REVIEW

SINGLE-CELL ANALYSES OF NITRERGIC NEURONS IN SIMPLE NERVOUS SYSTEMS

LEONID L. MOROZ[1,3,*], RHANOR GILLETTE[2] AND JONATHAN V. SWEEDLER[1]

[1]*Department of Chemistry and Beckman Institute,* [2]*Department of Molecular and Integrative Physiology, University of Illinois, Urbana, IL 61801, USA* and [3]*Department of Neuroscience, The Whitney Laboratory, University of Florida, St Augustine, FL 32086, USA*

*e-mail: moroz@whitney.ufl.edu

Summary

Understanding the role of the gaseous messenger nitric oxide (NO) in the nervous system is complicated by the heterogeneity of its nerve cells; analyses carried out at the single cell level are therefore important, if not critical. Some invertebrate preparations, most especially those from the gastropod molluscs, provide large, hardy and identified neurons that are useful both for the development of analytical methodologies and for cellular analyses of NO metabolism and its actions. Recent modifications of capillary electrophoresis (CE) allow the use of a small fraction of an individual neuron to perform direct, quantitative and simultaneous assays of the major metabolites of the NO–citrulline cycle and associated biochemical pathways. These chemical species include the products of NO oxidation (NO_2^-/NO_3^-), L-arginine, L-citrulline, L-ornithine, L-argininosuccinate, as well as selected NO synthase inhibitors and cofactors such as NADPH, biopterin, FMN and FAD. Diverse cotransmitters can also be identified in the same nitrergic neuron. The sensitivity of CE methods is in the femtomole to attomole range, depending on the species analysed and on the specific detector used. CE analysis can be combined with prior *in vivo* electrophysiological and pharmacological manipulations and measurements to yield multiple physiological and biochemical values from single cells. The methodologies and instrumentation developed and tested using the convenient molluscan cell model can be adapted to the smaller and more delicate neurons of other invertebrates and chordates.

Key words: nitric oxide synthase, NADPH diaphorase, mollusc, invertebrate, *Aplysia californica*, *Lymnaea stagnalis*, feeding, capillary electrophoresis.

Introduction

In contrast to other intercellular signal molecules, gaseous nitric oxide (nitrogen monoxide or NO) is synthesized and released without special storage and transfer mechanisms. It is one of the smallest and most diffusible signal molecules known, and it freely crosses membrane barriers to exert its effects through direct binding and/or by reaction with its target proteins (Stamler et al., 1992). Its diffusion from a point source can affect targets a relatively long distance away from its origin (Wood and Garthwaite, 1994). NO is also a highly reactive radical that can give rise to toxic secondary radical species (Stamler et al., 1992), and overproduction of NO contributes to numerous pathological conditions, such as septic shock, and to postischemic brain and heart injury (Dawson and Dawson, 1996; Moncada and Higgs, 1995). The reasons for the evolutionary choice of this versatile and dangerous molecule as a major modulator of physiological processes in animal tissues are not yet apparent, suggesting that a large part of our understanding of nitrergic physiology is yet to come.

To date, NO, its synthesis and its actions have been most extensively investigated in mammalian systems in literally scores of laboratories devoted to various aspects of biochemistry, molecular biology, physiology and behavior. Studies performed on the nervous system are a considerable portion of these efforts, as it has become obvious that NO is a prominent neuromodulatory molecule with significant relevance to neural excitability, synaptic transmission, network function arousal and learning and memory mechanisms (Garthwaite and Boulton, 1995).

In mammals, three main isoforms of NO synthase (NOS) have been identified, characterized and cloned, all of which are found in the central nervous system (CNS) (Griffin and Stuehr, 1995). The enzymatic synthesis of NO proceeds according to the following reaction:

$$\text{L-Arginine} + O_2 \rightarrow NO + \text{L-citrulline},$$

$$NO \rightarrow NO_2^- \rightarrow NO_3^-,$$
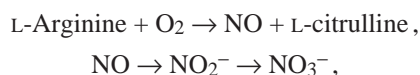
where nitrites (NO_2^-) and nitrates (NO_3^-) are the sequential breakdown products of NO oxidation.

Essential questions for understanding the biochemistry and physiology of NO in neurons and glia concern the actual concentrations of NO, its precursors and its breakdown products in single cell types. However, the complexity of the CNS, the equally great heterogeneity of cell types and the small sizes of the cells have for the most part greatly impeded the development and application of techniques necessary to answer such questions, despite the large amount of effort directed at mammalian studies.

In the study of the manifold roles of NO in the CNS, those studies reported from invertebrate model systems are relatively few in number. However, despite their small number, a certain depth of understanding may be emerging from the versatile nature of the preparations, their accessibility to varied experimental approaches and the high rates of data extraction in studies on small nervous systems with individually identifiable neurons.

NO synthase and identified nitrergic neurons in invertebrates

Selection of a useful model system

Among the many invertebrate groups tested, reliably identified and relatively large NOS-containing neurons have only been demonstrated in annelids and molluscs: the medicinal leech *Hirudo medicinalis* (Leake et al., 1996; Leake and Moroz, 1996), the freshwater pond snail *Lymnaea stagnalis* (Moroz et al., 1994a) and the marine sea slugs *Aplysia californica* (Jacklet and Gruhn, 1994b; Moroz et al., 1996a) and *Pleurobranchaea californica* (Moroz et al., 1996a; Moroz and Gillette, 1996b). The gastropod molluscs are especially useful models for single-neuron studies of NOS regulation and NO action. The large size and robustness of their neurons make these cells easily accessible to isolation and penetration with multiple electrodes, while their identifiability has allowed the roles of specific neurons in neural networks and behavior to be characterized. The few dozen identified 100–600 µm diameter nitrergic neuronal somata openly displayed on the surfaces of ganglia in *Pleurobranchaea*, *Lymnaea* and *Aplysia* (Fig. 1) offer a far more approachable situation than the thousands of 10–60 µm diameter diffusely distributed nitrergic neurons, surrounded by millions of other cells, in the mammalian brain. The genetic sequences of neuronal NOS isoforms of the fruitfly *Drosophila melanogaster* (Regulski and Tully, 1995), the hawkmoth *Manduca sexta* (Nighorn et al., 1998) and the pond snail *Lymnaea stagnalis* (Korneev et al., 1998) share substantial homology with mammalian NOS.
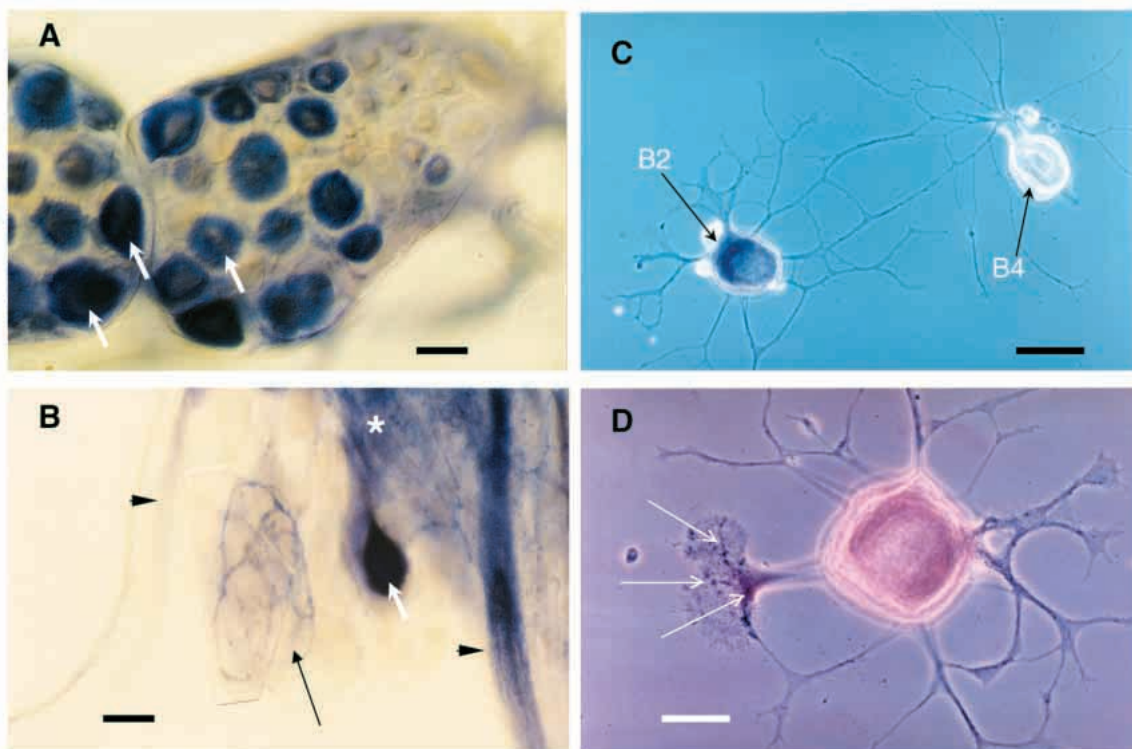

Fig. 1. Putative nitric oxide synthase (NOS)-containing neurons (arrows, dark blue in color) in molluscan central nervous systems stained using NADPH-diaphorase (NADPH-d) histochemistry. These representative preparations show the position of selected identified neurons. (A) The buccal ganglia of *Pleurobranchaea californica* (a whole-mount preparation; for experimental details, see Moroz and Gillette, 1996b). Scale bar, 150 µm. (B) The left caudo-lateral area of the cerebral ganglion of *Aplysia californica* (a whole-mount preparation). Scale bar, 200 µm. The black arrow indicates NADPH-d-reactive terminals, asterisks point out NADPH-d labeling in the neuropil and arrowheads indicate cerebral nerves. The white arrow points to the NADPH-d-positive cell. (C,D) Identified buccal neurons of *Lymnaea stagnalis* in culture. (C) An NADPH-d-reactive B2 esophageal motoneuron and an unlabelled B4 buccal motoneuron. Scale bar, 100 µm (modified from Moroz et al., 1994). (D) An individual B2 neuron in culture. Note the enhanced density of NADPH-d reactivity (white arrows) in the growth cone of the neuron. Scale bar, 100 µm.

Mapping nitrergic neurons

Histochemical location of NOS-containing neurons is generally a useful first step in studying the physiology of NO in any system. Immunohistochemistry using mammalian NOS antibodies has been helpful in some invertebrate preparations (Cooke et al., 1994; Martinez et al., 1994; Moroz et al., 1994b; Scholz et al., 1998). However, invertebrates may well have NOS isoforms that are quite different from mammalian ones, and caution must be exercised because of the absence of specific cross-reactivity of some antibodies. As a general NOS indicator, fixative-resistant NADPH-diaphorase (NADPH-d) histochemistry has proved useful for both mammals and invertebrates (Bredt et al., 1991; Dawson et al., 1991; Hope et al., 1991; Huang et al., 1997; Matsumoto et al., 1993; Muller, 1994). The appropriate fixation protocols for NADPH-d labeling vary among species, and some procedural comparisons of different conditions are required for a new preparation.

Using NADPH-d histochemistry, putative nitrergic neurons have been examined in more that 30 molluscan species (Moroz and Gillette, 1996a), including gastropods, amphineurans and cephalopods, and such information has been published in detail for *Pleurobranchaea californica* (Moroz and Gillette, 1996b), *Aplysia californica* (Jacklet and Gruhn, 1994a), *Helix aspersa* (Cooke et al., 1994; Huang et al., 1997) and *Lymnaea stagnalis* (Moroz et al., 1994a), with examples shown in Fig. 1. Almost all these species have a few NADPH-d-positive giant neurons, located mainly in the buccal and/or cerebral ganglia, and many relatively small NADPH-d-reactive cells at the periphery. Since many putative NOS-containing cells have been identified as characterized neurons of feeding networks (Jacklet, 1995; Moroz et al., 1994a; Moroz and Gillette, 1996a; Park et al., 1998), it is no surprise that NO may have significant roles in the activation of the feeding motor patterns (Moroz et al., 1993) and in chemosensory pathways (Elphick et al., 1995; Gelperin, 1994). In contrast to many other molluscs tested, the CNS of *Pleurobranchaea* contains several dozen large intensely-NADPH-d-positive cells distributed among all the ganglia (Moroz and Gillette, 1996a), whereas peripheral NADPH-d-reactive neurons have not been observed.

Among the many characterized NADPH-d-positive neurons, some suitable for single-cell analysis have been identified in the feeding network, including (i) five pairs of giant radular protractor motoneurons (the buccal A-group) in the buccal ganglion of *Pleurobranchaea californica* (Fig. 1A), (ii) a pair of giant serotonergic metacerebral (C1) cells in *Pleurobranchaea californica*, (iii) a symmetrical pair of esophageal motoneurons (B2) in the buccal ganglia of *Lymnaea stagnalis* (Fig. 1C) and (iv) the cerebral histamine-containing C2 neurons in *Aplysia californica* (Fig. 1B) (see Chiel et al., 1990; Jacklet, 1995). It is of interest that only in *Pleurobranchaea* were the serotonergic metacerebral cells labeled by NADPH-d, whereas their homologs in *Lymnaea* and *Aplysia* were not (Moroz and Gillette, 1996a,b). Curiously, the metacerebral neurons in *Lymnaea* are among several neurons

exhibiting mRNA for a putative NOS, as shown by *in situ* hybridization with a *Lymnaea*-derived specific probe (Korneev et al., 1998). Potentially, the NOS mRNA message is either untranslated in the soma or the enzyme is inactive or only present at a low concentration.

Since expression of NADPH-d reactivity is species-dependent, one must relate the observed staining to NOS activity using other methods. One of the most important tests is a functional verification of NO-mediated transmission. It has recently been shown that electrical stimulation of NADPH-d-positive neurons in the feeding network of *Aplysia* (C2 cells) and *Lymnaea* (B2 cells) induced a slow excitatory postsynaptic potential (EPSP) in follower neurons which was suppressed by both NOS inhibitors and NO scavengers (Jacklet, 1995; Park et al., 1998). Buccal B2 motoneurons also expressed NOS-immunoreactivity (Moroz et al., 1994b) and preserved histochemical properties in cell culture (Moroz et al., 1994a) (see Fig. 1C,D). It is of interest that NO-mediated transmission has been reconstructed *in vitro*, as shown in recent experiments on co-cultured B2/B7nor neurons of *Lymnaea* (Park et al., 1998).

In spite of the notable progress in our understanding of NO-mediated signaling, nitrergic neurons themselves are still a poorly characterized population in the nervous system. Intracellular concentrations of NO-related metabolites have not been reported, and verification of the nitrergicity of many other NADPH-d-reactive neurons has been difficult. Direct, single-cell analysis of the NO/NOS-associated metabolites is highly desirable.

Methods for single-cell NO/NOS analysis

NO has a short half-life and is difficult to measure directly in a complex chemical microenvironment such as nervous tissue; however, the NOS coproduct L-citrulline and the breakdown products of NO oxidation can be used as indicators of NOS activity. A number of analytical methods have recently been developed for measurements of NO and related metabolites in biological tissues (Archer, 1993), but problems arise when they have to be applied at the single-cell level.

Experimental approaches

Of the various methods employed in NO analyses, only three can be used directly for single-cell analysis: (i) chemiluminescence (Archer, 1993; Kikuchi et al., 1996; Leone et al., 1996) and fluorescence detection of NO formation using fluorescent probes (Kojima et al., 1997, 1998; Nakatsubo et al., 1998), (ii) NO-sensitive electrodes (Malinski and Czuchajowski, 1996; Malinski et al., 1996; Malinski and Taha, 1992; Shibuki and Okada, 1991), and (iii) capillary electrophoresis (CE) (Beale, 1998; Camilleri, 1993; Landers, 1997).

The first two methods have been developed to detect authentic NO gas (Archer, 1993). The NO electrode was successfully applied to isolated individual cells with useful results (Malinski and Taha, 1992), and some commercially
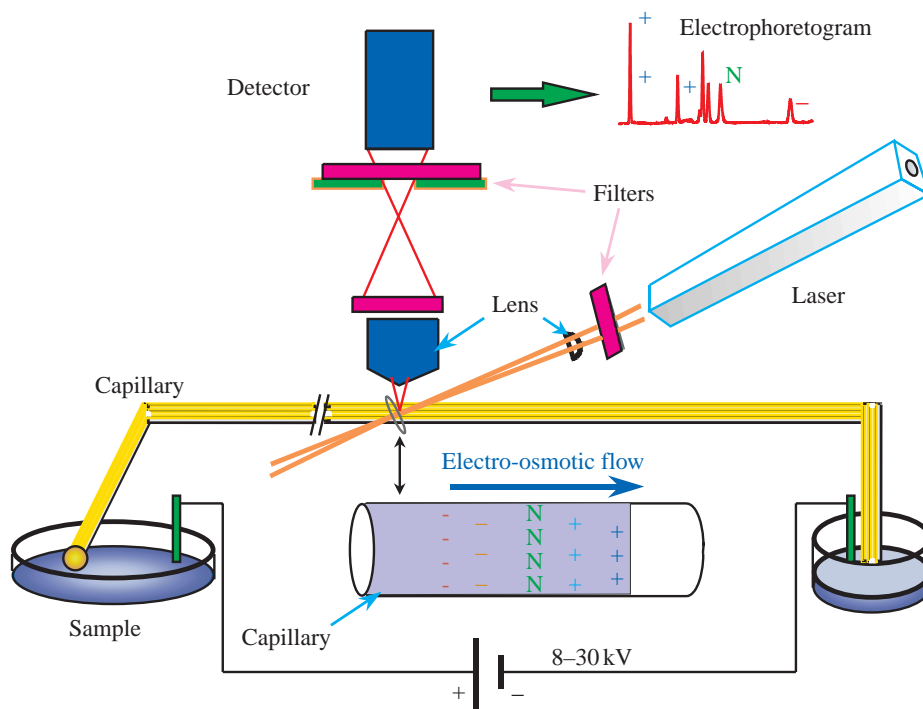
available electrodes indicate surprisingly high signal levels both at nitrergic cell bodies and in the neuropil of molluscan ganglia (Moroz et al., 1996b). However, the large sensor tip diameter of the commercially available NO electrodes (>30 µm) as well as the limited selectivity of both NO electrodes and chemiluminescence methods against various redox species (e.g. monoamines, H_2O_2) and pH restrict their applications for single-cell analysis within heterogeneous neuronal populations. Recent fluorescent indicators of NO show promise (Kojima et al., 1997, 1998; Nakatsubo et al., 1998). Useful methodologies for these applications are not yet available, and control studies are still required to gauge their effectiveness. If effective, they promise the advantage of multi-cell optical assays for NO and may offer the ability to follow its time-dependent generation.

In contrast, CE cannot be used for direct NO detection but, as a microseparation technique, it allows analysis of many essential metabolites associated and interacting with the NO–citrulline cycle. We emphasize the versatility of CE for single-neuron measurement in greater detail; it has not been reviewed previously, and CE involves significantly different methodology from the more familiar electrochemical and fluorescent approaches.

Capillary electrophoresis

Separation of metabolites by CE is based on the differential electrophoretic migration of compounds in an electric field. CE is similar to conventional gel electrophoresis except that the separation takes place in a small-diameter (10–75 µm) fused-silica capillary, allowing the use of a higher separative voltage (up to 30 kV) because of the efficient removal of current-induced heat (Fig. 2). This provides effective and rapid separation of metabolites from pico- and nanoliter samples. Recent revie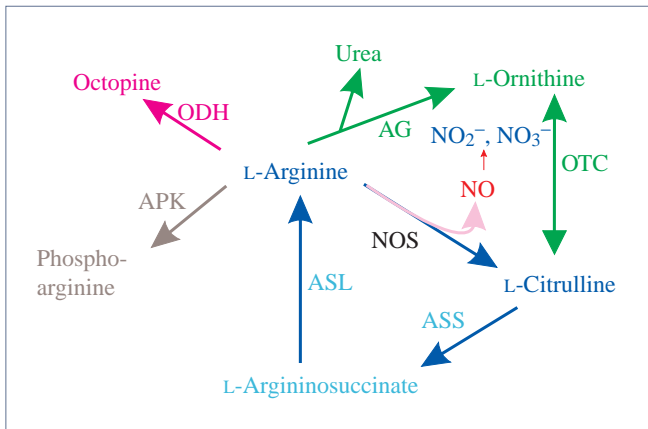ws provide more details of the capabilities and operation of CE (Beale, 1998; Camilleri, 1993; Jones, 1997; Khaledi, 1998; Landers, 1997; Righetti, 1996).

Detection using CE can be challenging owing to the small path length and small volumes. However, detection systems have been developed for most types of biologically active molecules and ions (Bryant et al., 1998; Cruz et al., 1998; Swanek et al., 1997; Tomer et al., 1998). Laser-induced fluorescence detection in CE has the best performance characteristics compared with other detection modes in terms of sensitivity, limits of detection and linearity (Lillard and Yeung, 1997). Most metabolites are not fluorescent; however, they can often be derivatized using various fluorescent or fluorogenic reagents. CE has been successfully used to assay amino acids, neurotransmitters, peptides and some enzymes in small biological samples, including individual cells such as erythrocytes (Yeung, 1994), chromaffin cells (Chang and Yeung, 1995) and single neurons (Jankowski et al., 1995; Kennedy et al., 1989). Examples of the detection of low femtomole, attomole and even zeptomole amounts of selected low-molecular-mass species and some peptides in cells, as well as descriptions of methods, have been reported elsewhere (Fuller et al., 1998; Gilman and Ewing, 1995; Jankowski et al., 1995; Kennedy and Jorgenson, 1989; Kennedy et al., 1989; Lillard and Yeung, 1997; Lillard et al., 1996; Shippy et al., 1995; Swanek et al., 1997; Tong and Yeung, 1997). Thus, the application of CE to analyses of NO metabolites is a natural evolution in studies of NO-mediated signaling at the level of the single cell.

Capillary electrophoresis analysis of putative molluscan nitrergic neurons

On the basis of the mechanisms of enzymatic synthesis of NO and its degradation (see Fig. 3), three groups of assays of

Fig. 2. Diagram of the capillary electrophoresis system with laser-induced fluorescence detection. A few nanoliters of the sample is injected into the capillary inlet, and a 5–30 kV potential is applied between the capillary inlet and outlet, causing charged substances to migrate at different rates down the capillary. As the metabolites pass through the illuminated zone near the outlet end of the capillary (excited by the laser radiation), fluorescent compounds are excited, and the fluorescence emission is collected, spectrally and spatially filtered, and detected by a photomultiplier tube. The elution order is dictated by the charge and size of the metabolite and the electro-osmotic flow in the capillary. In the arrangement shown in this diagram, the smallest positively charged metabolites have the fastest migration time, followed by larger positively charged species, neutral (N) and negatively charged analytes respectively.

NOS, nitric oxide synthase
ASS, argininosuccinate synthase
ASL, argininosuccinate lyase
AG, arginase
OTC, ornithine transcarbamylase
APK, arginine phosphokinase
ODH, octopine dehydrogenase

NO–citrulline cycle
Urea cycle

Fig. 3. Schematic diagram of several of the major biochemical pathways involved in the arginine/citrulline cycle showing the interactions between the NO–citrulline and urea cycles.
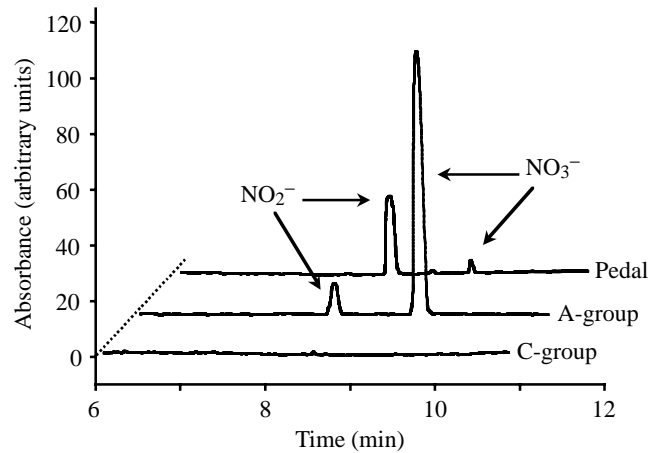

Fig. 4. Single-cell nitrite and nitrate detection by capillary electrophoresis (modified from Cruz et al., 1997). Representative electrophoretograms are shown for individual cell injections where the *x*-axis is time and the *y*-axis is absorbance at 214 nm. The diagram shows results for injections of an individual A-group motoneuron from the buccal ganglion (an NADPH-diaphorase-positive cell), for a single C-group buccal neuron (an NADPH-diaphorase-negative cell) and for an unidentified pedal neuron, all from a 300 g *Pleurobranchaea californica*. There are few interfering anions that elute during this time window so that the NO_2^- and NO_3^- peaks are easily measured. The electrophoretograms are offset for clarity.

NO metabolism in single cells have been developed recently. These are (1) quantification of the nitrite/nitrate breakdown products of NO by indirect ultraviolet absorbance detection after CE separation; (2) quantification of L-arginine, L-citrulline and related metabolites following fluorescent derivatization, CE and fluorescence detection; and (3) quantification of intrinsically fluorescent NOS cofactors (NADPH, biopterin, FAD and FMN) by CE and ultraviolet fluorescence detection. These methods are particularly useful when combined with NADPH-d histochemistry for identifying putatively nitrergic somata for analyses. The following examples illustrate such assays in selected individual molluscan neurons. In all cases, single cells were manually dissected, placed in a nanovial or microvial, and homogenized for analysis. Multiple NOS species have been studied both to provide a confirmation of NOS activity and to estimate the levels of NOS-related metabolites.

Nitrite/nitrate capillary electrophoresis analysis

The products of NO oxidation, NO_2^- and NO_3^-, which are not appreciably membrane-permeant, are potentially accumulated in NOS-containing cells (Salter et al., 1996). Although dietary factors can also contribute to nitrite/nitrate levels (Rhodes et al., 1995), these anions can be used, with appropriate controls, as stable and reliable markers of NO synthase (NOS) activity (Meulemans and Delsenne, 1994; Meulemans et al., 1995; Salter et al., 1996; Yokoi et al., 1996). Single isolated neuron somata from the CNS of *Pleurobranchaea californica* were directly injected into a 75 μm diameter capillary, disrupted and, after CE separation, intracellular NO_2^-/NO_3^- levels were measured by ultraviolet absorption at 214 nm (Fig. 4; see also Cruz et al., 1997). The

limits of detection for NO_2^- and NO_3^- were less than 200 fmol (less than 4 μmol l^{-1} for the neurons under study; volumes were determined optically), which was significantly lower than the actual intracellular concentrations. The NO_2^- and NO_3^- levels in individual neurons varied from 2 mmol l^{-1} (NO_2^-) and 12 mmol l^{-1} (NO_3^-) in neurons histochemically positive for NADPH-d activity (e.g. A-group buccal motoneurons) down to undetectable levels in many NADPH-d-negative cells (e.g. buccal C-group cells), confirming the previous identification of NADPH-d-reactive buccal cells in *Pleurobranchaea* as nitrergic neurons. NO_2^- was not detected in whole-ganglion homogenates or in hemolymph, while hemolymph NO_3^- concentration averaged $1.8 \times 10^{-3} \pm 0.2 \times 10^{-3}$ mol l^{-1}. Hemolymph NO_3^- concentration in *Pleurobranchaea* was appreciably higher than values measured for the freshwater pulmonate *Lymnaea stagnalis* ($3.2 \times 10^{-5} \pm 0.2 \times 10^{-5}$ mol l^{-1}). The high levels of intracellular nitrite measured in these cells suggest that the nitrites might function as a source of non-enzymatic NO production. It was shown that nitrites themselves generate NO in acidified and reducing environments (Benjamin, 1994; Moroz et al., 1997; Weller et al., 1996; Zweier et al., 1995).

Arginine, citrulline and related intermediate analysis

L-Arginine/L-citrulline conversion is a second complementary and widely used assay for NOS activity (Archer, 1993). However, both L-arginine and L-citrulline interact with other metabolic pathways (see Fig. 3) such as the

urea cycle and phosphagen synthesis (Bredt and Schmidt, 1996; Hecker and Billiar, 1996), which can significantly complicate the detailed analysis of NOS activity *in vivo*. CE provides reliable simultaneous detection of major intermediates of both NOS-associated and other pathways not related to NO (Fig. 5A; see also Floyd et al., 1998). In this type of assay, the primary or secondary amine metabolites from single-cell samples were converted into highly fluorescent species by either pre- or on-column derivatization with fluorescamine. The products were separated by CE and
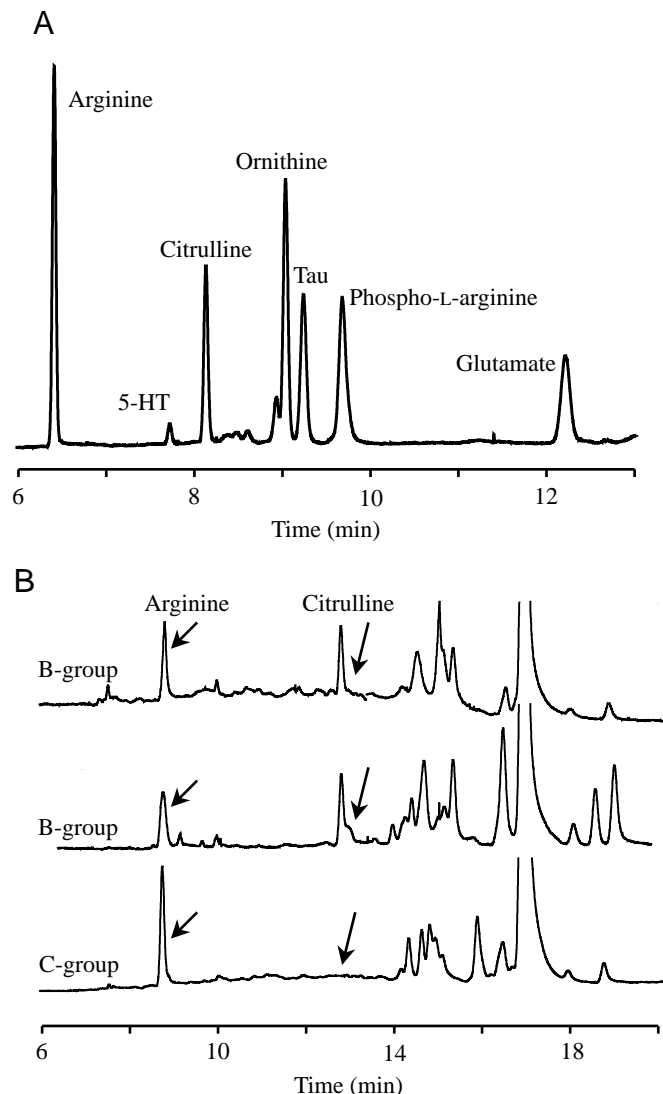
Fig. 5. Single-cell L-arginine/L-citrulline detection by capillary electrophoresis (modified from Floyd et al., 1998). (A) Standards. Electrophoretogram of representative components of the NO–citrulline cycle. Total injection volume was 10 nl, with the concentration of each species being $2.8\,\mu mol\,l^{-1}$ arginine, $1.5\,\mu mol\,l^{-1}$ citrulline, and $0.3\,mmol\,l^{-1}$ of each of the following: serotonin (5-HT), taurine (Tau), ornithine, phospho-L-arginine and glutamate. (B) Electrophoretograms from identified *Pleurobranchaea californica* neurons. Top two traces, NADPH-diaphorase-reactive B-group neurons; bottom trace, NADPH-diaphorase-negative buccal C-group neuron.

measured by laser-induced fluorescence (Fig. 5A). The limits of detection for L-arginine, L-citrulline, L-argininosuccinate, L-ornithine and L-arginine-phosphate ranged from 50 amol to 17 fmol, which corresponds to $5\,nmol\,l^{-1}$ to $17\,\mu mol\,l^{-1}$ in the neurons under study. Thus, the detection limits for this analytical technique are significantly lower than actual intracellular amounts of the metabolites. This sensitivity allows the use of only a small (1–5 %) fraction of the individual neurons for determination of intracellular metabolite concentrations (Fig. 5B, see also Floyd et al., 1998). Levels of NOS metabolites in individual neurons varied from $6\,mmol\,l^{-1}$ (arginine) and $4\,mmol\,l^{-1}$ (citrulline) in putative NOS-containing neurons down to less than $1\,\mu mol\,l^{-1}$ (undetectable levels) in many NADPH-d-negative cells. These results have also upheld the use of NADPH-d staining as a useful indicator of nitrergic cells in the molluscan CNS.

The millimolar concentrations of intracellular L-arginine measured in these cells has immediate significance for experiments in which competitive inhibitors of NOS are to be used: appropriate concentrations of inhibitory compounds can also be millimolar in many cases. Moreover, care must be used in the selection of these compounds (Greenberg et al., 1995), because some of these inhibitors can actually act as substrates for the enzyme-independent formation of appreciable amounts of NO in the presence of cellular reducing compounds such as NADPH, L-cysteine and L-ascorbate (Moroz et al., 1997, 1999).

While CE is quantitative, measuring cell volumes accurately can be problematic. Taking the ratio of amounts of arginine:citrulline (Arg/Cit) has the advantage that it is independent of cell volume. Low values of Arg/Cit (0.2–4) were found to be characteristic of NADPH-d-reactive cells, in accordance with expected NOS activity; in NADPH/NOS-negative cells, however, the ratio was very high (9–40), and in many cells L-citrulline was not detected (Floyd et al., 1998). The Arg/Cit ratio was strongly enhanced by the presence of NOS inhibitors, mostly because of a decrease in the level of the NO coproduct citrulline, consistent with depressed NOS activity. The intermediate of the restorative conversion of L-citrulline to L-arginine, L-argininosuccinate (Fig. 3), could also be readily identified. The substrate for citrulline production in reactions of the urea cycle (Fig. 3), L-ornithine, was not detectable in the neurons assayed, although it was found in the hemolymph. This excludes false positive conclusions of NOS activity in the molluscan CNS due to NOS-independent L-citrulline production by the urea cycle enzyme. Levels of arginine and citrulline were also determined in blood samples of *Pleurobranchaea* and *Aplysia*, and these values were orders of magnitude lower than the measured intracellular concentrations (Floyd et al., 1998).

NO synthase cofactors

Several cofactors, NADPH, FMN, FAD and biopterin, are required for NOS activity, and their availability may be involved in regulation of the enzyme (Griffin and Stuehr, 1995). Thus, the technique for single-cell detection of NOS

cofactors is a complementary assay of the metabolism of a nitrergic neuron. Although the cellular sample matrix is chemically complex, the combination of electrophoretic migration time and fluorescence spectral information allows positive identification of aromatic monoamines, aromatic amino acids and peptides containing them, flavins, adenosine- and guanosine-nucleotide analogs, and other natively fluorescent compounds (Chang and Yeung, 1995; Lillard et al., 1996; Timperman et al., 1995; Tong and Yeung, 1997). Most of the NOS cofactors possess unique spectral properties and can be directly characterized after CE separation without additional derivatization. Using wavelength-resolved native fluorescence detection, direct quantitative and simultaneous assays of NADPH, biopterin, FAD and FMN can be performed on samples amounting to only 1 % of the total single-neuron extract (Fuller et al., 1998). This methodology was also applied to single molluscan neurons for analysis of potential cotransmitters, including serotonin, dopamine, noradrenaline, adrenaline, octopamine and neuropeptides containing aromatic amino acids, which can be performed simultaneously with measurements of the NOS cofactors. Single-cell assays of these cofactors may provide a potent tool to probe their roles in dynamic regulation of NOS activity.

Future directions

Combinations of different methodologies can be brought to bear on single cells for powerful cross-correlation of physiological and biochemical parameters, both to analyze the physiology of the nitrergic neuron and to estimate possible differences among various types of NOS-containing cells. As the obvious next step in this direction, live, isolated or intact neurons can be voltage-clamped for measurement of ion currents while NO production is monitored *via* NO electrode/chemiluminescence techniques, a real-time measure of NOS activity. Following pharmacological or voltage manipulations and recording of the effects on current and NO production, the cell could be rapidly frozen and prepared for multiple measures of indices of NOS activity, reflected in NO breakdown products, NO precursor and coproducts and cofactor molecules.

We now know that neurons have distinct and specific complements of transmitters, with which NO might be acting as a cotransmitter or accessory modulator. Understanding the functional consequences of this complexity is likely to be best approached through the development and combination of techniques and instrumentation for multifactorial analyses in physiology and biochemistry. In this endeavor, molluscs continue to contribute their large and hardy nerve cells as usefully as they have done since the days of Hodgkin, Huxley, Young, Chalazonitis and Arvanitaki.

References

Archer, S. (1993). Measurement of nitric oxide in biological models. *FASEB J.* **7**, 349–360.

Beale, S. C. (1998). Capillary electrophoresis. *Analyt. Chem.* **70**, 279R–300R.

Benjamin, N. E. A. (1994). Stomach NO synthesis. *Nature* **368**, 502.

Bredt, D. S., Glatt, C. E., Hwang, P. M., Fotuhi, M., Dawson, T. M. and Snyder, S. H. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal population of the mammalian CNS together with NADPH diaphorase. *Neuron* **7**, 615–624.

Bredt, D. S. and Schmidt, H. H. H. W. (1996). The citrulline assay. In *Methods in Nitric Oxide Research* (ed. M. Feelisch and J. S. Stamler), pp. 249–255. Chichester, New York: John Wiley & Sons.

Bryant, B. R., Swanek, F. D. and Ewing, A. G. (1998). Electrochemical detection in high-performance capillary electrophoresis. In *High Performance Capillary Electrophoresis*, vol. 146 (ed. M. G. Khaledi), pp. 355- 374. New York: John Wiley & Sons.

Camilleri, P. (1993). History and development of capillary electrophoresis. In *Capillary Electrophoresis: Theory and Practice* (ed. P. Camilleri), pp. 1–23. Boca Raton, FL: CRC Press.

Chang, H. T. and Yeung, E. S. (1995). Determination of catecholamines in single adrenal medullary cells by capillary electrophoresis and laser-induced native fluorescence. *Analyt. Chem.* **67**, 1079–1083.

Chiel, H. J., Weiss, K. R. and Kupfermann, I. (1990). Multiple roles of a histaminergic afferent neuron in the feeding behavior of *Aplysia*. *Trends Neurosci.* **13**, 223–227.

Cooke, I. R. C., Edwards, S. L. and Anderson, C. R. (1994). The distribution of NADPH-diaphorase activity and immunoreactivity to nitric oxide synthase in the nervous system of the pulmonate mollusc *Helix aspersa*. *Cell Tissue Res.* **277**, 565–572.

Cruz, L., Moroz, L. L., Gillette, R. and Sweedler, J. V. (1997). Nitrite and nitrate levels in individual molluscan neurons: single-cell capillary electrophoresis analysis. *J. Neurochem.* **69**, 110–115.

Cruz, L., Shippy, S. A. and Sweedler, J. V. (1998). Capillary electrophoretic detector based on light. In *High Performance Capillary Electrophoresis*, vol. 146 (ed. M. G. Khaledi), pp. 303–354. New York: John Wiley & Sons.

Dawson, T. M., Bredt, D. S., Fotuhi, M., Hwang, P. M. and Snyder, S. H. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci. USA* **88**, 7797–7801.

Dawson, T. M. and Dawson, V. L. (1996). Nitric oxide synthase: role as a transmitter/mediator in the brain and endocrine system. *Annu. Rev. Med.* **47**, 219–227.

Elphick, M. R., Kemenes, G., Staras, K. and O'Shea, M. (1995). Behavioural role for nitric oxide in chemosensory activation of feeding in a mollusc. *J. Neurosci.* **15**, 7653–7664.

Floyd, P. D., Moroz, L. L., Gillette, R. and Sweedler, J. V. (1998). Capillary electrophoresis analysis of nitric oxide synthase related metabolites in single identified neurons. *Analyt. Chem.* **70**, 2243–2247.

Fuller, R. R., Moroz, L. L., Gillette, R. and Sweedler, J. V. (1998). Single neuron analysis by capillary electrophoresis with fluorescence spectroscopy. *Neuron* **20**, 173–181.

Garthwaite, J. and Boulton, C. L. (1995). Nitric oxide signaling in the central nervous system. *Annu. Rev. Physiol.* **57**, 683–706.

Gelperin, A. (1994). Nitric oxide, odor processing and plasticity. *Neth. J. Zool.* **44**, 159–169.

Gilman, S. D. and Ewing, A. G. (1995). Recent advances in the application of capillary electrophoresis to neuroscience. *J. Capillary Electrophoresis* **2**, 1–11.

Greenberg, S. S., Xie, J., Spitzer, J. J., Wang, J.-F., Lancaster, J., Grisham, M. B., Powers, D. R. and Giles, T. D. (1995). Nitro containing L-arginine analogs interfere with assays for nitrate and nitrite. *Life Sci.* **57**, 1949–1961.

Griffin, O. W. and Stuehr, D. J. (1995). Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol.* **57**, 707–736.

Hecker, M. and Billiar, T. R. (1996). Detection of L-arginine, related metabolites and guanidino compounds. In *Methods in Nitric Oxide Research* (ed. M. Feelisch and J. S. Stamler), pp. 259–270. Chichester, New York: John Wiley & Sons.

Hope, B. T., Michael, G. J., Knigge, K. M. and Vincent, S. R. (1991). Neuronal NADPH-diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **88**, 2811–2814.

Huang, S., Kerschbaum, H. H., Engel, E. and Hermann, A. (1997). Biochemical characterization and histochemical localization of nitric oxide synthase in the nervous system of the snail, *Helix pomatia*. *J. Neurochem.* **69**, 2516–2528.

Jacklet, J. W. (1995). Nitric oxide is used as an orthograde cotransmitter at identified histaminergic synapses. *J. Neurophysiol.* **74**, 891–895.

Jacklet, J. W. and Gruhn, M. (1994a). Co-localization of NADPH-diaphorase and myomodulin in synaptic glomeruli of *Aplysia*. *NeuroReport* **5**, 1841–1844.

Jacklet, J. W. and Gruhn, M. (1994b). Nitric oxide as a putative transmitter in *Aplysia*: neuronal circuits and membrane effects. *Neth. J. Zool.* **44**, 524–534.

Jankowski, J. A., Tracht, S. and Sweedler, J. V. (1995). Assaying single cells with capillary electrophoresis. *Trends Analyt. Chem.* **14**, 170–178.

Jones, W. R. (1997). Capillary ion electrophoresis. In *Handbook of Capillary Electrophoresis* (ed. J. P. Landers), pp. 155–187. Boca Raton, FL: CRC Press.

Kennedy, R. T. and Jorgenson, J. W. (1989). Quantitative analysis of individual neurons by open tubular liquid chromatography with voltammetric detection. *Analyt. Chem.* **61**, 436–441.

Kennedy, R. T., Oates, M. D., Cooper, B. R., Nickerson, B. and Jorgenson, J. W. (1989). Microcolumn separations and the analysis of single cells. *Science* **246**, 57–63.

Khaledi, M. G. (1998). High performance capillary electrophoresis. In *Chemical Analysis Series*, vol. 146 (ed. J. D. Winefordner), pp. 3–1014. New York: John Wiley & Sons.

Kikuchi, K., Nagano, T. and Beckman, J. S. (1996). Determination of nitric oxide by chemiluminescence in the reaction with luminol and hydrogen peroxide. In *Methods in Nitric Oxide Research* (ed. M. Feelisch and J. S. Stamler), pp. 479–488. New York: John Wiley & Sons Ltd.

Kojima, H., Nakasubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagashi, H., Hirata, Y. and Nagano, T. (1998). Detection and imaging of nitric oxide with novel fluorescent indicators: Diaminofluoresceins. *Analyt. Chem.* **70**, 2446–2453.

Kojima, H., Sakurai, K., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y., Akaike, T. and Maeda, H. (1997). Development of a fluorescent indicator for the bioimaging of nitric oxide. *Biol. Pharmac. Bull.* **20**, 1229–1232.

Korneev, S. A., Piper, M. R., Picot, J., Phillips, R., Korneeva, E. I. and O'Shea, M. (1998). Molecular characterization of NOS in

a mollusc: expression in a giant modulatory neuron. *J. Neurobiol.* **35**, 65–76.

Landers, J. P. (1997). *Handbook of Capillary Electrophoresis*. Boca Raton, FL: CRC Press. 894pp.

Leake, L. D., Davis, M. P., Chen, D. and Moroz, L. L. (1996). An unique model for the analysis of neuronal nitric oxide signaling: the leech CNS. In *Neurobiology of Invertebrates: Simple and Complex Regulatory Systems* (ed. J. Salanki, K. S-Rozsa and K. Elekes), pp. 135–143. Budapest: Akademia Kiado.

Leake, L. D. and Moroz, L. L. (1996). Putative nitric oxide synthase (NOS)-containing cells in the central nervous system of the leech, *Hirudo medicinalis*: NADPH-diaphorase histochemistry. *Brain Res.* **723**, 115–124.

Leone, A. M., Furst, V. W., Foxwell, N. A., Cellek, S. and Moncada, S. (1996). Visualization of nitric oxide generated by activated murine macrophages. *Biochem. Biophys. Res. Commun.* **221**, 37–41.

Lillard, S. J. and Yeung, E. S. (1997). Capillary electrophoresis for the analysis of single cells: Laser-induced fluorescence detection. In *Handbook of Capillary Electrophoresis* (ed. J. P. Landers), pp. 523–544. Boca Raton, FL: CRC Press.

Lillard, S. J., Yeung, E. S. and McCloskey, M. A. (1996). Monitoring exocytosis and release from individual mast cells by capillary electrophoresis with laser-induced native fluorescence detection. *Analyt. Chem.* **68**, 2897–2904.

Malinski, T. and Czuchajowski, L. (1996). Nitric oxide measurement by electrochemical methods. In *Methods in Nitric Oxide Research* (ed. M. Feelisch and J. S. Stamler), pp. 319–339. New York: John Wiley & Sons Ltd.

Malinski, T., Mesaros, S. and Tomboulian, P. (1996). Nitric oxide measurement using electrochemical methods. *Meth. Enzymol.* **268**, 58–69.

Malinski, T. and Taha, Z. (1992). Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor. *Nature* **358**, 676–678.

Martinez, A., Riveros-Moreno, V., Polak, J. M., Moncada, S. and Sesma, D. (1994). Nitric oxide (NO) synthase immunoreactivity in the starfish *Marthasterias glacialis*. *Cell Tissue Res.* **275**, 599–603.

Matsumoto, T., Nakane, M., Pollock, J. S. J. E. K. and Forstermann, U. (1993). A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative. *Neurosci. Lett.* **155**, 61–64.

Meulemans, A. and Delsenne, F. (1994). Measurement of nitrite and nitrate levels in biological samples by capillary electrophoresis. *J. Chromatogr.* **660**, 401–404.

Meulemans, A., Mothet, J. P., Schirar, A., Fossier, P., Tauc, L. and Baux, G. (1995). A nitric oxide synthase activity is involved in the modulation of acetylcholine release in *Aplysia* ganglion neurons: a histological, voltammetric and electrophysiological study. *Neuroscience* **69**, 985–995.

Moncada, S. and Higgs, E. A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* **9**, 1319–1330.

Moroz, L. L., Bulloch, A. G. M., Lukowiak, K. and Syed, N. (1994a). Putative NO-synthesizing neurons of *Lymnaea in vivo* and *in vitro*. *Neth. J. Zool.* **44**, 535–549.

Moroz, L. L., Chen, D., Gillette, M. U. and Gillette, R. (1996a). Nitric oxide synthase activity in the molluscan CNS. *J. Neurochem.* **66**, 873–876.

Moroz, L. L. and Gillette, R. (1996a). From Polyplacophora to Cephalopoda: comparative analysis of nitric oxide signalling in

Mollusca. In *Neurobiology of Invertebrates: Simple and Complex Regulatory Systems* (ed. J. Salanki, K. S-Rozsa and K. Elekes), pp. 169–182. Budapest: Akademia Kiado.

Moroz, L. L. and Gillette, R. (1996b). NADPH-diaphorase localization in the CNS and peripheral tissues of the predatory sea-slug *Pleurobranchaea californica*. *J. Comp. Neurol*. **367**, 607–622.

Moroz, L. L., Norby, S. W., Clarkson, R., Sweedler, J. V. and Gillette, R. (1997). Non-enzymatic formation of nitric oxide in the central and peripheral tissues. *Soc. Neurosci. Abstr.* **23**, 1780.

Moroz, L. L., Norby, S. W., Cruz, L., Sweedler, J. V., Gillette, R. and Clarkson, R. B. (1999). Non-enzymatic production of nitric oxide (NO) from NO synthase inhibitors. *Biochem. Biophys. Res. Com.* (in press).

Moroz, L. L., Park, J. H. and Winlow, W. (1993). Nitric oxide activates buccal motor patterns in *Lymnaea stagnalis*. *NeuroReport* **4**, 643–646.

Moroz, L. L., Radbourne, S. and Winlow, W. (1996b). The use of NO-sensitive microelectrodes for direct detection of nitric oxide (NO) production in molluscs. In *Neurobiology of Invertebrates: Simple and Complex Regulatory Systems* (ed. J. Salanki, K. S-Rozsa and K. Elekes), pp. 155–167. Budapest: Akademia Kiado.

Moroz, L. L., Winlow, W., Turner, R. W., Bulloch, A. G., Lukowiak, K. and Syed, N. I. (1994b). Nitric oxide synthase-immunoreactive cells in the CNS and periphery of *Lymnaea*. *NeuroReport* **5**, 1277–1280.

Muller, U. (1994). Ca^{2+}/calmodulin-dependent nitric oxide synthase in *Apis mellifera* and *Drosophila melanogaster*. *Eur. J. Neurosci.* **6**, 1362–1370.

Nakatsubo, N., Kojima, H., Kikukchi, K., Nagoshi, H., Hirata, Y., Maeda, D., Imai, Y., Irimura, T. and Nagano, T. (1998). Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins. *FEBS Lett.* **427**, 263–266.

Nighorn, A., Gibson, N. J., Rivers, D. M., Hildebrand, J. G. and Morton, D. B. (1998). The nitric oxide–cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*. *J. Neurosci.* **18**, 7244–7255.

Park, J. H., Straub, V. A. and O'Shea, M. (1998). Anterograde signaling by nitric oxide: characterization and *in vitro* reconstruction of an identified nitrergic synapse. *J. Neurosci.* **18**, 5463–5476.

Regulski, M. and Tully, T. (1995). Molecular and biochemical characterization of dNOS: a *Drosophila* Ca^{2+}/calmodulin-dependent nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **92**, 9072–9076.

Rhodes, P. M., Leone, A. M., Francis, P. L., Struthers, A. D. and Moncada, S. (1995). The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans. *Biochem. Biophys. Res. Commun.* **209**, 590–596.

Righetti, P. G. (1996). *Capillary Electrophoresis in Analytical Biotechnology*. Boca Raton, FL: CRC Press.

Salter, M., Duffy, C., Garthwaite, J. and Strigbos, P. J. L. M. (1996). *Ex vivo* measurement of brain tissue nitrite and nitrate accurately reflects nitric oxide synthase activity *in vivo*. *J. Neurochem.* **66**, 1683–1690.

Scholz, N. L., Chang, E. S., Graubard, K. and Truman, J. W. (1998). The NO/cGMP pathway and the development of neural networks in postembryonic lobsters. *J. Neurobiol.* **34**, 208–226.

Shibuki, K. and Okada, D. (1991). Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature* **349**, 326–328.

Shippy, S. A., Jankowski, J. A. and Sweedler, J. A. (1995). Analysis of trace level peptides using capillary electrophoresis with UV laser-induced fluorescence. *Analyt. Chim. Acta* **307**, 163–171.

Stamler, J. S., Singel, D. J. and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox activated forms. *Science* **258**, 1898–1902.

Swanek, F. D., Feris, S. S. and Ewing, A. G. (1997). Capillary electrophoresis for the analysis of single cells: Electrochemical, mass spectrometric and radiochemical detection. In *Handbook of Capillary Electrophoresis* (ed. J. P. Landers), pp. 495–522. Boca Raton, FL: CRC Press.

Timperman, A. T., Oldenburg, K. E. and Sweedler, J. V. (1995). Native fluorescence detection and spectral differentiation of peptides containing tryptophan and tyrosine in capillary electrophoresis. *Analyt. Chem.* **67**, 3421–3426.

Tomer, K. B., Deteding, L. J. and Parker, C. E. (1998). High-performance capillary electrophoresis–mass spectrometry. In *High Performance Capillary Electrophoresis*, vol. 146 (ed. M. G. Khaledi), pp. 405–448. New York: John Wiley & Sons.

Tong, W. and Yeung, E. S. (1997). Monitoring single-cell pharmacokinetics by capillary electrophoresis and laser-induced native fluorescence. *J. Chromatogr. B Biomed. Appl.* **689**, 321–325.

Weller, R., Pattullo, S., Smith, L., Golden, M., Ormerod, A. and Benjamin, N. (1996). Nitric oxide is generated on the skin surface by reduction of sweat nitrate. *J. Invest. Dermatol.* **107**, 327–331.

Wood, J. and Garthwaite, J. (1994). Models of the diffusional spread of nitric oxide: Implications for neural nitric oxide signaling and its pharmacological properties. *Neuropharmac.* **33**, 1235–1244.

Yeung, E. S. (1994). Chemical analysis of single human erythrocytes. *Acc. Chem. Res.* **27**, 409.

Yokoi, I., Habu, H., Kabuto, H. and Mori, A. (1996). Analysis of nitrite, nitrate and nitric oxide synthase activity in brain tissue by automated flow injection technique. *Meth. Enzymol.* **268**, 152–159.

Zweier, J. L., Wang, P., Samouilov, A. and Kuppusamy, P. (1995). Enzyme-independent formation of nitric oxide in biological tissues. *Nature Medicine* **1**, 804–809.