1992) and invertebrates (Bicker et al., 1996; Elphick et al., 1996; Ott and Burrows, 1998). NO can modulate neurotransmitter release and has thus been implicated in synaptic plasticity in the central

nervous systems of many animals (Gelperin, 1994; Mothet et al., 1996; Schuman and Madison, 1994). Furthermore, NO–cGMP signalling is also implicated in the normal postembryonic development of the nervous system (Gibbs and Truman, 1998; Scholtz et al., 1998; Truman et al., 1996).

Many histochemical studies have pointed to an important role for NO in the nervous system of invertebrates (Elphick et al., 1996; Müller and Hildebrandt, 1995; Ott and Burrows, 1998), including the cerebral ganglion of crayfish (Talavera et al., 1995; Johansson et al., 1996), but the action of NO at identified synapses remains to be analysed in detail. The local circuits controlling the movements of the paired abdominal appendages of crayfish, the uropods, have been extensively analysed at the cellular level, although nothing is known of the action of NO on these circuits or of the distribution of NOS in the terminal ganglion. Identified ascending interneurones receive direct synaptic input from cholinergic mechanosensory afferents innervating receptors on and in the tailfan (Nagayama and Sato, 1993; Ushizawa et al., 1996). They are crucial components of local circuits in the terminal abdominal ganglion and are known to have intersegmental (Aonuma et al., 1994; Zucker, 1972) and local (Nagayama et al., 1993) output effects.

Recent studies indicate that NO probably acts as a neuromodulator at neuromuscular junctions in crayfish (Aonuma et al., 2000). Here, we show that NO also has a significant effect on local circuits and modulates synaptic transmission from sensory afferents onto identified ascending interneurones.

Materials and methods

Adult male and female crayfish *Pacifastacus leniusculus* Dana, 8–12 cm in body length (rostrum to telson), were used in all experiments. They were obtained commercially (Riversdale Farm, Dorset, UK) and maintained in laboratory tanks before use. We observed no differences in results between males and females.

The distal part of the abdomen, from the fourth segment, was isolated from the thorax and pinned ventral side up in a small chamber, 10 ml in volume, containing physiological saline (van Harreveld, 1936) at 20–24 °C. The swimmerets of all abdominal segments were removed, and the terminal (sixth) abdominal ganglion was exposed by removing the overlying fifth ventral sternite, the surrounding soft cuticle, the ventral aorta and connective tissue. The ganglion was supported on a silver platform for intracellular recordings.

Intracellular recordings and pharmacological experiments

Intracellular recordings from neuropilar processes of interneurones were performed using microelectrodes filled with 3 % Lucifer Yellow CH (Stewart, 1978) dissolved in 0.1 mol l^{-1} lithium chloride. Electrode resistance was approximately 120 MΩ. Mechanosensory afferents in the second nerve of the terminal ganglion, which innervate hairs on the exopodite, were stimulated electrically using a suction electrode. The stimulus intensity was set just below the threshold for generating spikes in interneurones, so that only subthreshold excitatory postsynaptic potentials (EPSPs) were elicited, and this intensity remained unchanged throughout an experiment. Nerve 2 was stimulated five times with a 3 s interpulse interval. This stimulus was repeated at 1 min intervals throughout the entire recording period and did not normally give rise to any significant depression of the synaptic inputs to an interneurone. If the inputs to any interneurone were depressed during the course of an experiment, then the data obtained from these interneurones were omitted from our analysis. Statistical significance in changes in EPSP amplitude was analysed by comparing the amplitudes of five EPSPs evoked under normal saline with those of five EPSPs evoked during application of each test chemical using paired *t*-tests.

All recordings were stored on a digital tape recorder (RD-101T, TEAC) and analysed on a computer running Spike2 software (Cambridge Electronic Design Ltd). Data are based on 216 successful recordings of ascending interneurones from 234 crayfish. Results obtained from crayfish in which the original response did not recover after washing were excluded from our analysis (with the exception of responses to 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, ODQ). Each identified interneurone was tested only once with each compound, with the exception of control tests using D-arginine, N^G-nitro-D-arginine methyl ester (D-NAME), *N*-acetyl-penisillamine (NAP) and degassed *S*-nitroso-*N*-acetyl-penisillamine (SNAP), when each test was followed by one using the appropriate active isomer. Compounds were never re-applied to the same preparation. The numbers of ascending interneurones tested with each compound are given in parentheses in Table 1.

After physiological examination, interneurones were stained by ionophoretic injection of Lucifer Yellow using 5–10 nA hyperpolarising current pulses of 500 ms duration at 1 Hz for 5–10 min. Ganglia containing the stained interneurones were then dissected from the animal, fixed in 10 % formalin, dehydrated and cleared using methyl salicylate. Stained preparations were observed using a Nikon fluorescence microscope (E-800), and images were captured using a cooled CCD video camera (Sony microMax 1300) and stored on an IBM-compatible computer using MetaMorph image-acquisition software. Interneurones were identified on the basis of their morphological characteristics as described by Nagayama et al. (Nagayama et al., 1994).

Bath application of drugs

All pharmacological agents, D-arginine, L-arginine, N^G-nitro-D-arginine methyl ester (D-NAME), N^G-nitro-L-arginine methyl ester (L-NAME), 2-Phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl3-oxide (PTIO) *S*-nitroso-*N*-acetyl-penisillamine (SNAP), *N*-acetyl-penisillamine (NAP), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), were obtained from Sigma Chemical Co. Ltd. Each chemical was kept in the dark and only dissolved in normal saline to the required concentration immediately prior (2–5 min) to application, with the exception of degassed SNAP which was maintained in the dark at room

Table 1. *Summary of the effects of drugs on evoked EPSPs in ascending interneurones*

Interneurone	SNAP	PTIO	L-Arg	L-NAME	8-Br-cGMP	ODQ	Alternative name
Class 1							
CA-1	Ex(1)	In(1)	Ex(1)		Ex(2)		6D1
RC-3	Ex(1)		Ex(5)	In(2)			Not described
RC-5			Ex(2)	In(3)			6A3
RC-7	Ex(3)	In(1)	Ex(7)	In(7)	Ex(2)	In(1)	6E3
RC-8	Ex(1)	In(1)		In(2)		In(1)	6C1/intC
RO-6	Ex(1)		Ex(1)				Not described
RO-7	Ex(1)	In(1)	Ex(1)	In(1)		In(1)	Not described
NE-1	Ex(4)	In(2)	Ex(2)	In(1)			6B1/intA
NE-2	Ex(3)	In(1)					6A6
NE-4	Ex(3)	In(1)	Ex(4)		Ex(1)		6A1/CPR
Class 2							
CI-2	In(7)	Ex(3)	In(2)	Ex(4)	In(2)		6A7/6B7
RC-1	In(2)		In(2)				6C2
RC-2	In(2)	Ex(1)	In(2)	Ex(3)		Ex(1)	6B6
RC-4	In(3)						6A5
RC-6			In(5)				6P1
RO-2	In(1)		In(2)				6B3
RO-5	In(6)	Ex(2)	In(3)	Ex(1)	In(1)	Ex(1)	6A2
*Unidentified							
VE-1	Ex(7), In(4)	Ex(1)	Ex(7), In(4)	Ex(3), In(5)	In(2)	In(2)	6D2
CI-3/RO-1/RO-3	Ex(2), In(3)		Ex(5), In(4)	Ex(1), In(5)	Ex(1), In(4)	Ex(1)	6B2/6B5
Unknown	Ex(1), In(1)		Ex(1), In(1)	Ex(2), In(2)		Ex(1)	Not described

Each interneurone was tested with only one compound in any given experiment.

Numbers in parentheses indicate the number of times that a particular interneurone was tested with the specified chemical.

Ex, excitatory effect; In, inhibitory effect.

*These interneurones cannot be distinguished on the basis of anatomical structure alone. Alternative names for these interneurones are based on anatomical similarities with previous descriptions (Sigvardt et al., 1982; Wilkens and Larimer, 1972; Zucker, 1972).

All chemicals caused significant changes in the amplitude of evoked potentials in class 1 and class 2 interneurones at $P<0.05$ using paired *t*-tests.

The full names of the drugs are given in the Materials and methods section.

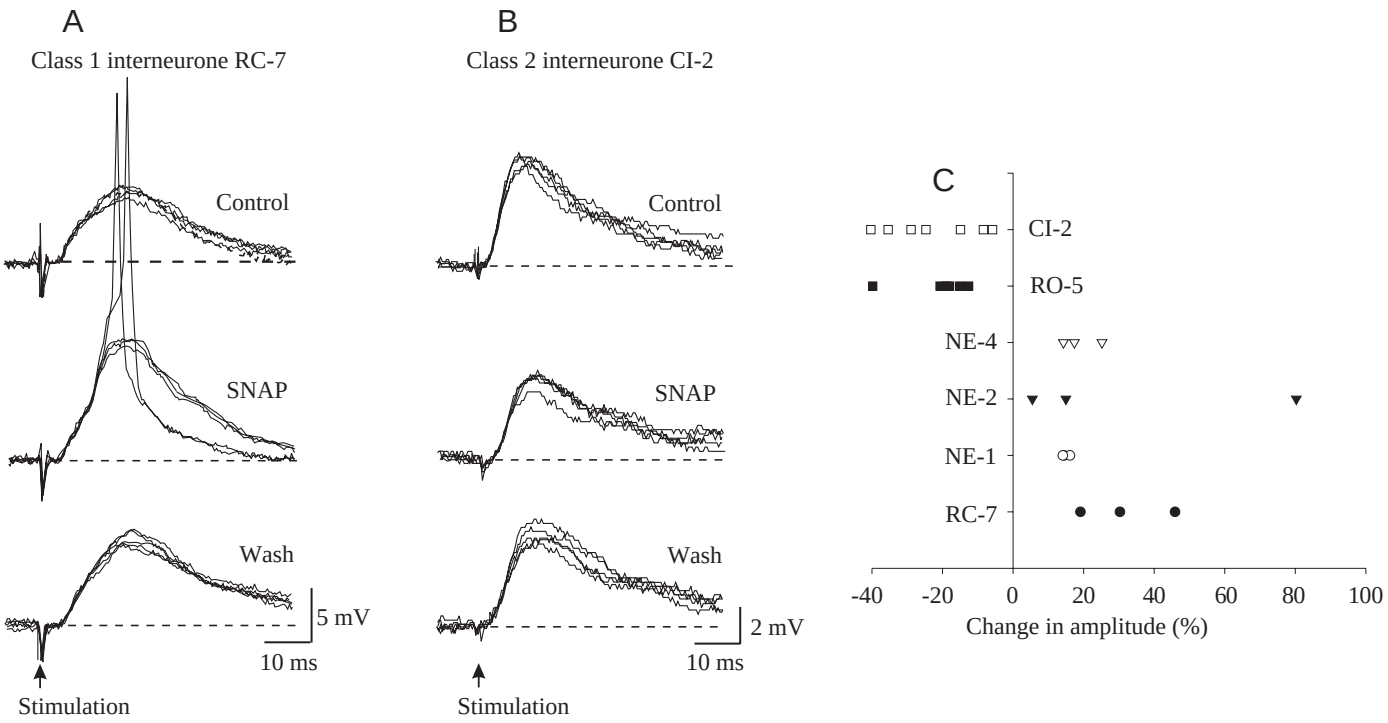
temperature (20–25 °C) for 24 h. The recording chamber was constantly perfused with cooled fresh saline using a microtube pump (502S, Watson-Marlow), and the bathing solution could be changed at a rate of 10 ml min^{-1} so that different drugs could be bath-applied. The latency between the onset of drug application and the achievement of 95 % of the applied concentration was measured by pumping a solution of Toluidine Blue through the recording chamber. A light source was directed through one side of the recording chamber, and its intensity was measured with a light-sensitive photodiode (Radio Spares) mounted on the opposite side of the chamber. The Toluidine Blue solution entering the chamber reduced the intensity of light detected by the photodiode, and the time taken to achieve 95 % of the applied concentration was calculated to be 81.4±3.0 s (mean ± S.E.M., N=4). The preparation was exposed to a drug for 5–6 min and then washed with normal saline.

Results

More than 30 pairs of intersegmental ascending interneurones have been identified on the basis of their morphological and physiological characteristics (Nagayama et al., 1994; Sigvardt et al., 1982). We have analysed the modulatory effects of nitric oxide on synaptic inputs from sensory afferents on 17 of these interneurones (Table 1). They are known to receive cholinergic inputs (Kennedy, 1971; Ushizawa et al., 1996) from exteroceptive water-motion- and touch-sensitive hairs on the uropods (Nagayama and Sato, 1993) and from a proprioceptive chordotonal organ that spans the joint between the two blade-like segments of the uropod, the exopodite and endopodite (Newland and Nagayama, 1993). Electrical stimulation of sensory neurones in nerve 2 of the terminal ganglion evokes EPSPs in all ascending interneurones. These EPSPs occur with a short and constant latency, increase in amplitude when an interneurone is hyperpolarised by constant current and gradually decrease in amplitude when the bathing saline is replaced by one containing no Ca^{2+}, suggesting that they are chemically mediated and monosynaptic (Nagayama and Sato, 1993).

Effects of exogenous NO on neurotransmission

To determine whether NO had any effect on the EPSPs that



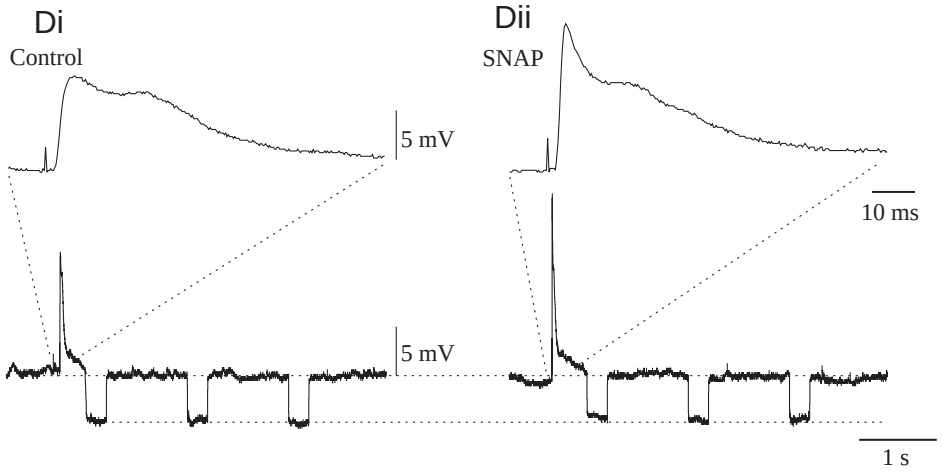


Fig. 1. The effects of the NO donor SNAP on synaptic potentials in ascending interneurones. (A) The response of a class 1 ascending interneurone, RC-7, to electrical stimulation. During bath application of SNAP, the amplitude of the evoked excitatory postsynaptic potentials (EPSPs) increased significantly and EPSPs sometimes gave rise to action potentials (middle trace). After a 20 min wash, the EPSP amplitude returned close to control levels. (B) The response of a class 2 ascending interneurone, CI-2, to electrical stimulation. The amplitude of EPSPs decreased significantly during bath application of SNAP (middle trace) and recovered to control levels after washing in normal saline for 20 min. (C) Graph showing the changes in amplitude of EPSPs caused by bath application of 200 μmol l^{-1} SNAP on specific interneurones. Each point represents the mean change caused by SNAP in different experiments, and the different symbols show the changes in amplitude caused in each type of interneurone tested. Note that the effects of SNAP were consistent for each interneurone: the inputs to class 1 interneurones were enhanced while those to class 2 interneurones were depressed. The standard errors of the mean (S.E.M.) have been omitted for clarity. (D) The responses of interneurone RC-3 to bath application of SNAP. (i) Electrical stimulation of the sensory nerve evoked a compound EPSP in the interneurone in control saline. The inset shows the EPSPs on a faster time scale. (ii) After a 5 min bath application of SNAP, the amplitude of the evoked potential increased. Neither the membrane potential of the interneurone nor the responses of the membrane to current pulses of −0.6 nA at 1 Hz was changed during bath application of SNAP. Three such current pulses can be seen during control tests and during the application of SNAP.

could be evoked in interneurones, we bath-applied the NO donor SNAP, which releases free-radical NO into solution and increases exogenous NO levels in the central nervous system. The application of SNAP had one of two effects on the EPSPs recorded in specific interneurones, either enhancing or depressing the evoked EPSPs (Fig. 1). These effects were consistent for the same identified interneurone recorded in different animals, i.e. the inputs to a particular identified interneurone were always modulated in the same way, as indicated in Table 1 where the values in parentheses indicate the number of times a given interneurone was tested with a particular drug.

On the basis of the change in amplitude of evoked synaptic inputs caused by SNAP, we have grouped the interneurones into

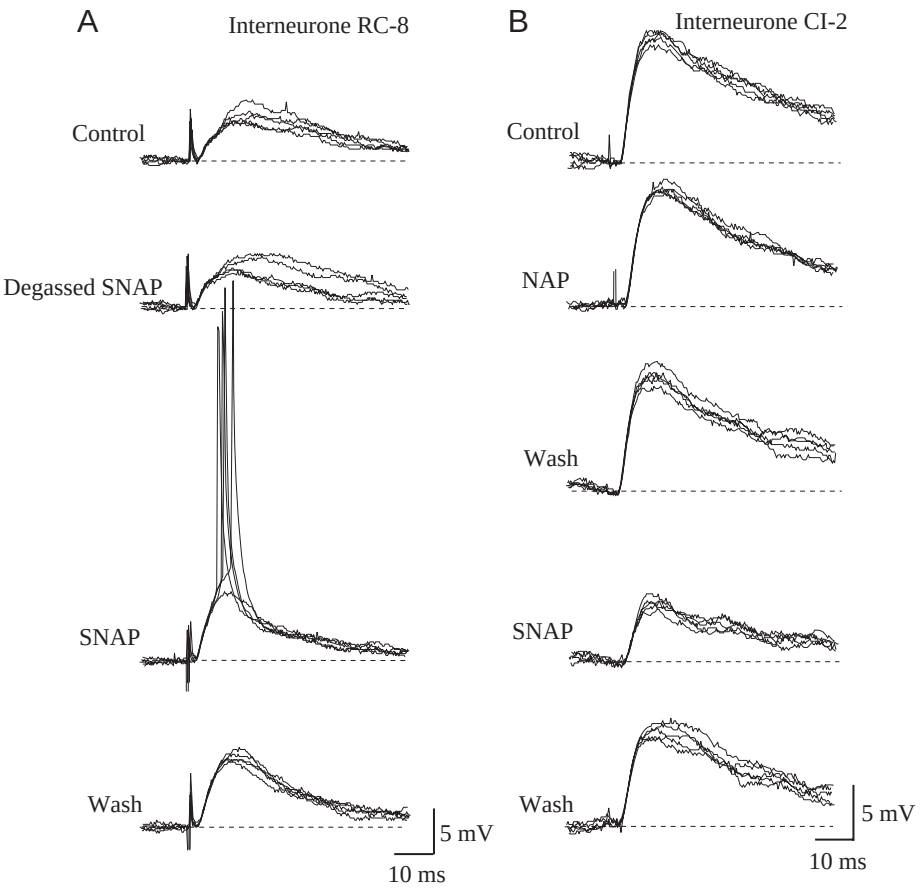


Fig. 2. Effects of degassed SNAP and NAP on synaptic inputs to interneurones. (A) Bath application of degassed 200 μmol l^{-1} SNAP caused no significant changes in the amplitude of evoked potentials in a class 1 interneurone, RC-8. After washing in normal saline (not shown) subsequent application of 200 μmol l^{-1} SNAP, for 5 min, during the same recording (third set of traces) significantly increased the amplitude of evoked potentials in the interneurone, which generally evoked spikes in the interneurone. The amplitude of the potentials during subsequent washing in normal saline gradually declined towards control levels (bottom set of traces). (B) Similarly, bath application of 200 μmol l^{-1} NAP (second set of traces) caused no significant changes in the amplitude of evoked potentials in a class 2 interneurone, CI-2. After washing in normal saline, application of 200 μmol l^{-1} SNAP caused a significant decrease in the amplitude of the evoked potentials (fourth set of traces), which gradually recovered to control levels after washing in normal saline.

two response classes: response class 1 interneurones, whose inputs were enhanced by NO, and response class 2 interneurones, whose inputs were depressed (this initial classification on the basis of the response to SNAP alone was extended to include all chemicals tested; see below). Thus, in nine class 1 interneurones, CA-1, RC-3, RC-7, RC-8, RO-6, RO-7, NE-1, NE-2 and NE-4, bath application of 200 μmol l^{-1} SNAP increased the amplitude of EPSPs by 26.16±3.8 % (mean ± S.E.M., N=21); the EPSPs sometimes gave rise to action potentials when the sensory neurones were stimulated electrically. During bath application of 200 μmol l^{-1} SNAP, the amplitude of EPSPs in interneurone RC-7, for example, increased significantly by 40 % (P<0.05) (Fig. 1A). After a 15 min wash in control saline, the EPSPs returned close to their initial amplitude. Where an interneurone was recorded more than once, SNAP always had the same significant excitatory effect on the inputs to that interneurone (Table 1). Inputs to interneurones CI-2 and RO-5 were always depressed by SNAP in different preparations, while those in RC-7, NE-1, NE-2 and NE-4 were always enhanced (Fig. 1C).

In contrast, in six class 2 ascending interneurones, CI-2, RC-1, RC-2, RC-4, RO-5 and RO-6 bath application of SNAP decreased the amplitude of EPSPs by 22.3±2.4 % (mean ± S.E.M., N=21). During bath application of 200 μmol l^{-1} SNAP, for example, the amplitude of EPSPs in interneurone CI-2 decreased significantly (P<0.05) to approximately 60 % of their initial amplitude (Fig. 1B). The effects of SNAP were completely reversible, and after washing for 15 min in normal saline the EPSP amplitude recovered to 80 % of the initial amplitude. The NO donor again had consistent inhibitory effects on the inputs to specific class 2 interneurones recorded in different animals (Fig. 1C; Table 1).

To determine whether NO acts on the pre- or postsynaptic neurone, we performed a series of experiments to examine the effects of SNAP on the input resistance of the ascending interneurones. For both class 1 and class 2 interneurones, the responses to 0.6 nA hyperpolarizing current pulses of 200 ms duration and passed through the recording electrode at 1 Hz were unaffected by bath application of SNAP, even though SNAP caused significant changes in the amplitude of the evoked potentials (Fig. 1D). Moreover, SNAP caused no consistent shifts in membrane potential (–55±1.7 mV, mean ± S.E.M., N=12) in any of the interneurones during the course of an experiment.

As controls, we bath-applied the compounds NAP, which is unable to release NO, and a degassed solution of SNAP and analysed their effects on the EPSPs evoked in the ascending interneurones during stimulation of nerve 2. Neither 200 μmol l^{-1} NAP (N=13) (Fig. 2B) nor 200 μmol l^{-1} degassed SNAP (N=7) (Fig. 2A) had any effect on EPSP amplitude in either class of interneurone, while subsequent bath application of SNAP following either of these control solutions caused significant changes in EPSP amplitude in the same interneurone (Fig. 2), indicating that NO was the active component in these experiments.

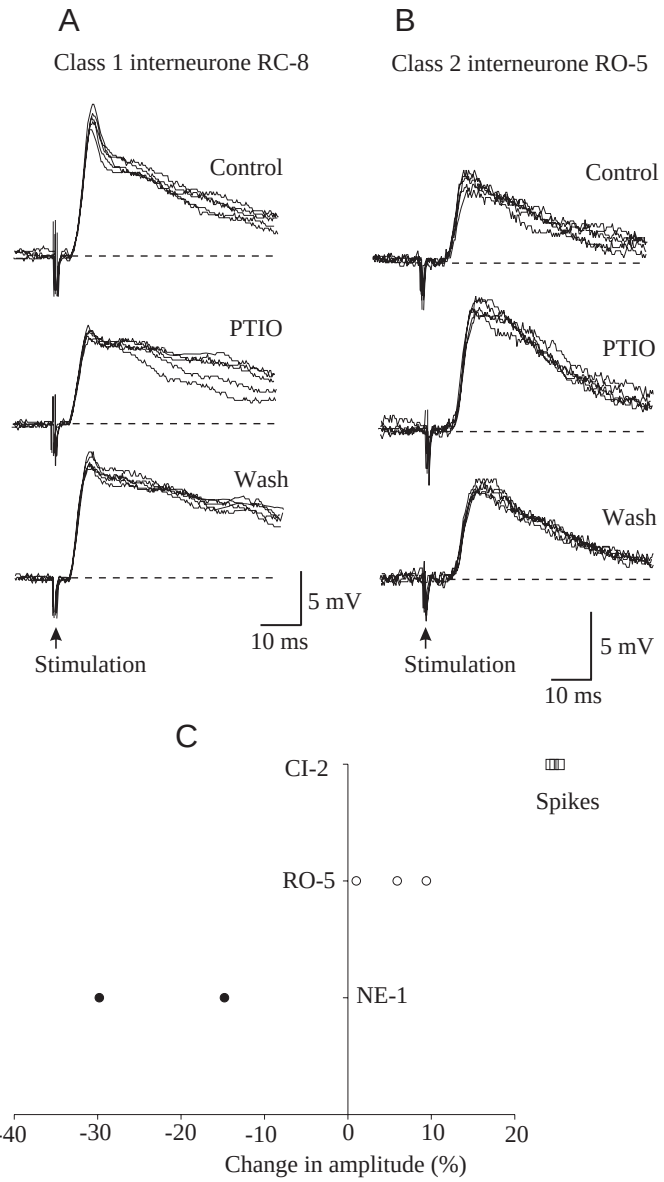


Fig. 3. Action of a NO scavenger, PTIO, on evoked synaptic potentials. (A) Bath applying 500 μmol l^{-1} PTIO during stimulation of mechanosensory nerve 2 reduced the amplitude of evoked potentials in a class 1 interneurone, RC-8. The effects of PTIO were completely reversible, and excitatory postsynaptic potentials (EPSPs) returned almost to control levels after a 20 min wash in normal saline. (B) The effects of PTIO on class 2 ascending interneurones were the opposite of those on class 1 interneurones, so that bath application of 500 μmol l^{-1} PTIO during stimulation of mechanosensory nerve 2 increased the amplitude of EPSPs recorded intracellularly from the interneurone, in this case RO-5. The effects of PTIO were significant (P<0.05). (C) Graph showing the changes in amplitude of EPSPs caused by bath application of PTIO on specific interneurones. Each point represents the mean change caused by PTIO in different experiments, and the different symbols show the changes in amplitude caused in each type of interneurone tested. The effects of PTIO were consistent for each interneurone, but were the opposite of those caused by SNAP. Note that EPSP amplitude could not be measured for interneurone CI-2 because application of PTIO led to an increase in excitability that caused each EPSP to lead to a spike.

Effects of a NO scavenger on evoked EPSPs

To analyse whether endogenous NO levels had any effect on EPSP amplitude, (PTIO), a scavenger of NO, was bath-applied during electrical stimulation of nerve 2. PTIO had the opposite effects to SNAP for any given interneurone (see Table 1) and for all class 1 interneurones caused a 33.2±4.6 % (mean ± S.E.M., N=8) reduction in EPSP amplitude. For example, after a 5 min bath application of PTIO, the EPSPs evoked in a response class 1 interneurone, RC-8, were reduced significantly by 38 % (P<0.05) (Fig. 3A). This effect was completely reversed after washing in normal saline for 20 min. In contrast, for the interneurones whose inputs were depressed by SNAP, class 2 interneurones, PTIO caused a 106±2.1 % (mean ± S.E.M., N=6) enhancement in EPSP amplitude. In the example shown in Fig. 3B, the inputs to interneurone RO-5 were enhanced significantly by 50 % (P<0.05) after a 5 min application of PTIO, but were again reversed after washing in normal saline. The effects of PTIO were consistent for the same identified interneurones recorded in different animals (Fig. 3C; Table 1).

Action of endogenous NO synthase

The synthesis of NO from L-arginine by the enzyme nitric oxide synthase (NOS) is inhibited by L-NAME, which had the opposite effects on the ascending interneurones to those of the NO donor SNAP. Thus, in six class 1 ascending interneurones, RC-3, RC-5, RC-7, RC-8, RO-7 and NE-1, bath application of L-NAME decreased the amplitude of EPSPs mediated by the stimulation of sensory neurones in nerve 2 by 26.7±4.3 % (mean ± S.E.M., N=14) (Fig. 4A) with no change in membrane potential. For example, before bath application of 10 mmol l^{-1} L-NAME, the amplitude of EPSPs in interneurone RC-7 elicited by the stimulation of root 2 were almost constant (Fig. 4B). During bath application of 10 mmol l^{-1} L-NAME, the amplitude of EPSPs decreased significantly to approximately 50 % of their initial size (P<0.05). After a 15 min wash with normal saline, the amplitude of EPSPs returned close to control levels (Fig. 4B).

In contrast, application of L-NAME increased the amplitude of EPSPs elicited by the same stimulation of sensory afferents (Fig. 4C) in three response class 2 ascending interneurones, CI-2, RC-2 and RO-5, by 20.4±3.6 % (mean ± S.E.M., N=8). Before bath application of 10 mmol l^{-1} L-NAME, the amplitude of EPSPs in the interneurone CI-2, for example, was approximately 9 mV. During bath application of 10 mmol l^{-1} L-NAME, the EPSP amplitude increased significantly (P<0.05) to approximately 13 mV (Fig. 4C), and EPSPs often gave rise to action potentials in the interneurone (Fig. 4D, middle traces). After washing for 5 min with normal saline, the action potentials disappeared, and after a 20 min wash in normal saline the amplitude of EPSPs returned gradually to control levels. The effects of L-NAME were consistent for the same identified interneurone recorded in different animals (Table 1). For example, the application of L-NAME during recording from interneurone CI-2 in different animals always led to an enhancement of the EPSP amplitude (Fig. 4E). In contrast,

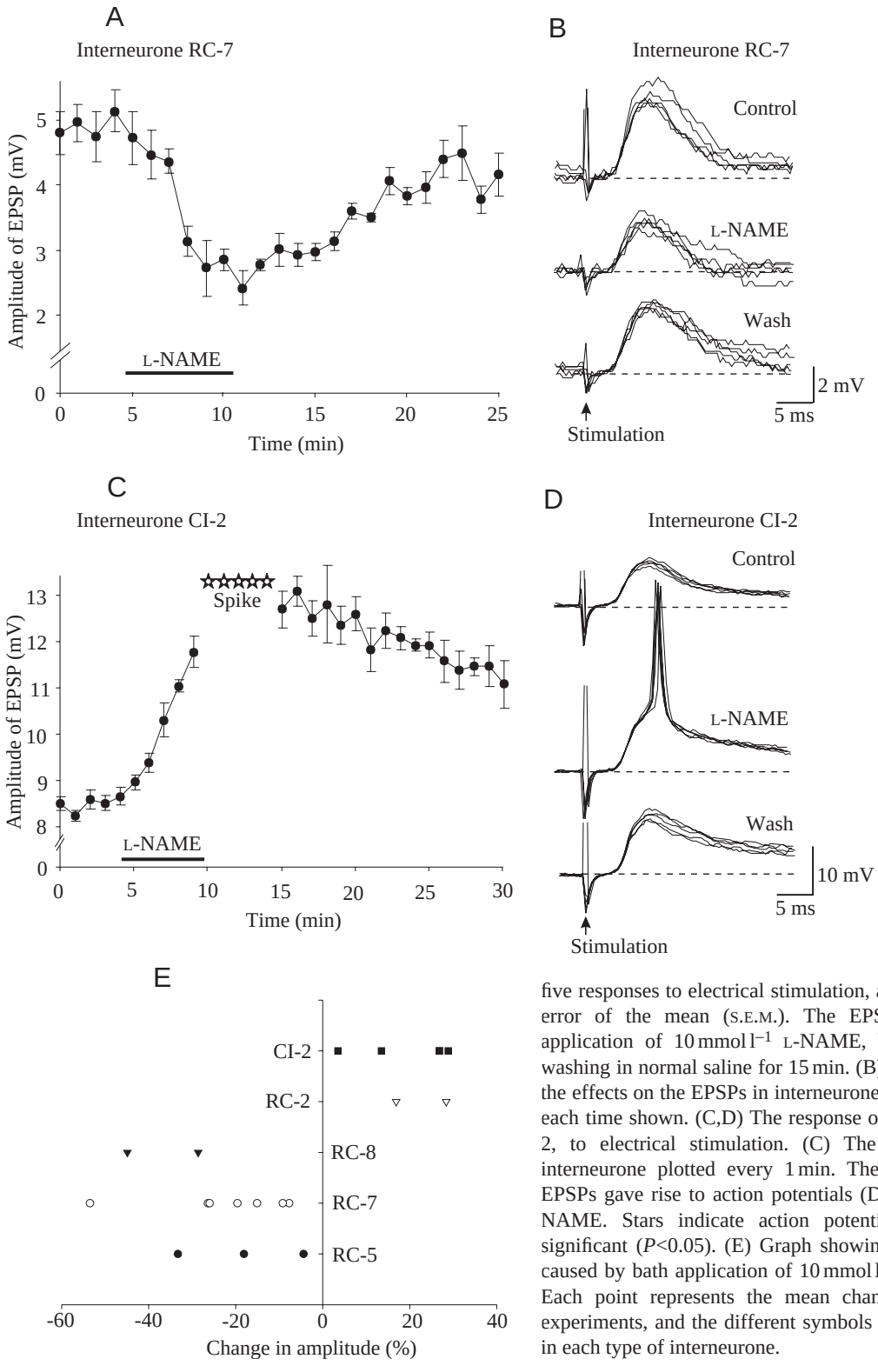


Fig. 4. Effects of 10 mmol l^{-1} L-NAME, a NO inhibitor, on excitatory postsynaptic potentials (EPSPs) evoked by the stimulation of sensory nerve 2. (A,B) The response of a class 1 ascending interneurone, RC-7, to electrical stimulation. (A) The change in EPSP amplitude of interneurone RC-7 was plotted every 1 min. Each point represents the mean of five responses to electrical stimulation, and the error bars indicate the standard error of the mean (S.E.M.). The EPSP amplitude decreased during bath application of 10 mmol l^{-1} L-NAME, but recovered to control levels after washing in normal saline for 15 min. (B) Examples showing the consistency of the effects on the EPSPs in interneurone RC-7. Five traces are superimposed at each time shown. (C,D) The response of a class 2 ascending interneurone, CI-2, to electrical stimulation. (C) The change in EPSP amplitude of the interneurone plotted every 1 min. The EPSP amplitude increased, and the EPSPs gave rise to action potentials (D) during application of 10 mmol l^{-1} L-NAME. Stars indicate action potentials. The effects of L-NAME were significant (P<0.05). (E) Graph showing the changes in amplitude of EPSPs caused by bath application of 10 mmol l^{-1} L-NAME on specific interneurones. Each point represents the mean change caused by L-NAME in different experiments, and the different symbols show the changes in amplitude caused in each type of interneurone.

EPSPs evoked in interneurone RC-7 in seven different animals were always depressed by L-NAME (Fig. 4E).

Bath application of the inactive D-isomer of NAME, D-NAME (Park et al., 1998), had no significant effect on the evoked sensory inputs in the identified interneurones (N=6) (Fig. 5). Subsequent application of L-NAME, however, increased the amplitude of the EPSPs by approximately 50 % (P<0.05).

To test further whether NO modulates transmission in the terminal ganglion, we tried to increase endogenous NO levels by increasing the concentration of L-arginine, the substrate for NO

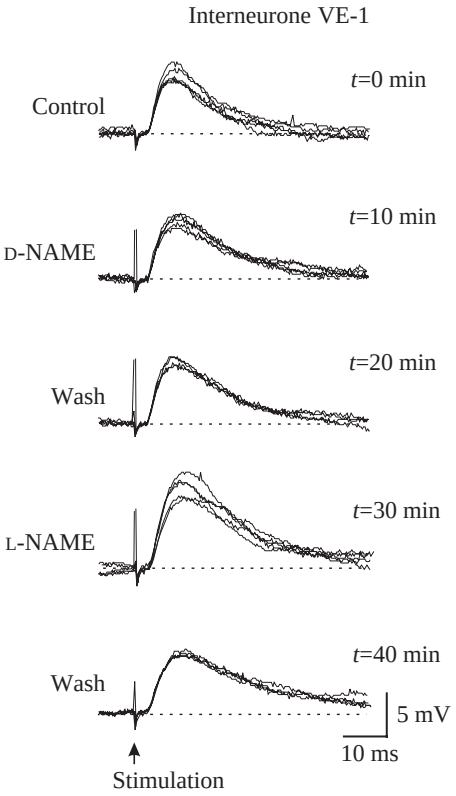


Fig. 5. Specificity of the effects of NAME on synaptic transmission. Bath application of the inactive isomer D-NAME (10 mmol l^{-1}) had no effect on interneurone VE-1. A further 10 min wash in normal saline followed by a 10 min application of 10 mmol l^{-1} L-NAME led to an increase in the amplitude of potentials evoked by electrical stimulation of nerve 2. This effect was reversible following a further wash in normal saline. *t*, time.

synthase (Mothet et al., 1996). While the use of L-arginine is an indirect test for the role of NO in modulating synaptic inputs to ascending interneurones, its effects are consistent with all the other test solutions we used (Table 1). Similar observations were made previously (Mothet et al., 1996) in an analysis of modulation of cholinergic transmission in *Aplysia californica*.

For eight response class 1 interneurones, CA-1, RC-3, RC-5, RC-7, RO-6, RO-7, NE-1 and NE-4, bath application of 1 mmol l^{-1} L-arginine increased the amplitude of EPSPs evoked during stimulation of nerve 2 by 25±7.6 % (mean ± S.E.M., N=15). L-Arginine caused no observable shift in the membrane potential of any of these interneurones, and its effect on any class 1 interneurone was consistent from animal to animal; it always enhanced the amplitude of synaptic inputs from the sensory neurones (see Table 1). For example, the amplitude of EPSPs in interneurone RC-7 elicited by stimulation of nerve 2 increased significantly by over 50 % (P<0.05) during bath application of 1 mmol l^{-1} L-arginine (Fig. 6A, middle traces) for 5 min, but returned close to control levels after a subsequent wash in normal saline for 15 min. In some preparations, the increased amplitude of the evoked EPSPs gave rise to action potentials in the ascending interneurone.

In the second response class of interneurones, the amplitudes of evoked EPSPs were consistently reduced, by 26.3±4.1 % (mean ± S.E.M., N=10), during bath application of 1 mmol l^{-1} L-arginine. This inhibitory effect on synaptic transmission was observed in six identified ascending interneurones, CI-2, RC-1, RC-2, RC-6, RO-2 and RO-5. In interneurone RO-5, for example, bath application of 1 mmol l^{-1} L-arginine for 5 min decreased significantly (P<0.05), and reversibly, the amplitude of EPSPs in interneurones by 37 % (Fig. 6B). The effects of L-arginine were always consistent for specific interneurones in different animals (Fig. 6E; Table 1). As in *Aplysia californica* (Mothet et al., 1996), bath application of the L-arginine enantiomer D-arginine had no effect on EPSP amplitude (N=6).

The responses of the remaining interneurones were variable from animal to animal (Table 1). The inputs to interneurone CI-3/RO-1/RO-3 and VE-1 were either enhanced and (N=5 and N=7, respectively) or reduced (N=4 and N=4, respectively) by bath application of L-arginine. This variability, however, is the result of problems in identifying these interneurones purely on the basis of anatomy. Interneurones CI-3, RO-1 and RO-3 all have similar morphologies but quite different physiological properties (Nagayama et al., 1993), and these physiological differences may account for the differing effects of L-arginine. Similarly, the anatomy of VE-1 is variable from crayfish to crayfish, indicating that there may be more than one interneurone of this type, which could again lead to the differing effects of NO found here. A similar variability of effects was also observed for SNAP and L-NAME.

The action of L-arginine on synaptic transmission was dose-dependent. The amplitude of evoked EPSPs in response class 1 ascending interneurones increased with increasing concentration during bath application of L-arginine. When 10 µmol l^{-1}, 100 µmol l^{-1}, 1 mmol l^{-1} and 10 mmol l^{-1} L-arginine were sequentially applied at 5 min intervals, the amplitude of the evoked EPSPs increased in a dose-dependent manner (Fig. 6C). The amplitude of the EPSPs during application of 10 mmol l^{-1} L-arginine was 52 % greater than that of EPSPs evoked by 10 µmol l^{-1} L-arginine. At higher concentrations, the EPSPs occasionally gave rise to action potentials, which were never seen at lower concentrations or during control tests. After approximately 10 min of washing, the amplitude of EPSPs in the interneurone recovered to near control levels.

These effects were consistent for interneurones of both response classes (Fig. 6D; Table 1). For class 1 interneurones, 1 mmol l^{-1} L-arginine increased the amplitude of synaptic inputs by 17.8±3.7 % (mean ± S.E.M., N=16), while 10 mmol l^{-1} L-arginine produced a greater increase of 56.9±24.7 % (mean ± S.E.M., N=3). In contrast, 1 mmol l^{-1} L-arginine reduced the amplitude of synaptic potentials in class 2 interneurones by 20.1±3.8 % (mean ± S.E.M., N=11), while 10 mmol l^{-1} L-arginine reduced the evoked potentials by 44.1±6.7 % (mean ± S.E.M., N=4).

NO may act through a NO–cGMP signalling pathway

One of the principal actions of NO is to activate soluble guanylate cyclase (sGC), which results in an increase in the level of the second messenger cGMP (Bredt and Snyder,

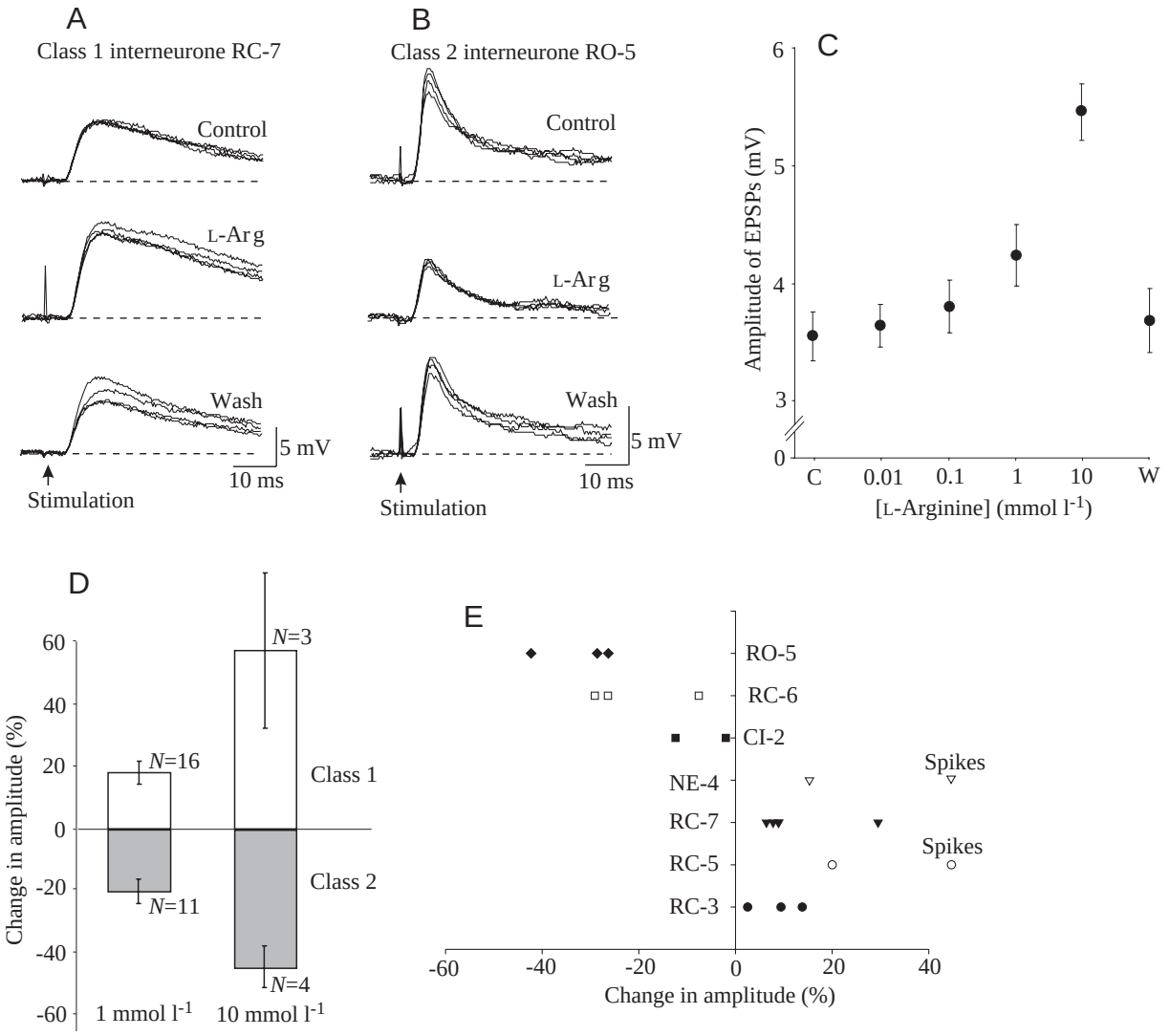


Fig. 6. Effects of L-arginine (L-Arg), a precursor of NO, on synaptic transmission. (A) The response of a class 1 ascending interneurone, RC-7, during electrical stimulation of the sensory nerve. The amplitude of excitatory postsynaptic potentials (EPSPs) increased during bath application of 1 mmol l^{-1} L-arginine (middle traces). After washing with normal saline for 15 min, the amplitude of EPSPs returned close to control levels. (B) The response of a class 2 ascending interneurone, RO-5, to stimulation of sensory nerve 2. During application of L-arginine, the amplitude of EPSPs decreased (middle traces). After a 15 min wash, the amplitude of EPSPs returned to control levels. (C,D) Dose-dependent effects of L-arginine on synaptic transmission. (C) The effects of L-arginine on interneurone RC-7. Each concentration of L-arginine was bath-applied sequentially, before the preparation was finally washed in control saline. *C*, control value; *W*, after washing. Each point represents the mean of 25 responses to stimulation, and the error bars indicate the standard error of the mean (S.E.M.). The data in this figure were collected from one animal. (D) The dose-dependent effects of L-arginine on class 1 and class 2 interneurones. Note that the higher concentration of L-arginine consistently caused larger changes in EPSP amplitude in the interneurones. The effects of L-arginine were significant ($P<0.05$). Values are means ± S.E.M. (E) Graph showing the changes in amplitude of EPSPs caused by bath application of 1 mmol l^{-1} L-arginine on specific interneurones. Each point represents the mean change caused by L-arginine in different experiments, and the different symbols show the changes in amplitude caused in each type of interneurone tested. Note that the effects of L-arginine on specific interneurones are consistent with the effects of the NO donor SNAP, but are the opposite of the effects of the NO scavenger PTIO and the NOS inhibitor L-NAME.

1989). To test whether a cGMP analogue had any effect on mechanosensory inputs to ascending interneurones, we bath-applied 8-Br-cGMP. Although 8-Br-cGMP is membrane-permeable, its molecular mass is much greater than those of the other chemicals used in this study. Therefore, in all experiments using 8-Br-cGMP, we desheathed the ventral surface of the terminal ganglion prior to drug application.

The effects of 8-Br-cGMP were similar to those of SNAP, again depending upon the response class of interneurone (Fig. 7). In three class 1 ascending interneurones, CA-1, RC-7 and NE-4, 8-Br-cGMP increased the amplitude of the EPSPs by 20.1±6.6 % (mean ± S.E.M., $N=5$). Bath application of 200 μmol l^{-1} 8-Br-cGMP significantly increased ($P<0.05$) the amplitude of EPSPs in interneurone RC-7 elicited by electrical

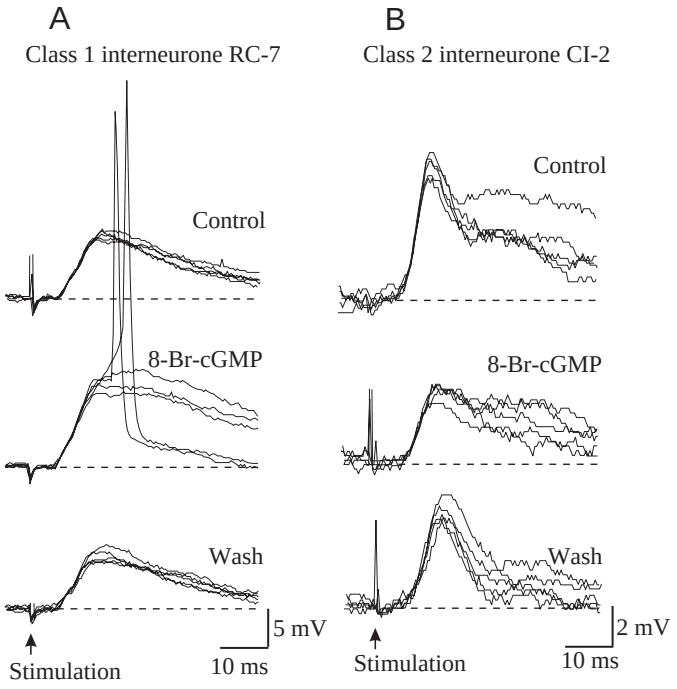


Fig. 7. The effects of 8-Br-cGMP on excitatory postsynaptic potentials (EPSPs) elicited by stimulation of sensory nerve 2. (A) The response of a class 1 interneurone, RC-7. The amplitude of EPSPs increased significantly during bath application of 200 μmol l^{-1} 8-Br-cGMP, and EPSPs sometimes gave rise to action potentials (middle trace). After a 20 min wash in normal saline, the amplitude of EPSPs returned to control levels. (B) The response of a class 2 interneurone, CI-2, to electrical stimulation. The amplitude of EPSPs decreased significantly during bath application of 200 μmol l^{-1} 8-Br-cGMP (middle trace), but recovered after washing.

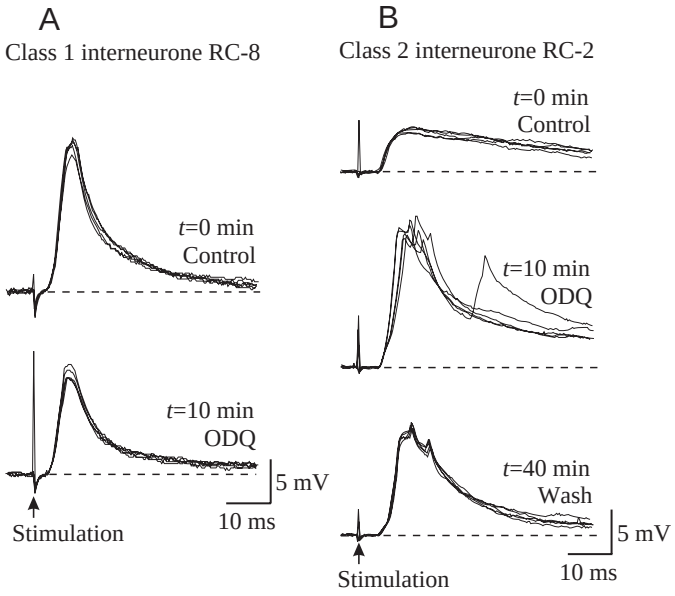


Fig. 8. Effects of the soluble guanylate cyclase inhibitor ODQ on synaptic transmission. (A) The responses of a class 1 interneurone, RC-8. The amplitude of excitatory postsynaptic potentials (EPSPs) decreased during bath application of 100 μmol l^{-1} ODQ (lower trace). The responses did not recover to control levels even after a 40 min wash with normal saline, when the electrode came out of the cell. (B) The responses of a class 2 interneurone, RC-2. The amplitude of EPSPs increased, and EPSPs gave rise to action potential during bath application of 100 μmol l^{-1} ODQ (middle trace). The effect of ODQ was partially reduced after a 40 min wash with normal saline. The effects of ODQ were significant (P<0.05). *t*, time.

stimulation (Fig. 7A), and EPSPs sometimes gave rise to action potentials (Fig. 7A, middle traces). After a 20 min wash with normal saline, the amplitude of EPSPs returned to their initial amplitude.

In two class 2 ascending interneurones, CI-2 and RO-5, 200 μmol l^{-1} 8-Br-cGMP significantly decreased the amplitude of the EPSPs mediated by stimulation of the sensory neurones by 22.3±3.5 % (mean ± S.E.M., N=9) (Fig. 7B). For example, the amplitude of EPSPs decreased significantly (P<0.05) by 40 % in interneurone CI-2 during bath application of 8-Br-cGMP (Fig. 7B). After a 15 min wash with normal saline, the amplitude of EPSPs returned to 80 % of their control levels. Thus, the synaptic inputs in interneurones that were enhanced by SNAP and L-arginine were also enhanced by 8-Br-cGMP, and those in interneurones that were depressed by SNAP and L-arginine were also depressed by 8-Br-cGMP (Table 1).

To prevent the synthesis of cGMP by sGC, we applied a membrane-permeable inhibitor of sGC, ODQ. Bath application of ODQ led to effects on the identified interneurones (Fig. 8) that were the opposite of those to 8-Br-cGMP. The actions of ODQ were consistent with those of the NOS inhibitor L-NAME, with the evoked inputs of class 1 interneurones (RC-7, RC-8 and RO-7) being depressed significantly (P<0.05) by 23.3±6.9 % (mean ± S.E.M., N=6) (Fig. 8A) and those of class 2 interneurones (RC-2 and RO-5) being enhanced significantly (P<0.05) by 113.8 % (N=2) (Fig. 8B). While the effects of ODQ are known to be reversible (Garthwaite et al., 1995), the amplitude of the EPSPs in the ascending interneurones only began to return to control levels after long periods of washing (Fig. 8B), a feature shared by metacerebral neurones in *Aplysia californica* (Koh and Jacklet, 1999). These long washing times meant that it was not always possible to maintain intracellular recordings for the time required to test reversibility for all interneurones encountered (Fig. 8A).

Discussion

NO–cGMP signalling modulates cholinergic synaptic transmission

This study shows that it is likely that NO is produced in the central nervous system of crayfish and that it can modulate synaptic transmission at central synapses between mechanosensory neurones and ascending interneurones through a NO–cGMP signalling pathway. We have shown that the efficacy of the synaptic transmission from sensory neurones to ascending interneurones is influenced by the NO donor SNAP and by the NO precursor L-arginine. The effects

of these chemicals on synaptic transmission were always similar, either enhancing the amplitude of evoked synaptic potentials (class 1 interneurones) or decreasing their amplitude (class 2 interneurones). While L-arginine acts indirectly, in that its application is used to increase the synthesis of endogenous NO (Mothet et al., 1996), its effects on the synaptic inputs of the intersegmental interneurones parallel those following the application of the NO donor SNAP, suggesting that the action of L-arginine is mediated through NO production.

In contrast, the NO scavenger PTIO and the NOS inhibitor L-NAME always had the opposite effects to those of SNAP and L-arginine on specific interneurones of both classes. Both compounds had significant modulatory effects on the inputs to these interneurones, suggesting that the enzyme NOS is present in the local circuits of the terminal abdominal ganglion and that it synthesizes NO.

A number of studies have reported a role for NO in activating sGC in many animals (Moncada et al., 1991; Southam and Garthwaite, 1991). Our analysis has shown that the application of the cGMP analogue 8-Br-cGMP mimicked the effects of SNAP and L-arginine, implying that NO could act on the inputs to these interneurones through a NO–cGMP signalling pathway. To confirm this, we applied an inhibitor of sGC, ODQ, which prevents the synthesis of cGMP. This also led to a significant modulatory effect on the inputs to the interneurones. These results indicate that NO is likely to be synthesised near the synaptic sites between the sensory neurones and ascending interneurones and that it may act on sGC to increase cGMP levels, through a NO–cGMP signalling pathway. In two-thirds of the ascending interneurones recorded in this study, the response class 1 interneurones, NO acted as an excitatory modulator to enhance the amplitude of the evoked potentials. In contrast, NO acted as an inhibitory modulator in one-third of the ascending interneurones, the response class 2 interneurones, to reduce the amplitude of evoked potentials. These opposing effects show that NO can regulate synaptic transmission in different ways at different synapses in local circuits.

NO acts as an intracellular messenger to stimulate sGC, which causes an accumulation of the second messenger cGMP in target cells. This NO–cGMP signalling pathway is found in both vertebrate (Arnold et al., 1977; Miki et al., 1977; Murad et al., 1978; Garthwaite et al., 1988; Garthwaite et al., 1989; East and Garthwaite, 1991) and invertebrate (Elphick et al., 1993) nervous systems. In crustaceans, NOS has been detected in the cerebral ganglion of crayfish using NADPH-diaphorase histochemical staining techniques (Johansson and Carlberg, 1994; Talavera et al., 1995; Johansson et al., 1996), but the actual effects of NO on the local circuits of the terminal ganglion have, until now, been the subject of much speculation.

Nitrergic modulation can be mediated by sequential changes in intracellular Ca^{2+} levels in presynaptic neurones. Excitatory modulation, therefore, could be mediated by an elevation of intracellular Ca^{2+} levels through NO-dependent pathways in the sensory neurones themselves. There are two main pathways in which NO increases intracellular Ca^{2+} levels to facilitate transmitter release. The first is an increase in cGMP levels by the activation of sGC through a NO–cGMP signalling pathway (Moncada et al., 1991). The second is *via* an increase in ADP ribosylation by NO (Brüne and Lapetina, 1989; Wroblewski et al., 1991; Williams et al., 1992), which causes an increased release of Ca^{2+} from internal stores (Lee et al., 1994). One explanation of our results is that NO acts to increase the level of cGMP, which in turn is acting to change Ca^{2+} levels, probably in the sensory neurones themselves.

The opposing effects of NO on synaptic transmission from sensory neurones to ascending interneurones could be due (i) to its action on different downstream enzymes, leading to opposite effects on voltage-dependent ionic fluxes, (ii) to its action on the regulation of intracellular Ca^{2+} concentration by presynaptic buffers or (iii) to an action on different molecular targets, such as presynaptic proteins, involved in the machinery for transmitter release. For example, the accumulation of cGMP has been thought to decrease intracellular Ca^{2+} levels through activation of a Ca^{2+}-ATPase and subsequent removal of Ca^{2+} from the cell (Rashatwar et al., 1987).

Possible role of NO in the central nervous system of the crayfish

NO has the ability to influence all neurones in close proximity to those generating it since it diffuses readily in three dimensions (Philippedes et al., 2000). It could act, at least in part, presynaptically on the sensory neurones to regulate transmitter release in the terminal ganglion. While NO is known to mediate synaptic plasticity, such as long-term facilitation and depression, in mammals (Schuman and Madison, 1994), its actions in the invertebrate nervous system remain to be examined in detail at the physiological level. Most studies on NO in invertebrates have concentrated on the localisation of NO-generating neurones or target cells of NO using histochemical methods. These studies strongly suggest the possibility that NO could act in the central nervous systems in invertebrates. In insects, for example, potential NOS-containing cells have been found in the cerebral ganglion, in particular in the mushroom bodies (Bicker et al., 1996; O'Shea et al., 1998). The mushroom bodies are thought to be involved in learning and memory (Heisenberg, 1980; Heisenberg, 1998; Erber et al., 1987), and therefore NO could potentially affect memory in the invertebrate nervous system. Moreover, NO mediates buccal motor patterns and is thought to be involved in the regulation of the feeding programme in *Lymnaea stagnalis* (Moroz et al., 1993).

Recent anatomical studies on the thoracic nervous system of locusts suggest that NO may play a major role in synaptic transmission in local circuits (Ott and Burrows, 1998; Ott and Burrows, 1999). Those studies suggest that sensory neurones and spiking interneurones are the main targets for the action of NO. Moreover, cGMP immunostaining in locusts has indicated the possibility of a parallel action of NO on different classes of interneurone within local circuits (Ott et al., 1999). Ott et al. (Ott et al., 1999) showed that all sensory neurones expressed

sGC throughout adult life. Taken together, these results strongly support the idea of a general role for NO–cGMP signalling in local circuits controlling limb movements, possibly by acting on the sensory neurones themselves. Our physiological studies on local circuit neurones in crayfish support those ideas since the lack of effect of any chemical used on the membrane potential and the lack of change in input impedance are indicative of a presynaptic site of action of NO. If this were the case, our results suggest that specific sensory neurones could be differentially modulated by NO either directly or indirectly through regulation of presynaptic inhibition (Newland et al., 1996).

Ascending interneurones receive many types of sensory input, including exteroceptive and proprioceptive information (Newland and Nagayama, 1993). They have outputs on both the uropod motor system (Nagayama et al., 1993) and on the abdominal postural motor system (Aonuma et al., 1994). Thus, these interneurones act not only as intrasegmental premotor neurones but also as intersegmental coordinators. Some ascending interneurones also have outputs onto the lateral giant interneurones, which activate motor giant neurones to produce tail-flip escape behaviour (Zucker, 1972). The consistent, but opposite, effects of NO on the two different classes of interneurone described in this study suggest that sensory information could be selectively enhanced or reduced at an early stage of input by nitrergic modulation.

Why the synaptic inputs to one group of interneurones should be enhanced while those of others should be depressed is not yet clear. For many of the interneurones described in this study, we know in detail their anatomy, their local output effects on uropod opener and closer motor neurones (Nagayama et al., 1993), their intersegmental output effects on abdominal superficial extensor motor neurones (Aonuma et al., 1994) and their patterns of projection along the ventral nerve cord (Kennedy, 1971; Sigvardt et al., 1982). Nevertheless, we have not yet found a correlation between the effects of NO modulation and any of these properties of the interneurones. Further anatomical analyses of NADPH-diaphorase staining and immunohistochemical analysis of sGC and cGMP localisation in the terminal ganglion may help to provide a basis for understanding the specific pharmacological effects we describe here. This study indicates, however, that endogenous NO modulates synaptic transmission in the crayfish central nervous system and represents a further step forward in our understanding of the ways in which neuromodulation can alter the processing of sensory signals in local circuits.

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