Injury-induced expression of endothelial nitric oxide synthase by glial and microglial cells in the leech central nervous system within minutes after injury

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It is known that nitric oxide (NO) is produced by injured tissues of the mammalian central nervous system (CNS) within days of injury. The aim of the present experiments was to determine the cellular synthesis of NO in the CNS immediately after injury, using the CNS of the leech which is capable of synapse regeneration, as a step towards understanding the role of NO in nerve repair. We report that within minutes after crushing the nerve cord of the leech, the region of damage stained histochemically for NADPH diaphorase, which is indicative of nitric oxide synthase (NOS) activity, and was immunoreactive for endothelial NOS (eNOS). On immunoblots of leech CNS extract, the same antibody detected a band with a relative molecular mass of 140 000, which is approximately the size of vertebrate eNOS. Cells expressing eNOS immunoreactivity as a result of injury were identified after freezing nerve cords, a procedure that produced less tissue distortion than mechanical crushing. Immunoreactive cells included connective glia and some microglia. Calmodulin was necessary for the eNOS immunoreactivity: it was blocked by calmodulin antagonist W7 (25 μ M), but not by similar concentrations of the less potent calmodulin antagonist W12. Thus in the leech CNS, in which axon and synapse regeneration is successful, an increase in NOS activity at lesions appears to be among the earliest responses to injury and may be important for repair of axons.

Keywords: nitric oxide; nitric oxide synthase; endothelial nitric oxide synthase; calmodulin antagonists; *Hirudo medicinalis*; nerve injury

1. INTRODUCTION

A delayed appearance of nitric oxide (NO) has been reported in injured and diseased tissues of the mammalian central nervous system (CNS) (Blottner *et al.* 1995). For example, after peripheral axotomy, nitric oxide synthase (NOS) messenger RNA (Verge *et al.* 1992) and protein (Vizzard *et al.* 1995) increase in some neuronal cell populations. Possible sources of NO may be macrophages that migrate to lesions and make an inducible nitric oxide synthase (iNOS), or neurons that express the Ca²⁺/calmodulin-dependent endothelial (eNOS) and neuronal (nNOS) isoforms of NOS (Paakkari & Lindsberg 1995).

The precise function of NO in the mammalian CNS is unknown. Its cytotoxicity is believed to be a line of defence against infection (Koprowski *et al.* 1993). In addition, NO activates guanylate cyclase and can reduce growth-cone motility through its inhibition of fatty acid acylation (Hess *et al.* 1993). These actions might be related to the transient

expression of NOS during the development of the CNS and peripheral nervous system (PNS), and its reported reappearance after injury. NO can also function as a neurotransmitter both in vertebrates and invertebrates (Gelperin 1994), which have corresponding isoforms.

The leech CNS comprises a chain of ganglia (figure 1a) and has been favourable for the study of nerve repair and synapse regeneration at the level of individual, identified neurons (Nicholls 1987). Only eight large glial cells ensheath all of the neurons of each ganglion and two span the distance of up to 5 mm between each pair of ganglia, ensheathing thousands of axons. Investigation of injury-induced NOS expression in a simple nervous system such as that of the leech has the potential to increase our understanding of this protein's role in the pathology of neurological injury. Portions of the work have been presented in abstract form (Shafer & Sahley 1996; Shafer *et al.* 1997).

2. MATERIALS AND METHODS

The CNS from 74 adult 4–6 g leeches (*Hirudo medicinalis*) were dissected in leech Ringers (Nicholls & Baylor 1968). In some nerve cords, the presence of NOS activity after injury was

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determined using NADPH diaphorase (NADPH-d) histochemistry (Gelperin 1994; Blottner *et al.* 1995; Leake & Moroz 1996). In brief, connectives were dissected from animals, crushed with fine forceps, fixed in 4% paraformaldehyde for 1–2 h at room temperature, incubated for 20 min in NADPH-d staining solution (1.5 mM nitro blue tetrazolium (NBT), 2.4 mM β -NADPH, 0.05% (v/v) dimethyl sulphoxide (DMSO), 5×10^{-5} M dicumarol in 0.1 M Tris buffer, pH 8), rinsed in 0.1 M Tris buffer and fixed for 5 min in 4% formaldehyde in methanol.

For immunohistochemistry, crushed and uncrushed connectives were kept in Ringers with glucose at 25 °C for between 10 min and 4 h, fixed in 4% paraformaldehyde for 20 min, and rinsed in phosphate buffered saline (PBS), pH 7.5. Primary monoclonal antibodies for eNOS, nNOS, or iNOS (Transduction Laboratories, Lexington, KY) were diluted 1:150 in blocking solution (10% (v/v) foetal-calf serum and 2% (v/v) Triton X-100 in PBS, pH 7.5). For the secondary antibody, a 1:200 dilution of Texas Red-conjugated rabbit anti-mouse IgG (H+L) was used.

Damaged adult leech CNS extracts were made by homogenizing nerve cords in liquid nitrogen and placing them in ice-cold extraction buffer (0.1 M PIPES, 2 mM MgSO_4 , 1 mM EGTA, 0.5 mM DTT, pH 6.9) with protease inhibitors (0.3 mM PMSF, 1 mM TAME, $2 \mu\text{g ml}^{-1}$ leupeptin, $2 \mu\text{g ml}^{-1}$ aprotinin). After centrifugation, the pellet was resuspended in SDS buffer containing 50 mM Tris-HCl (pH 6.8), 0.002% bromophenol blue, 1% SDS, 1% β -mercaptoethanol and 8% glycerol. Samples were boiled for 5 min and *ca.* 20 μg protein per lane were analysed by SDS-PAGE. Immunoblotting was with the eNOS antibody diluted 1:2500 in blocking solution (10% goat serum (v/v) and 0.15% Tween-20 (v/v) in PBS, pH 7.5) and a 1:2000 dilution of goat anti-mouse IgG conjugated to HRP as a secondary antibody. Protein bands were revealed with a chemiluminescent substrate (SuperSignal, Pierce).

To identify NOS immunoreactive cells most easily, whole cords were frozen at -70°C , thawed and fixed in 4% paraformaldehyde in PBS. Cells responding to freeze injury stained with eNOS immunohistochemistry and a 1:100 dilution of HRP-conjugated secondary antibody.

To determine the role of calmodulin in activation of the eNOS-like protein, we exposed crushed and uncrushed connectives to the calmodulin antagonists W7 and, as a control, the less potent antagonist W12 (Calbiochem, San Diego, CA). Connectives were incubated in the antagonist for 30 min at 25 °C, crushed and processed for eNOS immunoreactivity using Texas Red-conjugated secondary antibody. A series of images was recorded through each connective with a laser-scanning confocal imaging system (BioRad), equipped with a $\times 20$ Nikon objective ($\text{NA}=0.75$), without measurable bleaching. The mean pixel intensity at the injury was measured in a standard rectangle, subtracting background pixel intensity of the nerve cord away from the injured area and dividing by the mean pixel intensity at the injured region of a control nerve cord from the same animal, but not exposed to drug treatment. Four experiments were pooled and the normalized data analysed with an analysis of variance and a Fisher's PLSD (protected least significant difference).

3. RESULTS

(a) *Rapid induction of NADPH diaphorase (NADPH-d) activity after injury*

As with other NADPH-diaphorases, NOS catalyses the reduction of tetrazolium salts into an insoluble blue

Figure 1. Detection of NOS-like protein in damaged leech CNS using NADPH-d histochemistry and monoclonal antibodies for NOS. (a) Diagram of the leech CNS showing crush site (marked by an X) in the connectives linking segmental ganglia. In all of the figures, connectives are orientated in this way. (b) Segmental connective stained for NADPH-d activity 10 min after the delivery of a single crush. Panels (c–e) show immunohistochemical staining of crushed connectives using mouse monoclonal antibodies raised against eNOS, nNOS and iNOS, respectively. Monoclonal antibody raised against human eNOS recognized a molecule specific to the site of crush (c), whereas a monoclonal antibody raised against nNOS stained regions inside and outside the crush (d). No staining was visible in crushed connectives stained with an iNOS monoclonal antibody (e). Scale bar, 50 μm .

formazan precipitate at sites where it is present. After injury to the leech segmental connective, NADPH-d histochemistry stained the lesion and immediately adjacent regions, indicating NOS activity (figure 1b). The increase in NADPH-d activity was rapid, occurring within 10 min of injury. Little or no staining was discerned in uncrushed regions of the connectives (figure 1b). In control preparations without NADPH there was no staining at the crush (not shown).

(b) *NADPH-d activity and eNOS immunoreactivity co-localize at the injury site*

Coincident with the increase in NADPH-d activity at the crush site, intense endothelial NOS (eNOS) immunoreactivity appeared at the site of injury in crushed cords (figure 1c), but not outside the crush or in neuronal cell bodies. There was little staining in non-crushed controls (not shown). Another monoclonal antibody, made against human nNOS, produced little additional staining at the crush, but showed some binding outside the crush (figure 1d) and in uncrushed preparations. Crushed cords did not stain using anti-iNOS monoclonal antibodies, resembling controls (figure 1e).

To test further for the existence of an eNOS in the leech nervous system, immunoblot analysis of adult leech

Figure 2. A mouse monoclonal antibody for human endothelial cell nitric oxide synthase recognized a protein with an M_r of 140K in (a) human endothelial cell line extract (positive control; Transduction Laboratories) and in (b) leech CNS extract. Bands of lower molecular mass were also immunoreactive and may have been products of proteolytic degradation. Blots were from the same experiment but film exposure for chemiluminescent detection was different for each.

CNS homogenate was carried out using the monoclonal antibody raised against human eNOS. As indicated in figure 2, the monoclonal antibody recognized a protein with a relative molecular mass of 140 000 (M_r 140K), as well as several bands of lower molecular mass in the leech CNS extract. These smaller bands are assumed to be products of proteolytic degradation, as reported in other studies of NOS (Elphick *et al.* 1995). The existence of the 140K band, a size nearly identical to the human eNOS positive control, served as further evidence for the existence of an eNOS-like molecule in the CNS of *Hirudo*.

(c) Multiple cell types are immunoreactive for eNOS after freeze injury

The identity of the cells that were NOS-immunoreactive after injury was established by cryoneurolysis, a method that reliably severs axons in leech connective (A. Chen and K. Muller unpublished data) but maintains basic cord structure at the light-microscope level. Although the cord can be frozen focally, whole nerve cords were frozen at -70°C for the easiest identification of individual cell types, which could be recognized by their distinctive location, morphology and size. Staining was always present, but some variability existed in the types of cells that stained in each preparation.

The large glial cell and the microglia are two types of cells located among the nerve fibres of each leech connective. Figure 3a illustrates a freeze-damaged connective with an eNOS-immunoreactive large glial cell, easily identified by its large size, shape and location with a large nucleus at the midpoint along the lateral connective. Stretches of immunoreactivity were visible along the

Figure 3. Multiple cell types were immunoreactive for eNOS in freeze-injured cords. Whole connectives were frozen and then immunostained using a monoclonal antibody made against a fragment of human eNOS. Panel (a) is a Nomarski photomicrograph showing an immunostained large glial cell body (arrowhead) located at the midpoint along the lateral connective. Glial cytoplasm aligns along axons and occupies the medial portion of the connective that was immunostained in (b). The fibre-like appearance of the stain may derive from longitudinal folds of the ensheathing glial cell. As illustrated in (c), microglial cells (arrowhead) also stained with the eNOS antibody. Note the microglial cell's processes, its location among the nerve fibres and its small size compared to the large glial cell in (a). On the surface of the connective (d), eNOS immunoreactivity was visible in the cells (arrowheads) of the perineurial sheath. Scale bar, 20 μm .

medial portion of the connective (figure 3b). Cytoplasm of the large glial cell occupies the central portion of the connective and wraps the surrounding axons in longitudinal sheets of folded membrane. The location and uniform length of the immunoreactive, striated region indicated that a portion of the large glia was stained. We have not ruled out the possibility, however, that some of the striations in the cluster may have been immunoreactive axon segments, although the uniform border made this unlikely (not shown). Immunopositive microglial cells were also present among the axonal fibres of the connective. They were distinguishable from the connective glia by location, small cell bodies (less than 10 μm in diameter) and their processes (figure 3c). Thus, both the connective glia and microglia contain eNOS-like immunoreactivity after freeze injury.

Cells of the perineurial sheath surrounding the leech CNS also stained by eNOS immunohistochemistry in

Figure 4. Effect of calmodulin antagonist potency on eNOS immunoreactivity at the site of injury. The normalized intensity at the crush is presented as a function of drug treatment. Those significantly different as determined by a Fisher's PLSD at $p < 0.0001$ are indicated by *. For details of the analysis, see § 2.

freeze-damaged cords (figure 3*d*). In some preparations, the long spindle-shaped connective muscle cell that extended from each hemi-ganglion within the length of the perineurial sheath was lightly stained (not shown). Therefore, several different cell types in the injured leech nerve cord are immunoreactive for eNOS and may produce NO at lesions.

(d) *eNOS immunoreactivity requires calmodulin*

Because activation of eNOS is calcium-activated calmodulin dependent, we investigated whether the eNOS-like immunoreactivity was also regulated by calmodulin. The calmodulin antagonist W7 (25–100 µM) blocked the appearance of eNOS immunoreactivity at the site of injury (figure 4), whereas the weaker calmodulin antagonist W12 (50 µM) had little or no effect (figure 4).

Analysis of variance indicated that there was a significant difference among the groups ($F_{5,16} = 22.608$, $p < 0.0001$). Post-hoc analysis (Fisher's PLSD) indicated that there was a significant difference between the intensity of crushed cords and those injured cords treated with concentrations of W7 at 25–100 µM ($p < 0.0001$). However, there was not a significant difference between the injured cords and the injured cords treated with the less potent calmodulin antagonist W12 at 50 µM ($p = 0.2521$). These results are consistent with the presence in the leech of an injury-induced eNOS-like protein the immunofluorescence of which is regulated by calmodulin, similar to the regulation of eNOS activity in other systems.

4. DISCUSSION

The very rapid appearance at the lesion of NADPH-d activity and eNOS immunoreactivity indicate that eNOS present in the nerve cord is activated by injury. The immunoreactivity and activation of eNOS at the lesion

depend upon calmodulin. This is consistent with the calmodulin dependence of mammalian eNOS activity.

(a) *eNOS immunoreactive cells in injured leech CNS*

The cells containing eNOS when injured include connective glia, microglia, and cells of the perineurial sheath. This was shown by freeze damage, or cryoneurolysis, which is an established method that causes nervous system injury and axotomy without the full structural disruption associated with a mechanical crush (Collins *et al.* 1986).

As the rate of freezing and thawing can influence the damage (Mazur 1970) and may vary from one preparation to another, it could account for the variability in staining of cell types. However, this does not reflect a limitation in antibody access, as perineurial sheath cells and connective muscle, which are located closest to the surface, were not stained in some preparations that had immunostained glial cells at the core of the connectives.

In the mammalian CNS, injury induces after several days the production of iNOS in astrocytes and microglia (Murphy & Grzybicki 1996). In the leech CNS, although no cells were labelled with monoclonal antibodies to iNOS, it is possible that a leech iNOS-like molecule was present and not recognized by the antibody used, or that it appears much later after injury, as in mammals.

(b) *Injury-induced eNOS-like activity and immunoreactivity*

Several lines of evidence show that NADPH-d activity was due to the activation of NOS, rather than one of the other non-NOS NADPH hydrogenases reported to be in neural tissue (Blottner *et al.* 1995). First, staining from non-NOS NADPH-d in mammalian CNS is eliminated by fixation, whereas NOS is resistant to fixation (Matsumoto *et al.* 1993), as in the leech. Second, immunohistochemistry with a monoclonal antibody made against human eNOS demonstrated a pattern of staining at lesions that was coextensive with NADPH-d stain. Third, immunoblots of damaged leech CNS extracts revealed a protein band with an M_r that was *ca.* 130K–140K, which is the size of mammalian eNOS (Lamas *et al.* 1992).

In the mammalian CNS, NO is produced predominantly by nNOS (Huang *et al.* 1993), although eNOS may occur in neurons (Dinerman *et al.* 1994; O'Dell *et al.* 1994). In invertebrates, NOS-like mRNA, protein or activity is found in central neuron axons and cell bodies, in specific fibre tracts, and in the neuropil (Martinez 1995). Leake & Moroz (1996) determined that in the leech *H. medicinalis*, some neuronal cell bodies in ganglia exhibit NADPH-d activity and measurable NO production. Because in the present study eNOS monoclonal antibody did not stain neuronal cell bodies, and tissue outside the lesion stained using antibody to nNOS, multiple NOS isoforms must exist in the leech CNS, as in mammals.

(c) *Regulation of injury-induced eNOS-like protein*

The intense staining localized at the crush site was not due to differential penetration of the antibody at the crush site, because under similar staining conditions other antibodies, such as anti-myomodulin (Keating & Sahley 1996), penetrate the perineurial sheath and stain

cells in the connective, but not in a damage-specific manner. In addition, specific immunostain was not present in tissue damaged after fixation. Instead, the presence of staining at the crush site may have been due to unmasking of an antigenic site recognized by the eNOS antibody as a result of an injury-induced conformational change in the structure of eNOS-like protein or unbinding of an inhibitor (Jaffrey & Snyder 1996). Moreover, this conformational change could be regulated by calmodulin, as a calmodulin antagonist inhibited the appearance of eNOS immunoreactivity at sites of injury. Although there is no definitive proof that changes in antigenicity of an eNOS-like molecule reflect changes in activity, such mechanisms could at least account for the rapid appearance of eNOS immunoreactivity at the injury site.

To our knowledge, rapid appearance of NADPH-d activity and NOS immunoreactivity after nervous system injury has not been reported previously. Time periods reported for NOS upregulation in injured nervous tissue are hours to days (Verge *et al.* 1992; Vizzard *et al.* 1995), although in general it is not clear that shorter periods have been examined. Early production of NO may affect immediate events after damage. For example, NO may cause growth cones to collapse (Hess *et al.* 1993; Rentera & Constantine-Paton 1995). In the pruning of damaged axons that takes place before axonal outgrowth and regeneration (Muller 1984), NO may also be involved through a similar mechanism.

Our results describe an injury-induced NOS-like protein in the well studied and relatively simple nervous system of the leech, in which cellular and molecular events after injury to the CNS have been characterized (Nicholls 1987; von Bernhardt & Muller 1995). In addition, the rapid appearance of NADPH-d activity and eNOS immunoreactivity after injury may be important for early regenerative events. Although a role for NO is a matter of conjecture, it is clear that in the leech, where regeneration is successful, NOS activity is one of the earliest responses to injury, so that early NO production could influence axonal repair.

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REFERENCES

- Blottner, D., Grozdanovic, Z. & Gossrau, R. 1995 Histochemistry of nitric oxide synthase in the nervous system. *Histochem. J.* **27**, 785–811.
- Collins, G. H., West, N. R., Parmley, J. D., Samson, F. M. & Ward, D. A. 1986 The histopathology of freezing injury to the rat spinal cord. A light microscope study. I. Early degenerative changes. *J. Neuropath. Exp. Neurol.* **45**, 721–741.
- Dinerman, J. L., Dawson, T. M., Schell, M. J., Snowman, A. & Snyder, S. H. 1994 Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc. Natn. Acad. Sci. USA* **91**, 4214–4218.
- Elphick, M. R., Kemenes, G., Staras, K. & O'Shea, M. 1995 Behavioral role for nitric oxide in chemosensory activation of feeding in a mollusc. *J. Neurosci.* **15**, 7653–7664.
- Gelperin, A. 1994 Nitric oxide mediates network oscillations of olfactory interneurons in a terrestrial mollusc. *Nature* **369**, 61–63.
- Hess, D. T., Patterson, S. I., Smith, D. S. & Skene, J. H. 1993 Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* **366**, 562–565.
- Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. & Fishman, M. C. 1993 Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **75**, 1273–1286.
- Jaffrey, S. R. & Snyder, S. H. 1996 PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* **174**, 774–777.
- Keating, H. H. & Sahley, C. L. 1996 Localization of myomodulin-like immunoreactivity in the leech CNS. *J. Neurobiol.* **30**, 374–384.
- Koprowski, H., Zheng, Y. M., Heber-Katz, E., Fraser, N., Rorke, L., Fu, Z. F., Hanlon, C. & Dietzschold, B. 1993 *In vivo* expression of inducible nitric oxide synthase in experimentally induced neurological diseases. *Proc. Natn. Acad. Sci. USA* **90**, 3024–3027.
- Lamas, S., Marsden, P. A., Li, G. K., Tempst, P. & Michel, T. 1992 Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natn. Acad. Sci. USA* **89**, 6348–6352.
- Leake, L. D. & 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.
- Martinez, A. 1995 Nitric oxide synthase in invertebrates. *Histochem. J.* **27**, 770–776.
- Matsumoto, T., Nakane, M., Pollock, J. S., Kuk, J. E. & 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.
- Mazur, P. 1970 Cryobiology: the freezing of biological systems. *Science* **168**, 939–949.
- Muller, K. J. 1984 Regeneration of specific synapses and triggers of axon growth. In *Neuronal growth and plasticity* (ed. M. Kuno), pp. 71–82. Tokyo: Japan Scientific Societies Press.
- Murphy, S. & Grzybicki, D. 1996 Glial NO: normal and pathological roles. *Neuroscientist* **2**, 90–99.
- Nicholls, J. G. 1987 *The search for connections: study of regeneration in the nervous system of the leech. Magnes Lecture Series*, vol. 2, pp. 1–84. Sunderland, MA: Sinauer Associates Inc.
- Nicholls, J. G. & Baylor, D. A. 1968 Specific modalities and receptive fields of sensory neurons in the CNS of the leech. *J. Neurophysiol.* **31**, 740–756.
- O'Dell, T. J., Huang, P. L., Dawson, T. M., Dinerman, J. L., Snyder, S. H., Kandel, E. R. & Fishman, M. C. 1994 Endothelial NOS and the blockade of LTP by NOS inhibitors in mice. *Science* **265**, 542–546.
- Paakkari, I. & Lindsberg, P. 1995 Nitric oxide in the central nervous system. *Ann. Med.* **27**, 369–377.
- Rentera, R. C. & Constantine-Paton, M. 1995 Exogenous nitric oxide causes collapse of retinal ganglion cell axonal growth cones *in vitro*. *J. Neurobiol.* **29**, 415–428.
- Shafer, O. T. & Sahley, C. L. 1996 Nitric oxide synthase in the regenerating nervous system of the medicinal leech. *Soc. Neurosci. Abstr.* **22**, 1971.
- Shafer, O. T., Chen, A., Kumar, S., Muller, K., Scott, D. & Sahley, C. L. 1997 Regulation and roles of nitric oxide synthase in the injured leech CNS. *Soc. Neurosci. Abstr.* **23**, 978.
- Verge, V. M. K., Xu, Z., Xu, X. J., Wiesenfeld-Hallin, Z. & Hökfelt, T. 1992 Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies. *Proc. Natn. Acad. Sci. USA* **89**, 11617–11621.
- Vizzard, M. A., Erdman, S. L. & de Groat, W. C. 1995 Increased expression of neuronal nitric oxide synthase (NOS) in visceral neurons after nerve injury. *J. Neurosci.* **15**, 4033–4045.
- von Bernhardt, R. & Muller, K. J. 1995 Repair of the central nervous system: lessons from lesions in leeches. *J. Neurobiol.* **27**, 353–366.