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NITRIC OXIDE SYNTHESIS IN LOCUST OLFACTORY INTERNEURONES

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Summary

The brain of the locust *Schistocerca gregaria* contains a nitric oxide synthase (NOS) that has similar properties to mammalian neuronal NOS. It catalyses the production of equimolar quantities of nitric oxide (NO) and citrulline from L-arginine in a Ca^{2+}/calmodulin- and NADPH-dependent manner and is inhibited by the N^{ω}-nitro and N^{ω}-monomethyl analogues of L-arginine. In Western blots, an antiserum to the 160 kDa rat cerebellar NOS subunit recognises a locust brain protein with a molecular mass of approximately 135 kDa. NOS is located in several parts of the locust brain, including the mushroom bodies, but it is particularly abundant in the olfactory processing centres, the antennal lobes. Here it is present in two groups of local interneurones (a pair and a cluster of about 50) that project into the neuropile of the antennal lobes. The processes of these neurones terminate in numerous glomerulus-like structures where the synapses between primary olfactory receptor neurones and central interneurones are formed. NOS-containing local interneurones have also been identified in the mammalian olfactory bulb, suggesting that NO performs analogous functions in locust and mammalian olfactory systems. As yet, nothing is known about the role of NO in olfaction, but it seems likely that it is involved in the processing of chemosensory input to the brain. The locust antennal lobe may be an ideal 'simple' system in which this aspect of NO function can be examined.

Key words: *Schistocerca gregaria*, insect, antennal lobe, nitric oxide synthase, NADPH diaphorase.

Introduction

Nitric oxide (NO) is now recognised as a neuronal signalling molecule in the mammalian brain (Knowles *et al.* 1989; Bredt and Snyder, 1992). While its precise physiological roles in the brain are not yet known, NO has been proposed as a mediator in two models of synaptic plasticity, long-term potentiation in the hippocampus (Schuman and Madison, 1991) and long-term depression in the cerebellum (Shibuki and Okada, 1991).

NO is synthesised in nitrergic neurones by a Ca^{2+}/calmodulin-activated nitric oxide synthase (NOS) which catalyses the conversion of L-arginine and molecular oxygen to NO and citrulline. Neuronal NOS requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cosubstrate and is inhibited by analogues of L-arginine such as N^{ω}-nitro-L-arginine (L-NNA) and N^{ω}-monomethyl-L-arginine (L-NMMA). NOS has been purified from the brain of several mammalian species and in its native state exists as a homodimer of a protein that has a molecular mass of about 160 kDa (Steuhr and Griffith, 1992; Knowles and Moncada, 1994). Cloning and sequencing of the cDNA encoding this protein revealed recognition sites for a variety of cofactors, including NADPH, calmodulin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Bredt *et al.* 1991*b*). The cofactor (6R)-tetrahydro-L-biopterin (H_4B) has also been implicated in the regulation of NOS since it can stimulate activity of the enzyme (Mayer *et al.* 1990).

The distribution of NOS in the mammalian brain has been established using immunocytochemistry and *in situ* hybridisation (Bredt *et al.* 1991*a*). It is exclusively located in neurones, and particularly high densities of NOS-containing neurones are found in the cerebellum, the dentate gyrus of the hippocampus and the main and accessory olfactory bulbs. Another technique that has been used to localise NOS in the nervous system is NADPH diaphorase histochemistry. This method stains NOS-containing neurones in paraformaldehyde-fixed mammalian brain tissue (Hope *et al.* 1991; Dawson *et al.* 1991), and its apparent specificity may be due to inactivation of other cellular NADPH diaphorases during fixation (Matsumoto *et al.* 1993). It is not known whether this is unique to mammals or to what extent NADPH diaphorase staining can

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be used as an indicator of NOS in the nervous systems of other animal groups.

We are interested in establishing whether NO is utilised as a signalling molecule in the simpler nervous systems of invertebrates. To address this question, we have investigated the presence of NOS in the brain of an insect species, the locust *Schistocerca gregaria* (Elphick *et al.* 1993). This study provided evidence for an arginine-metabolising enzyme generating citrulline in a Ca^{2+}- and NADPH-dependent manner and inhibited by calmodulin antagonists and by L-NNA and L-NMMA. Furthermore, NO, which in the mammalian brain acts through the stimulation of guanylyl cyclase in target cells, causes an increase in cyclic guanosine monophosphate (cGMP) levels in the locust brain. It appears, therefore, that the NO–cGMP signalling pathway may be evolutionarily ancient.

We now demonstrate Ca^{2+}/calmodulin- and NADPH-dependent synthesis of both NO and citrulline from L-arginine in extracts of locust brains using specific detection methods. Futhermore, we show that the locust brain contains proteins that are recognised by antisera to mammalian NOS, pointing to similarities in structure between insect brain NOS and mammalian forms of this enzyme. In addition, we have used NADPH diaphorase histochemistry to examine the distribution of NOS in the locust brain as an indicator of possible functional roles for NO in the insect central nervous system. This information will provide the basis for development of 'simple' physiological preparations in which NO function can be studied.

Materials and methods

Animals and chemicals

Locusts (*Schistocerca gregaria* L.) used in this study were reared in our laboratory cultures or purchased from Blades Biological (Edenbridge, Kent, UK). Animals used were mature adults (male and female) unless otherwise stated. Chemicals used were purchased from Sigma unless otherwise stated.

Preparation of tissue extracts

Tissue extracts were prepared from homogenates of locust brains (without optic lobes) or from parts of brains. Tissue was homogenised using either a Teflon–glass or Ystral homogeniser at 4 °C in one of two buffers. Buffer A contained 50 mmol l^{-1} Tris–HCl (pH 7.4), 5 mmol l^{-1} dithiothreitol, 1 μg ml^{-1} leupeptin, 10 μg ml^{-1} chymostatin, 10 μg ml^{-1} bestatin, 1 μg ml^{-1} pepstatin A and 2 mmol l^{-1} phenylmethylsulphonyl fluoride. Buffer B contained 50 mmol l^{-1} Tris–HCl (pH 7.4), 0.5 mmol l^{-1} dithiothreitol, 20 μmol l^{-1} leupeptin, 2 μmol l^{-1} pepstatin A, 10 μmol l^{-1} *N*-[*N*-(L-3-*trans*-carboxyoxiram-2-carbonyl)-L-leucyl]-agmatine and 100 μmol l^{-1} 4-(amidophenyl)methanesulphonyl fluoride. Homogenates were centrifuged at 100 000 ***g*** for 1 h (4 °C) and the supernatant was collected and tested immediately. Total protein concentrations in tissue extracts were determined using the Coomassie Plus protein assay reagent (Pierce Chemical) with bovine serum albumin (BSA) as a standard.

Measurement of citrulline synthesis

Synthesis of citrulline from L-arginine in locust brain extracts was monitored by measuring conversion of [^{3}H]arginine to [^{3}H]citrulline in the presence of added NADPH, Ca^{2+}, FMN, FAD, H_4B and calmodulin, all cofactors required for maximal activity of the mammalian neuronal NOS. High-performance liquid chromatography (HPLC) combined with liquid scintillation counting was used to identify and quantify citrulline and arginine.

Samples of tissue extract (50 μl containing 10–50 μg of protein in buffer B) were incubated in a total volume of 100 μl with 65 nmol l^{-1} L-[2,3,4,5-^{3}H]arginine monohydrochloride (2.6 TBq $mmol^{-1}$, from Amersham International plc), 1 mmol l^{-1} NADPH, 1 mmol l^{-1} $CaCl_2$, 20 μmol l^{-1} arginine hydrochloride (Novabiochem), 25 mmol l^{-1} valine (to inhibit arginase activity), 5 μmol l^{-1} FMN, 5 μmol l^{-1} FAD, 10 μmol l^{-1} H_4B, 10 μg ml^{-1} bovine brain calmodulin and 50 mmol l^{-1} Tris–HCl (pH 7.4). The samples were incubated for 1 h at 37 °C and reactions were stopped by the addition of trifluoroacetic acid to 5 % (v/v). Each sample was then placed in a boiling water bath for 5 min, centrifuged to remove precipitated proteins and the supernatant stored at –80 °C until analysed. A quarter of the complete reaction mixture was fractionated by HPLC (ABI model 172 microbore system) using a C18 column (2.1 mm×250 mm; Hewlett-Packard) equilibrated in an aqueous buffer at pH 5.0 containing 25 mmol l^{-1} orthophosphoric acid (BDH), 10 mmol l^{-1} hexanesulphonic acid (Aldrich) and 1 % acetonitrile (Rathburn Chemicals, Scotland, UK). The column was eluted isocratically at 250 μl min^{-1}; fractions (45 s each) were collected for 18 min and further analysed by liquid scintillation counting. Fractions containing [^{3}H]arginine and [^{3}H]citrulline were identified by comparison with the elution times of standards.

Measurement of nitric oxide synthesis

NO synthesis in tissue extracts was measured using a method based on that of Feelisch and Noack (1987) and modified by Knowles *et al.* (1990). The oxidation of oxyhaemoglobin to methaemoglobin by NO is monitored by measuring the absorption difference between 401 and 421 nm at 37 °C in a dual-wavelength recording spectrophotometer (Shimadzu UV-3000) using a bandwidth of 2 nm. Tissue extract (25–100 μl) was incubated with 500 μl of a solution containing 1.5 μmol l^{-1} oxyhaemoglobin, 1.2 mmol l^{-1} magnesium chloride, 50 mmol l^{-1} potassium phosphate (pH 7.2) and 0.1 mmol l^{-1} L-arginine. $CaCl_2$ (0.2 mmol l^{-1}), bovine brain calmodulin (50 units) and NADPH (1 mmol l^{-1}) were then added sequentially. The rate of NO synthesis over a period of approximately 2 min was calculated by using the molar extinction coefficient of methaemoglobin for the wavelength pair 401 nm minus 421 nm (77 400 l mol^{-1} cm^{-1}). L-NMMA (1 mmol l^{-1}) or the calmodulin antagonist *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulphonamide (W-13, 0.5 mmol l^{-1}) was added to the incubation to establish whether NO synthesis could be terminated by known inhibitors of mammalian neuronal NOS.

Western blotting of locust brain nitric oxide synthase

NOS was partially purified from locust brain extracts using 2′,5′-adenosine dinucleotide phosphate (2′,5′-ADP) sepharose/agarose affinity chromatography (Elphick *et al.* 1994). In brief, a 3 ml extract (in buffer A) of 300 locust brains was continuously mixed with 2′,5′-ADP–sepharose (100 μl settled volume) for 30 min at 4 °C, then centrifuged for 3 s in a microcentrifuge. The pellet was successively washed (three times in 1 ml for each wash) by resuspension and centrifugation as above, first with buffer A, then with 0.5 mol l^{-1} NaCl in buffer A and again with buffer A. Proteins were eluted from the 2′,5′-ADP sepharose by mixing with 100 μl of 10 mmol l^{-1} NADPH in buffer A for 15 min. Samples of the eluate were analysed by electrophoresis on 7.5 % sodium dodecyl sulphate–polyacrylamide and then blotted onto a nitrocellulose membrane. After blocking with 3 % BSA in Tris-buffered saline (TBS; pH 7.5) overnight at 4 °C, the membrane was incubated for 1 h at room temperature with an antiserum to native rat cerebellar NOS (Springall *et al.* 1992) or an antiserum raised to purified mouse macrophage (J774 cell line) NOS (Hamid *et al.* 1993) at a dilution of 1:100. These antisera have been shown to be highly specific for NOS proteins and do not cross-react with other proteins at the dilution used here (Springall *et al.* 1992; Hamid *et al.* 1993). Detection of the immunoreactive proteins was performed as described by Springall *et al.* (1992).

Localisation of nitric oxide synthase

NOS was localised in the locust brain using NADPH diaphorase histochemistry and by immunocytochemistry using an antiserum raised to recombinant rat cerebellar NOS, expressed in a baculovirus system (Charles *et al.* 1993).

For NADPH diaphorase histochemistry, the exoskeleton was dissected from the anterior face of locust heads to expose the brain. Heads were then fixed with 4 % paraformaldehyde in phosphate-buffered saline (PBS; pH 7.3) at 4 °C for 4 h and processed for whole-mount NADPH diaphorase staining or for cryostat sectioning.

Whole mounts

After fixation, heads were left overnight at 4 °C in 50 mmol l^{-1} Tris–HCl (pH 7.5) containing 2 % Triton X-100. The next day, the heads were washed in Tris–HCl (3×15 min) and incubated with 1 mmol l^{-1} NADPH and 0.25 mmol l^{-1} Nitro Blue Tetrazolium (NBT) in Tris–HCl at room temperature (23–28 °C) in the dark, for 1 h. After washing in Tris–HCl, the brains were photographed and then dissected out of the head, dehydrated through an ethanol series, incubated in xylene and mounted in Fluormount (BDH).

Cryostat sections

After fixation, heads were cryo-protected with 10 % sucrose in PBS overnight at 4 °C. The brains were dissected out (with or without optic lobes), embedded in Tissue-Tek (Miles Inc.) and frozen in liquid N_2. Sections (20–40 μm) were cut using a Leica cryostat, mounted on chrome-alum/gelatin-coated glass slides, left to dry at room temperature, washed in 50 mmol l^{-1} Tris–HCl (pH 7.5), incubated in NADPH/NBT as described above, washed in deionized water and then dehydrated and mounted as above.

For immunocytochemistry, the locust brains were fixed with 1 % paraformaldehyde in PBS and processed for cryostat sectioning as described above. After washing in Tris-buffered saline (TBS; pH 7.6; 3×15 min), slides were incubated overnight at room temperature with an antiserum to recombinant rat cerebellar NOS diluted 1:500 in TBS/0.2 % Triton X-100. Slides were then washed with TBS (3×15 min), incubated for 1 h with peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO, 1:100 in TBS/0.2 % Triton X-100), washed with TBS (3×15 min), incubated for 1 h with peroxidase-conjugated rabbit anti-peroxidase complex (DAKO, 1:200 in TBS/0.2 % Triton X-100), washed with TBS (3×15 min) and then incubated with 0.5 mg ml^{-1} 3,3′-diaminobenzidine and 0.01 % hydrogen peroxide in TBS. After washing in water, slides were dehydrated and mounted as described above.

Results

Nitric oxide synthase in the locust brain

Citrulline synthesis in locust brain extracts was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline; in the presence of NADPH and the cofactors Ca^{2+}, calmodulin, H_4B, FAD and FMN, about 15 % of added [^{3}H]arginine was converted to [^{3}H]citrulline during a 1 h incubation (Fig. 1A). Under these conditions the conversion of arginine to citrulline was linear for at least 1 h (data not illustrated). The conversion of 15 % of added [^{3}H]arginine in 1 h corresponds to a citrulline synthesis rate of 0.13 nmol mg^{-1} protein min^{-1}. In the absence of NADPH and the cofactors, or in the presence of L-NMMA or L-NNA (0.5 mmol l^{-1}), only about 1.5 % of the [^{3}H]arginine was converted to [^{3}H]citrulline (Fig. 1B,C). Therefore, conversion of arginine to citrulline in locust brain extracts was dependent on the presence of NADPH and the usual NOS cofactors and was inhibited by two arginine analogues known to inhibit mammalian neuronal NOS.

Measurement of NO synthesis by monitoring oxidation of oxyhaemoglobin to methaemoglobin showed that the rate of synthesis was about 0.15 nmol NO mg^{-1} protein min^{-1} in extracts of locust brains following addition of L-arginine, NADPH and Ca^{2+}. Addition of L-NMMA or the calmodulin antagonist *N*-(4-aminobutyl)-5-chloro-2-naphthalene-sulphonamide (W-13) to the incubation mixture caused total inhibition of NO synthesis.

Western blots revealed a locust brain protein with a molecular mass of approximately 135 kDa that reacted strongly with an antiserum to rat cerebellar NOS (Fig. 2A). A similar protein band was weakly immunoreactive with an antiserum to mouse macrophage NOS (Fig. 2B). A number of lower molecular mass protein bands (55–65 kDa) were also immunoreactive (Fig. 2A,B). These might be degradation products of the 135 kDa protein, as similar products have been

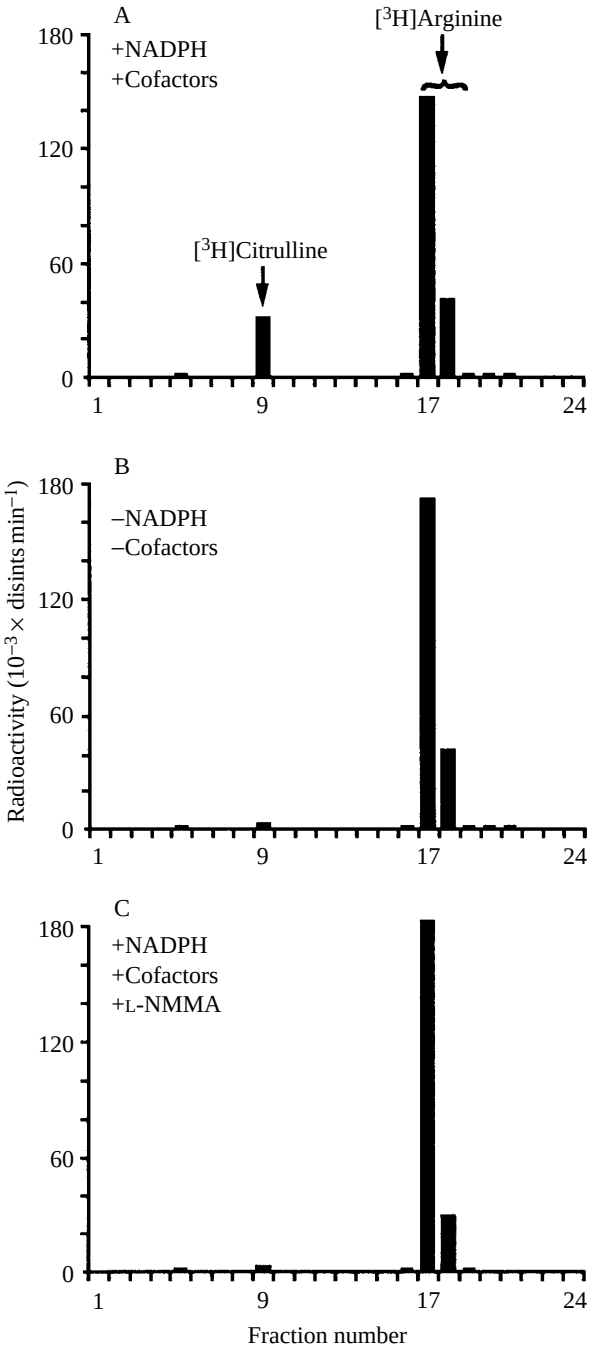


Fig. 1. Demonstration of nitric oxide synthase (NOS) activity in the locust brain. The graphs show HPLC separation of ^{3}H-labelled compounds (disints min^{-1}) after incubation of [^{3}H]arginine with locust brain extract. Fractions in which [^{3}H]arginine and [^{3}H]citrulline elute are indicated (A). The conversion of [^{3}H]arginine to [^{3}H]citrulline is dependent on the addition of NADPH and NOS cofactors (Ca^{2+}, FMN, FAD, H_4B, calmodulin) (A,B) and is inhibited by the NOS inhibitor L-NMMA (C).

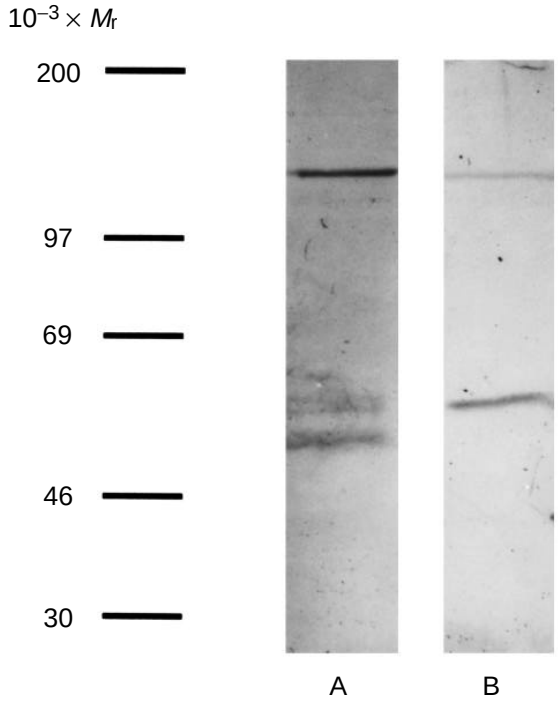


Fig. 2. Western blots showing an approximately 135 kDa locust brain protein that is strongly recognised by an antiserum to rat cerebellar NOS (A) and weakly recognised by an antiserum to mouse macrophage NOS (B). Lower molecular mass bands (55–65 kDa) are also immunoreactive.

shown to be generated from the mammalian enzyme (Springall *et al.* 1992).

Distribution of nitric oxide synthase in the locust brain

Whole-mount histochemistry shows NADPH-diaphorase-positive staining extensively throughout the brain (Fig. 3). Although diffuse staining was observed in the three primary subdivisions of the brain (the proto-, deuto- and tritocerebra), by far the most intense staining was associated with the antennal lobes of the deutocerebrum. Also, discrete staining within the protocerebrum defines two prominent structures, the mushroom bodies and the central body.

If NADPH diaphorase histochemistry is revealing the presence of NOS in the locust brain, the distribution of NOS activity measured biochemically should correspond with the distribution of NADPH diaphorase staining. In order to test this hypothesis, we measured NOS activity in the three parts of the locust brain illustrated in Fig. 4A: (1) the protocerebrum and deutocerebrum (without optic and antennal lobes attached), (2) the antennal lobes and (3) the tritocerebrum. NOS activity in extracts of these regions was measured using assays for both citrulline and NO production. In parallel with the distribution of NADPH diaphorase staining, the highest concentration of NOS activity was detected in the antennal lobes (approximately 0.7 nmol NO mg^{-1} protein min^{-1} and 0.6 nmol citrulline mg^{-1} protein min^{-1}), which was about six times higher than in other parts of the brain analysed (Fig. 4B,C). Within each part of the brain, the rates of NO and citrulline synthesis were very similar, indicating stoichiometric synthesis of these products, as expected for NOS. Furthermore, NOS activity in each part was also dependent on the presence of NADPH and Ca^{2+} and was inhibited by L-NNA and L-

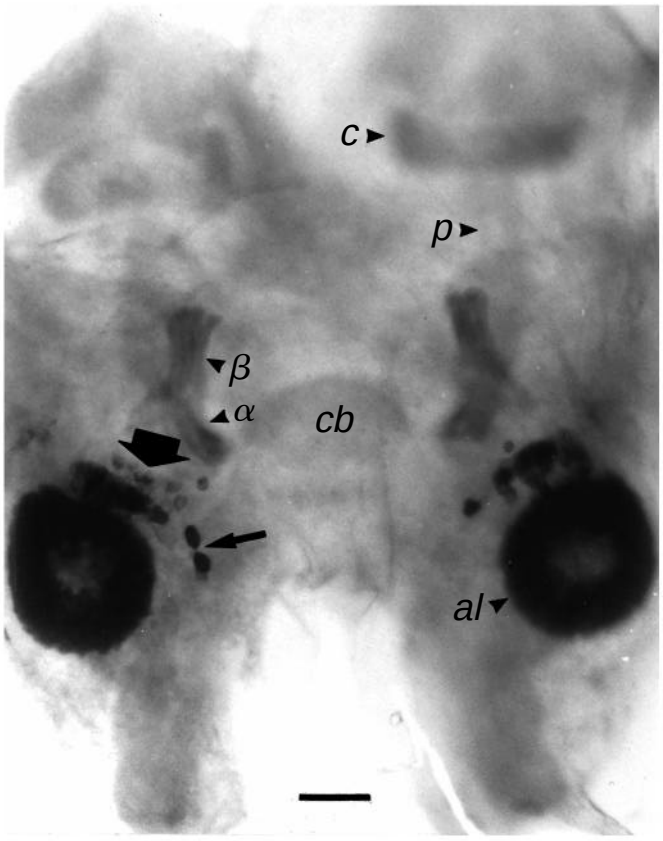


Fig. 3. NADPH diaphorase staining in the locust brain (fourth instar). Intense staining is present in the antennal lobes (*al*) and is localised in two groups of neurones that project into the antennal lobe neuropile. The main cluster of neuronal cell bodies is located in the anterior half of the antennal lobes (large arrow), but there is also a pair of neuronal cell bodies located at the periphery of the antennal lobe on its medial side (small arrow). Only one of this pair of neurones can be seen on the right-hand side of the figure because its partner is out of the plane of focus in this preparation. Less intense staining is associated with other brain structures, including the calyses (*c*), the peduncle (*p*), the alpha- (α) and beta- (β) lobes of the mushroom bodies and the central body (*cb*). Scale bar, 90 μm.

NMMA. These results show that, as in the mammalian brain, NADPH diaphorase histochemistry can be used as an indicator of the anatomical distribution of NOS in the insect nervous system.

Detailed analysis of the distribution of nitric oxide synthase in the antennal lobes

Since the antennal lobes contain the highest concentration of NADPH diaphorase and NOS activity in the locust brain, we focused our attention on this region and further characterised the cellular distribution of NOS. NADPH diaphorase staining was found to be localised in two groups of cells, a cluster of about 50 cells in the anterior (in relation to the neuro-axis) half of each antennal lobe and a pair of cells located at the periphery of the antero-medial quadrant of each antennal lobe. These two groups of cells can be seen in the whole-mount brain shown in Fig. 3. Intense NADPH diaphorase staining is also present in the neuropile of the antennal lobes.

Examination of cryostat sections of brains revealed that the main cluster and the pair of peripheral cells are unipolar interneurones that project into the neuropile of the antennal lobe (Fig. 5A,B). The staining in the neuropile is concentrated in spheroid structures with an unstained core and a diameter of about 25 μm (Fig. 5A). The size of these structures is similar to the glomerulus-like compartments that have been described in another locust species, *Locusta migratoria* (Ernst *et al.* 1977). About 1000 of these glomerulus-like compartments were counted in *Locusta migratoria*, and this figure is consistent with our estimation of the number of NADPH-diaphorase-positive spheroid structures in the *Schistocerca gregaria* antennal lobe neuropile.

The two groups of stained neurones that we have observed project into the neuropile of the antennal lobe and their processes are likely to be responsible for most of the neuropilar staining. It is possible, however, that the axons of neurones that project into the antennal lobe from the antennae or from other parts of the brain may contain NOS and may contribute to the NADPH diaphorase staining in the neuropile. The fibre tracts that contain the processes of such neurones, the antennal nerves (Fig. 5C) and antennal–protocerebral tracts (not shown), do not contain NADPH-diaphorase-positive fibres. These observations indicate that the staining in the antennal lobe neuropile is likely to be due entirely to the processes of the NADPH-diaphorase-positive neurones intrinsic to the antennal lobes.

NOS-immunoreactivity was observed in sections of lightly fixed (1% paraformaldehyde) brains. The intensity of the staining was quite weak, however, which probably reflects a not unexpected low cross-reactivity of insect NOS with an antiserum to a mammalian NOS. Nevertheless, the distribution of staining in the antennal lobes was clearly the same as that revealed by NADPH diaphorase histochemistry (Fig. 5D). This observation further supports our conclusion that NADPH diaphorase staining can be attributed to NOS in the insect nervous system.

Discussion

We have shown that the brain of the locust *Schistocerca gregaria* contains a NOS with properties similar to that of the mammalian neuronal NOS. It synthesizes both NO and citrulline from L-arginine in a Ca^{2+}/calmodulin- and NADPH-dependent manner and is inhibited by the N^{ω}-nitro and N^{ω}-monomethyl analogues of L-arginine. Although the methods used to measure citrulline and NO synthesis were quite different in nature, the rates of synthesis of these products measured in locust brain extracts were very similar. These data indicate, for the first time, that an invertebrate brain NOS functions like mammalian brain NOS, generating equimolar citrulline and NO from L-arginine.

The biochemical properties of insect and mammalian neuronal NOS appear to be similar, suggesting that they may

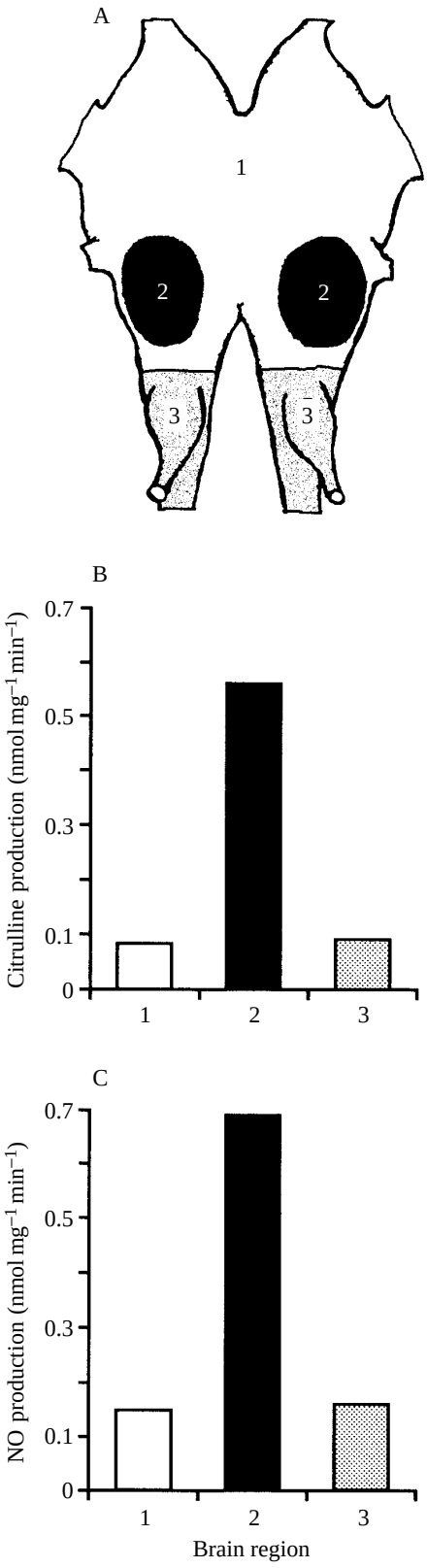


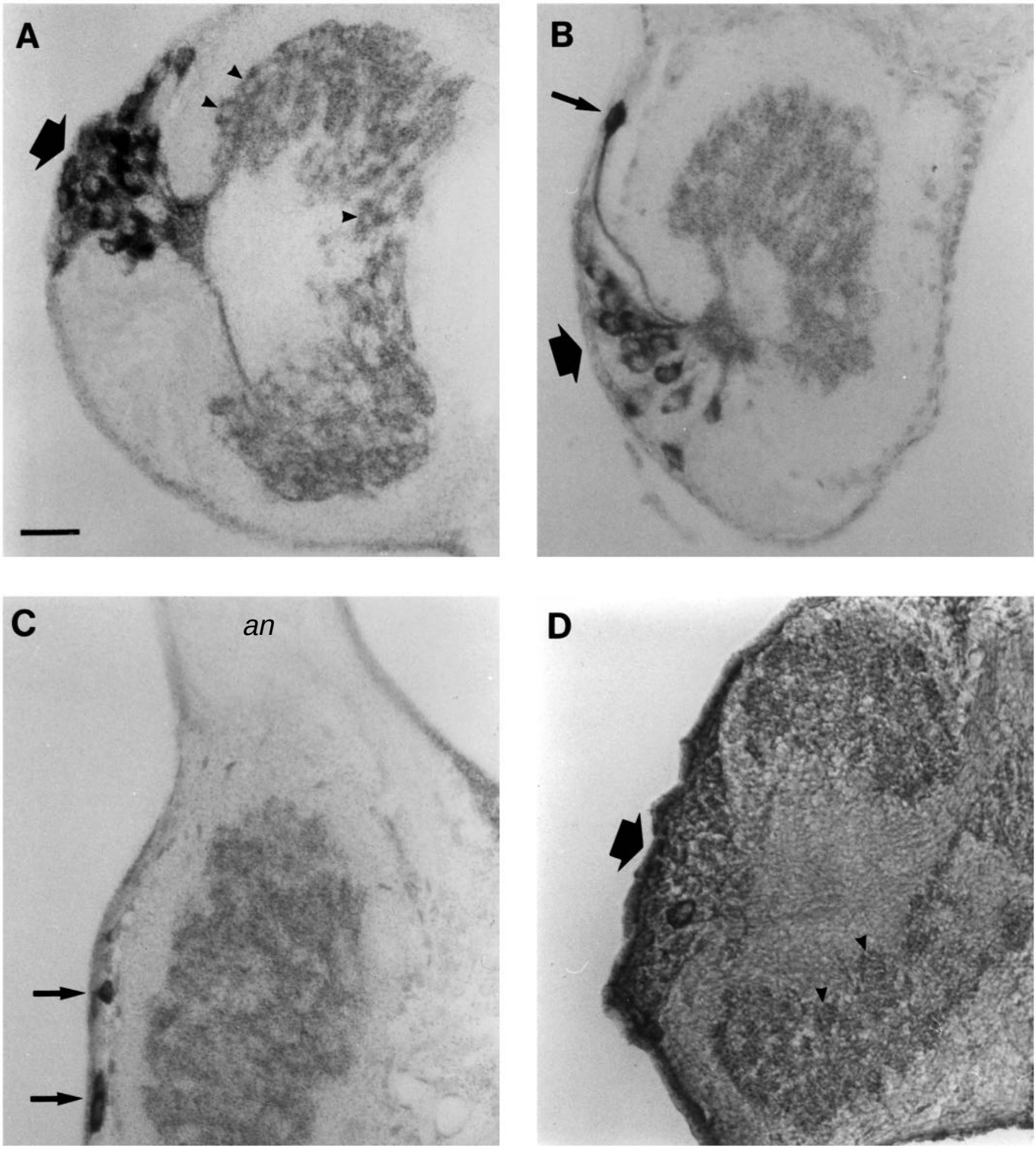
Fig. 4. Distribution of NOS in the locust brain. NOS activity was measured in the three regions of the locust brain illustrated in A: (1) the protocerebrum and deutocerebrum without optic and antennal lobes attached; (2) the antennal lobes; and (3) the tritocerebrum. NOS activity was determined by measurement of both citrulline (B) and NO (C) production.

have evolved from a common ancestral protein. This idea is further supported by our partial purification of a protein from the locust brain that is recognised by a specific antiserum to rat cerebellar NOS in Western blots. The same protein band was also weakly immunoreactive with an antiserum to mouse macrophage NOS (Fig. 2). These observations suggest that insect neural NOS is more similar to mammalian neuronal NOS than to mammalian macrophage NOS. However, the molecular mass of the insect protein (approximately 135 kDa) appears to be lower than that of the mammalian neuronal NOS subunit (160 kDa) but similar to those of mammalian endothelial and macrophage NOS subunits. To establish an evolutionary relationship between the insect and mammalian forms of NOS, it will be necessary to compare their amino acid sequences. Recently Regulski and Tully (1993) have reported the isolation of a 4.5 kb cDNA from *Drosophila melanogaster* with 40 % amino acid sequence identity to mammalian forms of NOS. This cDNA may encode the *Drosophila melanogaster* homologue of the NOS that we have characterised in the locust brain.

In order to gain an insight into possible roles of NO in the insect brain, we have localised NOS by combining NADPH diaphorase histochemistry, immunocytochemistry and regional measurements of NOS activity. What can be inferred about NO function from such a study of NOS distribution in the locust brain? NADPH diaphorase staining in the mushroom bodies indicates that NO may be involved in the mechanisms of learning and memory that occur in this part of the brain (Davis, 1993). The role of NO in this important aspect of insect brain function cannot be inferred from data available at present. A clearer indication of NO function, however, has emerged from analysis of the distribution of NOS in the deutocerebral

Fig. 5. (A) Sagittal section of the locust brain showing the main cluster of NADPH-diaphorase-positive neurones whose cell bodies are located in the anterior half of the antennal lobe (large arrow). The processes of these neurones project into the neuropile of the antennal lobe. NADPH diaphorase staining is concentrated in glomerulus-like spheroid regions of the neuropile (arrowheads). (B) Oblique section of the locust brain showing one of the pair of NADPH-diaphorase-positive neurones located at the periphery of the antennal lobe (small arrow) and the main cluster of NADPH diaphorase-positive neurones located in the anterior half of the antennal lobe (large arrow). Neurones in both groups project into the neuropile of the antennal lobe. (C) Horizontal section of the locust brain showing the absence of NADPH diaphorase staining in the antennal nerve (*an*). NADPH-diaphorase-positive neuronal cell bodies that belong to the main cluster are indicated by arrows. (D) NOS immunoreactivity in a horizontal section of the antennal lobe. The pattern of staining is identical to that revealed by NADPH diaphorase histochemistry. NOS immunoreactivity is localised in the main cluster of neuronal cell bodies located in the anterior part of the antennal lobe (arrow) and in the glomerulus-like compartments (arrowheads) of the neuropile. Scale bar, 47 μm.

antennal lobes, which are the principal olfactory processing centres of the insect brain (Homberg *et al.* 1989). Here NOS is localised in a population of interneurones that innervates the numerous glomerulus-like compartments of the antennal lobe neuropile. The glomeruli contain synapses between the axons of antennal primary olfactory receptor neurones, local interneurones and projection interneurones. Olfactory input from the antennae is processed here by local interneurones before it is relayed to other parts of the brain, including the mushroom bodies, by the projection interneurones. NADPH diaphorase staining was not observed in fibre tracts that contain the axons of projection interneurones, so we conclude that the NOS-containing neurones of the antennal lobe are likely to be local interneurones. NO released by these neurones may be



involved in olfactory processing at the level of the glomerulus and interglomerular integration.

Intriguingly, NO may have a very similar role in the mammalian brain. The principal olfactory processing centres in the mammalian brain are the main and accessory olfactory bulbs. Like the insect antennal lobes, they have glomerular neuropiles. Both subdivisions of the mammalian olfactory bulb also contain a high concentration of NOS (Bredt *et al.* 1991*a*). In the accessory olfactory bulb, NOS is localised in a class of local interneurones known as granule cells. The processes of these cells are not, however, associated with the glomeruli but, instead, they synapse with a class of projection interneurones known as mitral cells. In the main olfactory bulb, NOS is localised in a portion of a population of local interneurones known as periglomerular (PG) cells, whose processes terminate in the glomeruli. The role of NO in the accessory and main olfactory bulbs in mammals is as yet unknown, although one study shows that inhibition of NOS activity in the accessory olfactory bulb by infusion of L-NNA does not prevent formation of an olfactory memory to male pheromones in mice (Brennan and Kishimoto, 1993). Breer and Shepherd (1993) have proposed that NO released from the PG cell dendrites modulates intraglomerular synaptic integration of sensory inputs and that PG cell axons provide a system for interglomerular modulation. Clearly an understanding of the roles of NO in the olfactory system will only be elucidated by detailed electrophysiological and pharmacological experiments. The identification of a population of NOS-containing local interneurones in the locust antennal lobe opens up the possibility of also using simpler insect preparations to explore NO function in olfactory processing.

While this study was in progress Müller and Bucher (1993) reported the distribution of NADPH diaphorase in the brain of another insect species, the fruit fly *Drosophila melanogaster*, and they also detected intense staining in the antennal lobes. Staining in *Drosophila melanogaster*, however, is associated with the processes of the olfactory receptor neurones rather than local interneurones and this is in contrast with our findings in the locust. Therefore, interestingly, it appears that the distribution of NADPH diaphorase and, presumably, the role of NO in the olfactory system may be quite different amongst insects.

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