British Journal of Pharmacology (2002) 135, 1079–1095

www.nature.com/bjp

REVIEW

NO as a signalling molecule in the nervous system

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British Journal of Pharmacology (2002) **135,** 1079–1095

Keywords: NO; CNS; NANC; NOS synthases; stroke; neurotransmission; signalling

Abbreviations: CAM, calmodulin; CAPON, carboxy-terminal PDZ ligand of nNOS; cyclic AMP, cyclic adenosin monophosphate; cyclic GMP, cyclic guanosin monophosphate; eNOS, endothelial NO synthase; iNOS, inducible NO synthase; LPS, lipopolysaccharides; LTD, long-term depression; LTP, long-term potentiation; NADPH, α-nicotinamide adenine dinucleotide phosphate; NANC, non-adrenergic non-cholynergic; NO, nitric oxide; nNOS, neuronal NO synthase; $ONOO^-$, peroxynitrite; PIN, protein inhibitor of nNOS; PSD, postsynaptic density protein; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; tSXV, Ser/Thr-X-Val motif; VIP, vasoactive intestinal peptide

The discovery that nitric oxide (NO) functions as a signalling molecule in the nervous system has radically changed the concept of neural communication. Indeed, the adoption of the term nitrergic for nerves whose transmitter function depends on the release of NO or for transmission mechanisms brought about by NO (Moncada *et al*., 1997) emphasizes the specific characteristics of this mediator. The physical properties of NO prevent its storage in lipid-lined vesicles and metabolism by hydrolytic degradatory enzymes. Therefore, unlike established neurotransmitters, NO is synthesized on demand and is neither stored in synaptic vesicles nor released by exocytosis, but simply diffuses from nerve terminals. The distance of this NO diffusion (40–300 μm in diameter) implies that structures in the vicinity of the producing cell, both neuronal and non-neuronal, are influenced following its release. This suggests that, as well as acting as a neurotransmitter, NO has a neuromodulatory role (Garthwaite & Boulton, 1995). In addition, it diffuses into rather than binds with protein receptors on adjacent cells, and most of its known actions are the consequence of interplay with intracellular targets that would usually be regarded as secondary messengers. The activity of conventional neurotransmitters is terminated either by re-uptake mechanisms or enzymatic degradation while inactivation of NO follows reaction with a substrate. There are multiple points at which biological control can be exerted over the production and activity of conventional neurotransmitters. However, control of the synthesis of NO is the key to regulating its activity.

Endothelial NOS (eNOS) and inducible NOS (iNOS) are present in the nervous system and will be duly addressed here. However, neuronal NOS (nNOS) is the principal isoform present in said system and will be the main focus of this review. All nNOS positive neurones exhibit α-nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity, which has become the histochemical marker of nitrergic neurones. However, early results demonstrating this may have been limited by inappropriate fixation procedures and should be viewed with caution (Wolf, 1997). The original cloning of full-length nNOS produced what is now designated as nNOSα, and which accounts for the majority of nNOS activity in nervous tissue (Bredt *et al*., 1991). In addition, four splice variants have recently been identified (nNOSβ, nNOSγ, nNOSμ and nNOS-2) and these appear to exhibit distinct cellular and tissue locations (Gibson, 2001; Nakane *et al*., 1993; Silvagno *et al*., 1996; Alderton *et al*., 2001). In particular, there is growing evidence that nNOS biosynthesis in excitable tissues is not restricted to neurones while substantial amounts of this enzyme have been identified in skeletal muscle, where it is involved in the regulation of metabolism and muscle contractility (Stamler & Meissner, 2001).

The magnitude of literature dealing with the role of NO in the nervous system is so great that it would be impossible to include in this review the entirety of the research carried out. For logistical reasons, only groundbreaking references have been quoted, but when necessary, recent reviews dealing with specific areas within the field have been included and are intended to act as a guideline for further reading.

Regulation of nNOS

The most important regulator of nNOS activity seems to be free cytosolic Ca^{2+}, which stimulates nNOS through interaction with calmodulin. Arrival of action potentials activates voltage-dependent Ca^{2+} channels situated in the neurolemma, and stimulates the release of Ca^{2+} from intracellular stores. This elevates cytosolic Ca^{2+} concentrations above the 400 nM required for calmodulin to bind to nNOS, thereby activating the enzyme. When the concentration of Ca^{2+} falls, it dissociates from the calmodulin, which in turn dissociates from the nNOS, thus acting as a switch that turns the enzyme on and off (Knowles *et al*., 1989; Sheng *et al*., 1992). Phosphorylation, although less well analysed, constitutes an additional mechanism for regulating nNOS activity. The catalytic activity of the enzyme is decreased following phosphorylation by cyclic adenosin monophosphate (cyclic

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AMP)-dependent protein kinase (Bredt *et al*., 1992; Brüne & Lapetina, 1991), protein kinase C (Bredt *et al*., 1992; Nakane *et al*., 1991) or Ca^{2+}/calmodulin-dependent protein kinase II (Bredt *et al*., 1992; Hayashi *et al*., 1999; Komeima *et al*., 2000; Nakane *et al*., 1991; Schmidt *et al*., 1992b).

This process occurs in the majority of peripheral and in some central nitrergic neurones (Figures 1 and 2). However, in the CNS, NO synthesis seems predominantly regulated by the influx of Ca^{2+} through receptor-dependent channels, in particular following postsynaptic stimulation of NMDA receptors by the excitatory neurotransmitter, glutamate (Bredt & Snyder, 1989; Garthwaite *et al*., 1989). The amino acid terminal of nNOS possesses a PDZ domain which is not present in the β and γ splice variants (Alderton *et al*., 2001; Brenman *et al*., 1996). The aforementioned domains are modular structures of approximately 100 amino acids, which occur in a number of proteins, anchoring them to cytoskeletal elements such as synaptic densities and related membrane-associated guanylate kinases (Tomita *et al*., 2001). In the case of nNOS, its PDZ domain interacts with the postsynaptic density protein PSD-95, whereas the N-Methyl-D-Aspartate (NMDA) receptor contains a Ser/Thr-X-Val motif (tSXV) that also binds with PSD95. By facilitating the proximity of NMDA receptors to the enzyme, the scaffolding protein, PSD95 directly exposes nNOS to the flux of Ca^{2+} entering the ion channel of activated NMDA receptors (Kornau *et al*., 1995; Tomita *et al*., 2001). Transient Ca^{2+} fluxes following the activation of other receptors would be too diluted to have a similar effect by the time they reach the vicinity of nNOS. It is possible that NO bioactivity feeds back to control the activity of the channel as S-nitrosylation of critical cysteines seems to down-regulate the NMDA receptor (Choi *et al*., 2000). There are many other potential regulators of the NMDA receptor/nNOS coupling and downstream signalling pathways. The protein carboxy-terminal PDZ ligand of nNOS (CAPON) is thought to be selectively associated with nNOS and to exhibit a similar regional distribution. CAPON competes with nNOS for PDZ domains, binding to the enzyme and forcing it to disassociate itself from the plasma membrane (Jaffrey *et al*., 1998). Therefore, CAPON determines the amount of nNOS tethered to the plasma membrane and, in this way, regulates NO formation in neurones of the CNS. Furthermore, CAPON anchors nNOS to other macromolecules, such as the small G-protein Dexras-1 (Fang *et al*., 2000), although the importance of this relationship remains to be determined. At this point, it must be said that recent proteomic analysis has not identified CAPON in the vicinity of the NMDA receptor, which raises some doubts about the above hypotheses (Husi *et al*., 2000).

Various other receptors and domains contain the tSXV motif and are also potentially associated with central nNOS and regulated by this multifunctional protein–protein interaction (Tomita *et al*., 2001). nNOS may also be inhibited through an interaction with protein inhibitor of nNOS (PIN), a highly conserved small protein that was originally thought to destabilize nNOS dimers and thus act as an endogenous inhibitor of nNOS (Jaffrey & Snyder, 1996). However, recent reports suggest that PIN is an axonal transport protein for nNOS, rather than its regulator (Hemmens *et al*., 1998; Rodriguez-Crespo *et al*., 1998). nNOS may also be inhibited through an interaction with caveolin-1 and caveolin-3 that, in a way similar to the effect of caveolin-1 on eNOS, could

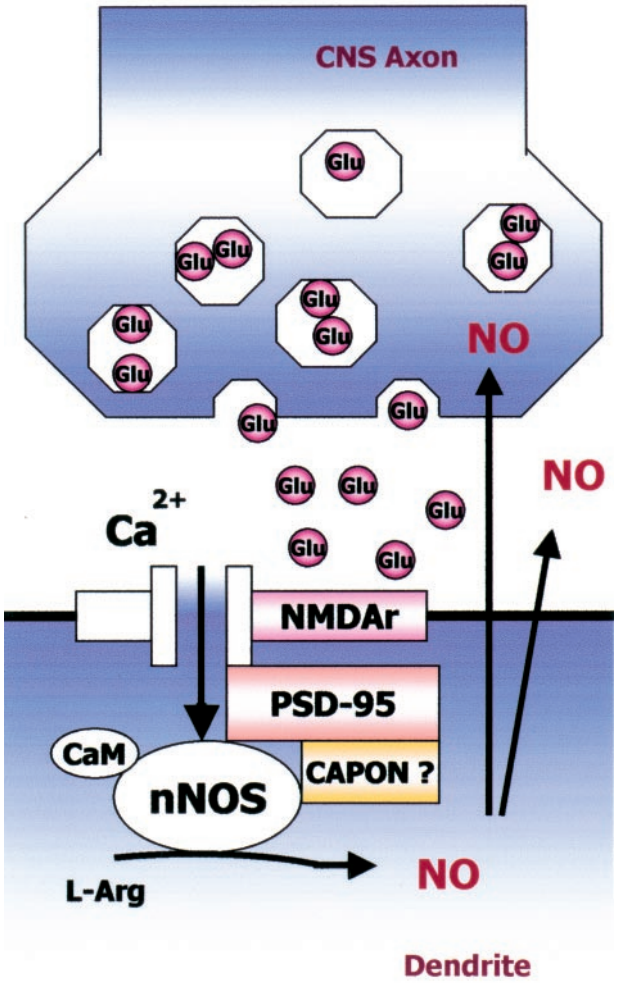

Figure 1 Activation of nNOS in the CNS. Release of glutamate activates NMDA receptors (NMDAr), and the consequent flux of Ca^{2+} entering the ion channel activates nNOS, which is linked to the receptor *via* the postsynaptic density protein PSD-95. It is possible that NO bioactivity feeds back to control the presynaptic neuron and the activity of the channel. The protein CAPON is thought to be selectively associated with nNOS and regulates NO formation in neurones.

displace calmodulin from nNOS (García-Cerdeña *et al*., 1997; Venema *et al*., 1997). Furthermore, these members of the caveolin family interact with other signalling molecules such as c-*src*, Ha-ras and $G_S\alpha$, which suggests a potential role for nNOS in some signalling complexes (Couet *et al*., 1997). Finally, a role for heat shock protein NOS-hsp90/hetero-complexes in the modulation of the haem's interaction with nNOS has recently been suggested (Bender *et al*., 1999).

In skeletal muscle, nNOS activity is related to muscle ACh receptors and membrane depolarization (Figure 3). Again, nNOS is targeted to membrane structures due to the association of its PDZ domain with α_1-syntropin, a dystrophin-associated protein that shares homology with postsynaptic density proteins PDS95 y PDS93. Interactions with PIN, highly expressed in skeletal muscle, and with caveolin-3 are also possible (Brenman *et al*., 1995; Chao *et al*., 1996; Stamler & Meissner 2001; Venema *et al*., 1997).

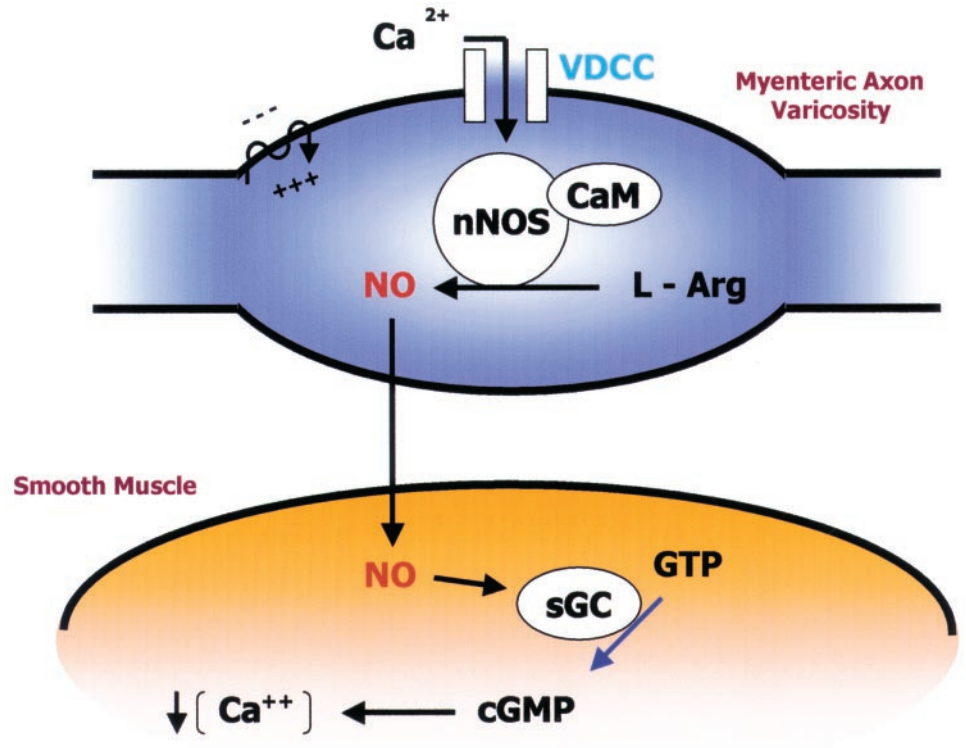

Figure 2 nNOS in mysenteric neurones is regulated by the flux of Ca^{2+} through voltage-dependent calcium channels (VDCC). NO relaxes the adjacent smooth muscle following activation of sGC.

Although considered to be constitutive, levels of nNOS activity and expression appear to be subject to dynamic up- or down-regulation induced by a large variety of stimuli, including nerve (Steel *et al*., 1994; Verge *et al*., 1992) and brain injury (Kitchener *et al*., 1993; Regidor *et al*., 1993), aging (Carrier *et al*., 1997; Mollace *et al*., 1995), pharmacological treatment (Bagetta *et al*., 1993), lactation (Ceccatelli & Eriksson, 1993), hypoxia (Guo *et al*., 1997), stress (Cazal *et al*., 1993), gonadectomy (Ceccatelli *et al*., 1993), light exposure (Schaad *et al*., 1994) and exercise (Tidball *et al*., 1998).

The existence of presynaptic automodulation in nitrergic neurones has also been proposed. Although not yet fully characterized, this action may result from the combination of NO with the haem group of NOS, which inhibits the enzyme (Klatt *et al*., 1992; Rogers & Ignarro, 1992). Finally, there is evidence that cells maintain low levels of cyclic guanosin monophosphate (cyclic GMP) while producing NO. This occurs because increases in Ca^{2+} levels, similar to those needed to stimulate nNOS, also activate a Ca^{2+}/calmodulin-dependent cyclic GMP phosphodiesterase that facilitates the degradation of cyclic GMP (Mayer *et al*., 1992).

Signalling

The actions of NO are a consequence of its influence on a variety of protein functions which it exerts through its reaction with cysteine thiol, S-nitrosylation, and transition metal centres (Drapier & Bouton, 1996; Jaffrey *et al*., 2001; Lane *et al*., 2001). The enzyme soluble guanylyl cyclase (sGC) has long been considered to be the major physiological target for neuronal NO, and there is ample evidence that increases in cyclic GMP levels mediate a large number of the physiological actions of NO. Thus, immunohistochemical techniques have found that the distribution of sGC and cyclic GMP is complementary to that of nNOS (Schmidt *et al*., 1992a; Southam & Garthwaite, 1993; Young *et al*., 1993). Functionally, either nitrergic nerve stimulation or administration of NO-donors increases intracellular cyclic GMP concentrations (Bredt & Snyder, 1989; Torphy *et al*., 1986). In both cases, these responses are mimicked by analogues of cyclic GMP (Gibson & Mirzazadeh, 1989), whereas inhibition of the destruction of this intracellular mediator potentiates the results of nitrergic stimulation (Barbier & Lefebvre, 1995; Bayguinov & Sanders, 1993). The mechanisms linking the rise in cyclic GMP content to the various effects of NO in the CNS and peripheral smooth muscle are not fully understood, although in both cases the final step seems to be a reduction of ($[Ca^{2+}]_i$). Alternative targets of cyclic GMP may involve direct channel gating with the opening of inward Ca^{2+} and Na^{2+} channels, activation of cyclic GMP-dependent kinases, actions related to cyclic adenosin diphosphate (ADP) ribose, and interactions with cyclic AMP resulting from regulation of cyclic GMP-dependent phosphodiesterases (Hunter, 2000; Jaffrey *et al*., 2001; Lincoln *et al*., 2001).

NO modulates oxygen consumption in the mitochondria. In particular, nanomolar concentrations of NO inhibit cytochrome oxidase, the terminal haem-containing enzyme in the mitochondrial respiratory chain. Recent evidence demonstrates that this effect is reversible and competitive

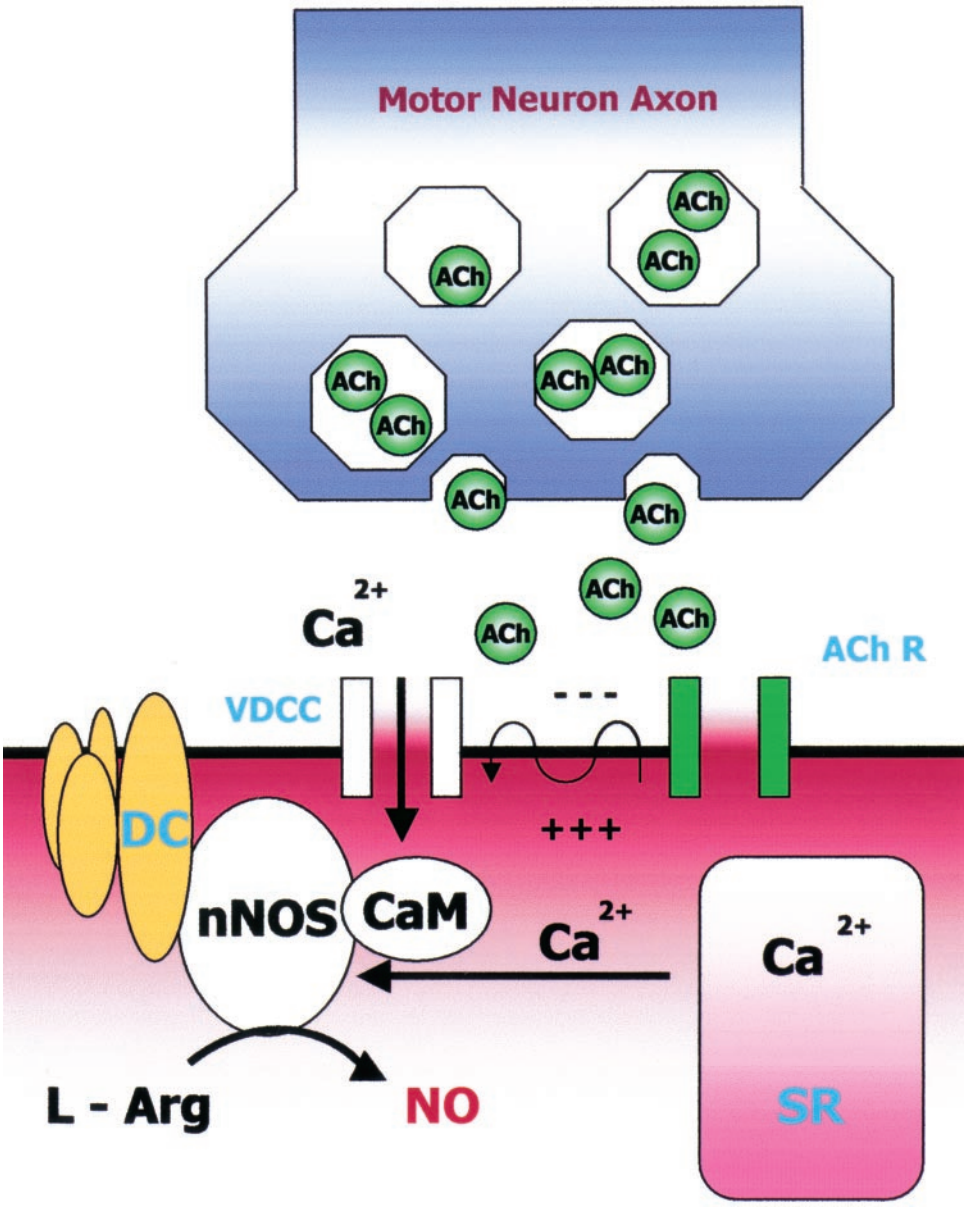

Figure 3 Activation of nNOS in the skeletal muscle follows the influx of Ca^{2+} through voltage-dependent calcium channels (VDCC) induced by activation of ACh receptors (AChr) and membrane depolarization. The release of Ca^{2+} from the sarcoplasmic reticulum (SR) is also implicated. nNOS targets the membrane due to its association with α_1-syntropin, a component of the dystrophin complex (DC).

with oxygen, and suggests that NO is a crucial regulator in the generation of energy and the mediation of cell death by mitochondria (Beltrán *et al*., 2000). The consequences of such an activity are still to be evaluated but, obvious physiological implications aside, they could clarify the mechanisms by which NO is involved in cell or tissue damage. Furthermore, and in conjunction with differences in glycolytic capacity, this activity may explain why neurones and glia show variations in sensitivity to NO-induced damage (Brown, 2000; Almeida *et al*., 2001).

NO has been linked to the release (Meffert *et al*., 1996) of other neurotransmitters and the effects which they produce, in particular acetylcholine (Gustafsson *et al*., 1990; Li & Rand, 1989b), noradrenaline (Boeckxstaens *et al*., 1993; Li & Rand, 1989a) dopamine (Hanbauer *et al*., 1992), glutamate (Montague *et al*., 1994; Sorkin, 1993), γ-aminobutyric acid (GABA) (Beltran *et al*., 1990; Kuriyama & Ohkuma, 1995), serotonin (Bogers *et al*., 1991; Reiser, 1990b), adenosin triphosphate (ATP) (Boeckxstaens *et al*., 1991a), bombesin (Beltran *et al*. 1999), carbon monoxide (Xue *et al*., 2000), opioids (Barnette *et al*., 1990) and endothelin (Reiser, 1990a). The mechanisms responsible for these interactions are still not fully understood, but direct S-nitrosylation of receptors, activation of cyclic GMP-dependent protein phosphorylation cascades, regulation of neuronal energy dynamics and a modulating effect on transporters are potentially involved (Choi *et al*., 2000; Kiss & Vizi, 2001; Pieper *et al*., 2000). In addition, a presynaptic modulation of NO release through the activation of α_2-adrenoceptors, nicotinic receptors, purinergic receptors etc. has also been

proposed (Boeckxstaens *et al*., 1993). Finally, it has been suggested that NO modulates gene transcription and translation in neurones and glia (Hess *et al*., 1993; Peunova & Enikolopov, 1993; 1995). However, these effects would seem to be indirect since there is little evidence of the existence of DNA elements within the promotor regions of eukaryotic cells that respond directly to NO (Morris, 1995).

Nitric oxide in the central nervous system

Introduction

NO was first characterized in the CNS as the intercellular messenger mediating the increase in cyclic GMP levels that follows activation of glutamate receptors (Garthwaite *et al*., 1988). The majority of the information available deals with nNOS, of which the brain contains the highest activity found in any tissue, and which, although present in some cerebral vessels and in glial cells, is predominantly found in neurones (Bredt *et al*., 1990; Salter *et al*., 1991). nNOS-containing neurones are present in many areas of the CNS (Figure 4), with the highest densities occurring in the accessory olfactory bulb and granule cells of the cerebellum. Although nNOS neurones represent only roughly 1% of cell bodies in the cerebral cortex, virtually every neurone in the cortex is exposed to nNOS nerve terminals. From a morphological point of view nNOS neurones display a great heterogeneity in their localization within the CNS, constituting a small population of varying interneurones. Furthermore, the number and chemical characteristics of nNOS neurones vary considerably depending on the area of the brain while the enzyme itself does not co-localize with any single neurotransmitter (Braissant *et al*., 1999; Iwase *et al*., 1998; Vincent, 1995; Wolf, 1997). nNOS can be located either pre- or postsynaptically and is particularly implicated in neural signalling, neurotoxicity, synaptic plasticity and modulation of behavioural pathways such as learning or expression of pain.

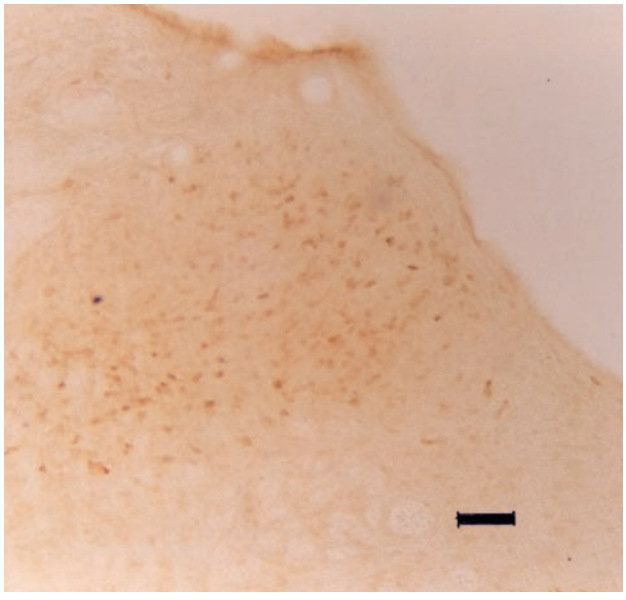

Figure 4 Representative microphotograph of basal nNOS immunoreactivity (monoclonal antibody) in the dorsal vagal complex (DVC) of the brainstem. Scale bar = 100 μm.

eNOS is mainly involved in the regulation of vascular function and, although also present in some populations of neurones (Dinerman *et al*., 1994) and glia (Wiencken & Casagrande, 1999), is predominantly located in the endothelial cells of cerebral vessels. Finally, induction of iNOS in glial cells is implicated in the unspecific immune response of the brain and is usually associated with pathological conditions (Murphy, 2000).

Effects of centrally released NO

Modulation of synaptic plasticity NO has been proposed as the retrograde messenger which co-ordinates the enhancement of both pre- and post-synaptic mechanisms involved in two forms of synaptic plasticity; namely long-term potentiation (LTP) and long-term depression (LTD). LTP is a property of many central excitatory synapses characterized by a prolonged enhancement of synaptic transmission, or an activity-dependent increase in synaptic strength, lasting from hours to weeks or even longer. The process by which LTP is induced is not completely clear, but it involves glutamate acting on amino-3-hydroxy-5-methylisooxazole-4-propionic acid (AMPA) or NMDA-receptors. This activates a series of events in which Ca^{2+}/calmodulin-dependent protein kinase II, NOS and protein tyrosine kinases are implicated. LTP is thought to be a synaptic correlate of learning and memory, and is most pronounced in higher brain centres involved in cognitive functions, particularly in the cerebral cortex and hippocampus. The basic evidence for said involvement in LTP stems from *in vitro* studies in which inhibition of NOS prevented the development of LTP (Bohme *et al*., 1991; O'Dell *et al*., 1991; Schuman & Madison, 1991). Gene targeting suggests that both nNOS and neuronally located eNOS are implicated in LTP. Thus, while LTP is only slightly reduced in nNOS or eNOS null mice (O'Dell *et al*., 1994), animals deficient in both NOS isozymes exhibit a substantially decreased LTP (Son *et al*., 1996). Guanylate cyclase seems to be the main effector of NO in the induction of LTP (Bohme *et al*., 1991; Haley *et al*., 1992), however, ADP-ribosylation (Brüne & Lapetina, 1989) and activation of calmodulin-dependent kinases (Soderling, 2000; Tomita *et al*., 2001) have also been implicated.

LTD is characterized by a long lasting depression of parallel fibre synapses, which follows repeated excitation of the climbing fibres of Purkinje cells. The reduction in synaptic strength appears to result from a diminished sensitization of postsynaptic AMPA receptors which is mediated by activation of protein kinases C and G and of the NO-cyclic GMP signalling pathway (Daniel *et al*., 1993; Shibuki & Okada, 1991). LTD can be observed in higher regions of the brain, although it has been particularly well studied in the cerebellum where it has been proposed as a model for the learning of motor movements. Finally, the role of NO in both forms of synaptic plasticity involves interaction with PSD-95 and related membrane-associated guanylate kinases, underlined by the fact that both LTP and LTD are significantly modified in mice with targeted disruption of PSD-95 (Migaud *et al*., 1998).

Involvement in central and peripheral functions

NO has complex influences on brain development, memory formation and behaviour through regulation of synaptic plasticity. Inhibition of NO synthesis produces amnesia (Holscher & Rose, 1992), disrupts spatial learning and olfactory memory (Bohme *et al*., 1993; Kendrick *et al*., 1997), blunts behavioural performance during task acquisition and decreases locomotor activity in habituation tasks (Yamada *et al*., 1995). NO has also been implicated in neuronal targeting and brain development (Contestabile, 2000; Okere & Kaba, 2000; Wu *et al*., 1994a), visual processing (Cudeiro & Rivadulla, 1999), discriminative learning (Groll-Knapp *et al*., 1988), food and drinking behaviour (Calapai *et al*., 1992; Morley & Flood, 1992), thermoregulation (De Luca *et al*., 1995), opiate tolerance and withdrawal (Mao, 1999; Zhu & Barr, 2001), circadian rhythm (Watanabe *et al*., 1995), sleep (Kapas *et al*., 1994) and respiratory pattern generation (Ling *et al*., 1992). Likewise, behavioural responses mediated by oxytocinergic and serotoninergic pathways are thought to involve NO generation or stimulation of central nitrergic neurones (Melis *et al*., 1994; 1995). The participation of NO in behavioural mechanisms seems likely after being confirmed in knockout nNOS mice. These animals show no overt behavioural disorders when housed individually, but if kept together males exhibit exceptionally aggressive and hypersexual behaviour. In contrast, nNOS deficiency reduces aggression in female mice (Mani *et al*., 1994; Nelson *et al*., 1995).

There is an ever-growing list of peripheral functions in which a role for central NO has been proposed, although the exact physiological relevance of these observations warrants further investigation. Without intending to provide an exhaustive list, there is evidence implicating central NO in the regulation of blood pressure (Togashi *et al*., 1992), heart rate (Sakuma *et al*., 1992), stimulated renal sympathetic nerve activity (Sakuma *et al*., 1992), gastric acid secretion and motility (Esplugues *et al*., 1996; García-Zaragoza *et al*., 2000; Quintana *et al*., 2001) and motor disruption associated with alcohol abuse (Sandor *et al*., 1995). Likewise, NO in the CNS appears to be involved in reflexes leading to a diminished sympathetic output to the periphery and the modulation of various neuroendocrine responses, including the production of oxytocin, luteinizing hormone-releasing hormone, osmoregulator peptide corticotropin-releasing hormone and adrenocorticotrophic hormone (Aguilla, 1994; Ceccatelli *et al*., 1993; Costa *et al*., 1993; Rivier & Shen, 1994).

Perception of pain

NO has been implicated at various levels of the nociceptive neural pathways, both peripherally (primary afferent neurones and dorsal root ganglia) and centrally (brainstem and several sensory structures of the thalamus) (Mao, 1999). Functionally, most nociceptive reflexes involve the interaction of NO and NMDA receptors, and it is established that synthesis of NO enhances spinal facilitation of the afferent input conveyed to the cortex and subsequently manifested in behavioural responses (Mayer *et al*., 1999). However, the role of NO changes according to the pain stimuli. Inhibition of NO has antinociceptive effects when pain stems from chemically stimulated peripheral nerve terminals and in models of thermal hyperalgesia or visceral pain (Kitto *et al*., 1992; Malmberg & Yaksh, 1993; Moore *et al*., 1991; 1993), while intrathecal administration of L-arginine induces allodynia by converting a non-noxious to a noxious mechanical stimulus (Minami *et al*., 1995). In contrast, blockade of NO synthesis exacerbates pain in models of mechanical hyperalgesia (Zhuo *et al*., 1993). The use of nNOS knockout mice has not clarified such contradictory results; for example, these animals display a normal sensitization to some types of damage unmodified by NOS inhibitors (Crosby *et al*., 1995). The implication of NO in the antinociceptive effects of drugs is also controversial. For instance, inhibitors of the NO/guanylate cyclase system potentiate the antinociceptive actions of morphine while attenuating the antinociceptive effects of β-endorphin (Xu & Tseng, 1995; Zhu & Barr, 2001). Furthermore, there is evidence which suggests that the splice variants of nNOS, nNOS-2 modulates morphine analgesia but not morphine tolerance (Kolesnikov *et al*., 1997).

Neuronal damage and protection

The neuronal damage that accompanies cerebral ischaemia involves an excessive release of glutamate and a subsequent activation of NMDA receptors that, if maintained for a sufficient period of time, induces a massive influx of Ca^{2+} into the postsynaptic neurone which, in turn, triggers the activation of nNOS and overproduction of NO. In contrast, NO produced by activation of eNOS (Marks *et al*., 1996; Stagliano *et al*., 1997), and even NMDA receptors (Fergus & Lee, 1997; Wilderman & Armstead, 1997), plays a protective role in brain ischaemia by maintaining regional cerebral blood flow. The first indications that NO could mediate neurotoxic effects came with the discovery that inhibition of NOS attenuates glutamate toxicity in primary neuronal cultures from the rat cerebral cortex (Dawson *et al*., 1991) and induces neuroprotection in animal models of stroke (Nowicki *et al*., 1991). These studies were soon followed by others which showed that inhibition of NO synthesis attenuated NMDA neurotoxicity both *in vivo* (Nagafuji *et al*., 1992; Tominaga *et al*., 1993) and *in vitro* (Kollegger *et al*., 1993), and which demonstrated enhanced concentrations of NO in various stroke models (Kader *et al*., 1993; Malinski *et al*., 1993). These concepts were initially controversial due to the appearance of contradictory *in vitro* and *in vivo* reports in which NO-mediated neurotoxicity was not observed (Bolaños & Almeida, 1999; Samdani *et al*., 1997). However, such discrepancies stem from variations in experimental conditions and doses of NOS inhibitors. Thus, inhibition of nNOS with concentrations of NOS inhibitors that do not suppress eNOS activity reduces infarct volume, whereas the use of selective nNOS inhibitors is consistently neuroprotective in models of focal ischaemia (Yoshida *et al*., 1994; Zhang *et al*., 1996). Exacerbation of injury seems to occur through inhibition of eNOS with high doses of non-selective NOS inhibitors, which results in deleterious alterations of cerebral blood flow and a subsequent increase in infarction volume.

A better understanding of the role of nNOS and eNOS in neuronal damage has been obtained using transgenic animals. nNOS knockout mice develop substantially less brain damage following ischaemia than those of the wild-type strain (Huang *et al*., 1994), while neuronal cultures from such animals are

more resistant to damage by glutamate and hypoxia hypoglycaemia (Dawson *et al*., 1996). The reduction of infarct volume in nNOS null transgenic mice does not occur when non-specific NOS inhibitors are administered at concentrations that inhibit NO-dependent relaxation of pial vessels. On the other hand, eNOS knockout mice exhibit more extensive damage following ischaemia, an effect associated with a sharp reduction of blood flow in the affected area, and administration of NOS inhibitors to these animals reduces injury (Huang *et al*., 1996).

The interactions and signalling mechanisms involved in these NO-related effects are complex. Vascular protection is linked to cyclic GMP-mediated mechanisms (Utepbergenov *et al*., 1998). In addition, s-nitrosylation of glutathione by NO has been implicated in the antioxidative neuronal defence system, while NO is thought to scavenge reactive oxygen species and partially offset ischaemia induced oxidative damage (Wink *et al*., 1993). Likewise NO could be directly neuroprotective by interacting with a specific site of the NMDA-receptor channel, resulting in a decreased binding of glutamate or a diminished flow of Ca^{2+} through the channel after activation (Choi *et al*., 2000; Hoyt *et al*., 1992).

Generation of peroxynitrite seems to be the leading cytotoxic mediator in glutamate-induced damage (Fukuyama *et al*., 1998; Lafon-Cazal *et al*., 1993; Tanaka *et al*., 1997). The production of peroxynitrite has been detected in postischaemic brains (Fukuyama *et al*., 1998; Tanaka *et al*., 1997). Mice with overexpression of superoxide dismutase (SOD) and nNOS deletion exhibit decreased neurotoxicity following vascular stroke (Yang *et al*., 1994), accompanied by a strongly suppressed peroxynitrite ($ONOO^-$) production (Keller *et al*., 1998). Damage caused to DNA by NO and peroxynitrite appears to be an important neurotoxic mechanism. This is due to the subsequent activation of the nuclear repair enzyme polyADP-ribose synthase which is capable of triggering massive energy depletion resulting in cellular death if the DNA damage is severe (Pieper *et al*., 1999; Wallis *et al*., 1993; Zhang *et al*., 1994). Inhibition of nNOS reduces polyADP-ribosylation while inhibition of polyADP-ribose synthetase decreases infarction volume. Animals with a genomic deletion of polyADP-ribose synthetase exhibit greater resistance to cerebral ischaemia than nNOS knockout mice or those treated with NOS inhibitors or NMDA antagonists (Eliasson *et al*., 1997). In addition, inhibition of mitochondrial respiratory chain enzymes exacerbates the depletion of neuronal energy stores (Beltrán *et al*., 2000; Bolaños *et al*., 1994; 1995; Clementi *et al*., 1998; Moncada & Erusalimsky, 2002). High local concentrations of NO may also reduce cellular viability by nitrosylating several enzymes, including phosphokinases C (Hammer *et al*., 1993) and glyceraldehyde-3-phosphate dehydrogenase (Zhang & Snyder, 1992), or by interacting with the iron present in haem or non-haem complexes associated with enzymes such as cytochrome P450 or aconitases (Drapier & Bouton, 1996).

Inflammatory conditions and transient ischaemic periods induce the expression of iNOS in various populations of cerebral cells. However, iNOS expression occurs later than that of nNOS and eNOS, and the cellular site of this expression is dependent on the nature of injury (Iadecola *et al*., 1995a). iNOS immunoreactivity is present in the neutrophils which infiltrate the brain after permanent ischaemia, is predominant in vascular cells in transient ischaemia (Iadecola *et al*., 1995b), and is abundant in reactive astrocytes in global ischaemia (Endoh *et al*., 1994). NO produced by iNOS seems to exert a detrimental effect in the ischaemic brain, contributing to the progression of tissue damage and exacerbating glutamate neurotoxicity (Chao *et al*., 1992). Thus, NO produced *in vitro* after hypoxia induces apoptotic death in neurones (Boje & Akora, 1992), while studies with iNOS knock out mice have confirmed the induction of iNOS in the delayed neuronal damage following ischaemia (Iadecola, 1997).

Role in central disorders

There is growing evidence to support a role for NO in the aetiology of neurologic conditions, including autoimmune and chronic neurodegenerative diseases. Concentrations of NO present in inflamed tissue cause reversible conduction block in normal, demyelinated and early remyelinated axons. Thus, diseases such as multiple sclerosis and Guillain-Barre syndrome, characterized by widespread loss of myelin, may see their neuronal symptoms exacerbated *via* the release of NO that accompanies the severe inflammation in the central and peripheral nervous system occurring in these conditions. NO may also be important in secondary neuronal cell death following trauma. In the spinal cord, nNOS expression precedes the death of motorneurones, which follows avulsion of spinal nerve roots (Wu *et al*., 1994b), and pre-treatment with nNOS inhibitors substantially increases the number of surviving neurones (Wu & Li, 1993). NOS neurones are resistant to NMDA and NO neurotoxicity although the protective mechanism responsible is still not fully known (Koh & Choi, 1988). Likewise, the excessive release of both glutamate and NO, coupled with mitochondrial dysfunction and oxidative stress, has been implicated in a number of neurodegenerative diseases. This highlights a potential therapeutic role for specific NOS inhibitors in their pharmacological control (Hobbs *et al*., 1999). For instance, nNOS is induced in various cortical regions following epileptic seizures (Huh *et al*., 2000). NOS neurons are spared in Alzheimer's disease and NOS inhibitors provide neuroprotection against toxicity elicited by fragments of human B-amyloid in primary cortical cultures (Hyman *et al*., 1992). Inhibition of nNOS markedly reduces the loss of dopamine neurones and clinical symptoms in a baboon model of Parkinson's disease (Hantraye *et al*., 1996) and inhibition of NOS is protective in models of Huntington's disease (Deckel, 2001; Schultz *et al*., 1995). Finally, significant changes in nNOS activity in the cerebrum and cerebellum follow the administration of metals such as aluminium and mercury, and suggest an involvement of this mediator in cerebral diseases induced by metals (Cucarella *et al*., 1998).

The influence of peripheral systemic inflammatory conditions on the expression of central NOS isoforms is still controversial, however, overproduction of NO by iNOS is known to play a pathological role in acute inflammatory disorders of the CNS. There are increased levels of NO production in viral and bacterial infections such as meningitis, and a role for NO has been clearly indicated in the disruption of the blood-brain barrier during inflammatory conditions (Brian *et al*., 1995; Visser *et al*., 1994; Zheng *et al*., 1993). There is little data available as to how inhibitors of iNOS modulate the course of these diseases, but considering

the role played by NO in defences against infection, it is logical to assume that they exacerbate the disease. Furthermore, substantial progress has been made towards identifying NO as a potential toxic mediator in inflammatory encephalitic diseases (Parkinson *et al*., 1999). NO has been implicated as a potential mediator of microglia-dependent primary demyelination, a hallmark of multiple sclerosis, and iNOS induction has been noted in the brains of patients with this autoimmune disease (Bo *et al*., 1994). NO may also be involved in the pathogenesis of sporadic amyotrophic lateral sclerosis and that of AIDS dementia. In the latter condition, neurotoxicity induced by certain HIV coat proteins is partially mediated by activation of NOS (Dawson *et al*., 1993), whereas HIV patients who develop severe dementia exhibit a substantial increase in cortical iNOS (Adamson *et al*., 1996).

Nitric oxide in the peripheral nervous system

Introduction

First debated in the late 1980s (Bowman *et al*., 1986; Gillespie, 1987; Gillespie *et al*., 1989; Li & Rand, 1989a), it is now well established that NO has a leading role as an inhibitory neurotransmitter of peripheral non-adrenergic, non-cholinergic (NANC) nerves. Peripheral nitrergic nerves have a widespread distribution, and are particularly important in that they produce relaxation of smooth muscle in the gastrointestinal, respiratory, vascular and urogenital systems. It is generally assumed that free NO is the transmitter substance released by nitrergic nerves (Lilley & Gibson, 1997; Martin *et al*., 1994; Wood & Garthwaite, 1994). However, several controversial experimental observations (Barbier & Lefebvre, 1992; Gillespie & Sheng, 1990; Hobbs *et al*., 1991; Rajanayagam *et al*., 1993) have pointed to the possibility of obtaining a closely related redox product from NO (Stamler *et al*., 1992), and also suggest that the inhibitory nitrergic transmitter is a NO-releasing molecule (Myers *et al*., 1990; Thomas & Ramwell, 1989; Vedernikov *et al*., 1992).

Furthermore, there is evidence that NO is not the only mediator involved in NANC neurotransmission, and that the release of a combination of messengers is involved in inhibitory NANC responses. Vasoactive peptide (VIP) seems to be the most important of these but roles for ATP, neuropeptide Y, enkephalin, peptide histidine isoleucine, carbon monoxide, pituitary adenyl cyclase activating peptide, gastrin-releasing peptide and dynorphin have also been suggested (Furness *et al*., 1995). There is some evidence for the existence of 'solely nitrergic neurones' (Hohler *et al*., 1995; Smet *et al*., 1994), but most functional and morphological studies have demonstrated the existence of neurones and nerve fibres containing various neurotransmitters (D'Amato *et al*., 1992). The relative contribution of such mediators varies greatly depending on factors such as stimuli and the tissue innervated. There is still discrepancy as to whether neurones release mediators upon stimulation, most likely to be the case because of their co-localization, or whether they are activated to release, preferentially, a specific neurotransmitter (Furness *et al*., 1995; Schemann *et al*., 1995; Smet *et al*., 1994). Considerably more controversial is the proposal of a sequential link between NO and VIP (Boeckxstaens *et al*., 1991b; Ellis & Undem, 1992; Grider *et al*., 1992; Murthy *et al*., 1995). According to this hypothesis, VIP is the primary NANC neurotransmitter, but its release is dependent on NO synthesis. Furthermore, relaxation by VIP is produced partially *via* activation of adenylate cyclase and partially *via* stimulation of NO production in the smooth muscle. Said NO may relax the smooth muscle through activation of sGC and diffuse back to the nerve terminal to further enhance VIP release (Dick & Lefebvre, 2000; Teng *et al*., 1988).

Effects of peripherally released NO

Gastrointestinal system In the gastrointestinal tract the majority of NOS positive fibres are intrinsic, with smooth muscle cells containing sCG next to axon varicosities containing NOS (Bredt *et al*., 1990; Ekblad *et al*., 1994). This neuronally produced NO is implicated in many physiological and pathophysiological reflexes in which changes in gastrointestinal muscle relaxation are noted (Barrachina *et al*., 2001; Calatayud *et al*., 2001). Dysfunction of the inhibitory NANC nerves in the lower oesophageal sphincter results in the motility disorder, achalasia (Mearin *et al*., 1993), and is probably involved in oesophageal spasms and related primary motor disorders in the oesophageal body (Yamato *et al*., 1992). Gastric NANC-mediated relaxation following vagal stimulation, food intake or distension of the antrum or duodenum was among the first NANC effects described (Martinson, 1965). Indeed, pyloric hypertrophy and gastric distension are the most prominent abnormalities in nNOS knock out mice (Huang *et al*., 1993). The importance of such inhibitory NANC neurones in human gastric function is illustrated by the frequent complaint by vagotomized patients of epigastric bloating and discomfort after meals, which has been related to an abnormal gastric receptive relaxation (Koster & Madsen, 1979; Troncon *et al*., 1995). Infantile hypertrophic pyloric stenosis has been attributed to a lack of NOS-containing nerves at the pylorus (Vanderwinden *et al*., 1992) while diabetic gastropathy has been linked to a loss of nNOS that can be treated with insulin and the phosphodiesterase-5 inhibitor sildenafil (Watkins *et al*., 2000). A similar diminution in nNOS expression and activity in the myenteric plexus is associated with the delay in colonic transit appearing with advanced age (Takahashi *et al*., 2000). In contrast, increases in the activity of NANC nerves have been held responsible for the decreased gastrointestinal motility appearing during pregnancy (Shah *et al*., 2001). Similarly, the severe constipation characterizing Hirschsprung's disease results from the absence of intramural inhibitory NANC in the smooth muscle cells of the internal anal sphincter (Vanderwinden *et al*., 1993). Furthermore, *in vivo* topical application of nitroglycerin causes a reduction in human anal pressure, pointing to the use of NO-donors in conditions such as anal fissures, haemorrhoids and proctalgia (Loder *et al*., 1994).

Pulmonary system The density of extrinsic NOS-containing fibres increases progressively from the top of the trachea to the primary bronchi, and then diminishes as the bronchial diameter decreases (Fischer *et al*., 1993; Fischer & Hoffmann, 1996). Nitrergic nerves are believed to represent the main

nervous bronchodilator pathway in humans, and dysfunction of this system may be implicated in the increased tone and hyper-responsiveness observed in asthma (Belvisi *et al*., 1995). Furthermore, inhalation of NO has become an important therapeutic tool in the treatment of diseases such as acute respiratory distress syndrome, hypoxic respiratory failure, high pulmonary artery pressure, lung transplantation, sickle cell disease and specifically paediatric conditions such as neonatal pulmonary hypertension (Weinberger *et al*., 1999; 2001; Weinacker & Vaszar, 2001). Indeed, new approaches to the treatment of some of these diseases involve potentiation of NO responses with inhibitors of phosphodiesterase-5 (Fernandes *et al*., 1994; Gibson, 2001; Weinberger *et al*., 2001).

Vascular system nNOS is found in the perivascular nerves of various blood vessels and appears to constitute an alternative regional control mechanism for blood flow, independent of eNOS (Bredt *et al*., 1990; Huang *et al*., 1999; Martinez Cuesta *et al*., 1996; Figure 5). This neuronally produced NO seems to be particularly relevant in the regulation of cerebral blood flow (Estrada & Defelipe, 1998; Faraci & Heistad, 1998). High levels of nNOS are present in vasodilator nerves in cerebral blood vessels (Bredt *et al*., 1990; Thomsen *et al*., 1993), although, in most cases, nNOS is co-localized with different vasoactive neurotransmitters (Bredt *et al*., 1990; Estrada & Defelipe, 1998). In the brain, activity-dependent activation of nNOS is associated with a local increase in blood blow, and this response is prevented by inhibitors of NOS (Iadecola *et al*., 1993). The initial vascular response to neuronal ischaemia and the implication of nNOS in this condition have been discussed here previously. In addition to this relationship, it has been suggested that blockade of NANC vasodilatation by haemolysate or haemoglobin may contribute to the vasospasm observed in haemorrhages (Estrada & Defelipe, 1998). Abnormal dilatation of cerebral vessels appears to mediate vascular headaches. Furthermore, the finding that blockade of NO synthesis aborts acute attacks of migraine points to the use of the pharmacological manipulation of nNOS in the development of anti-migraine compounds (Thomsen & Olesen, 1998).

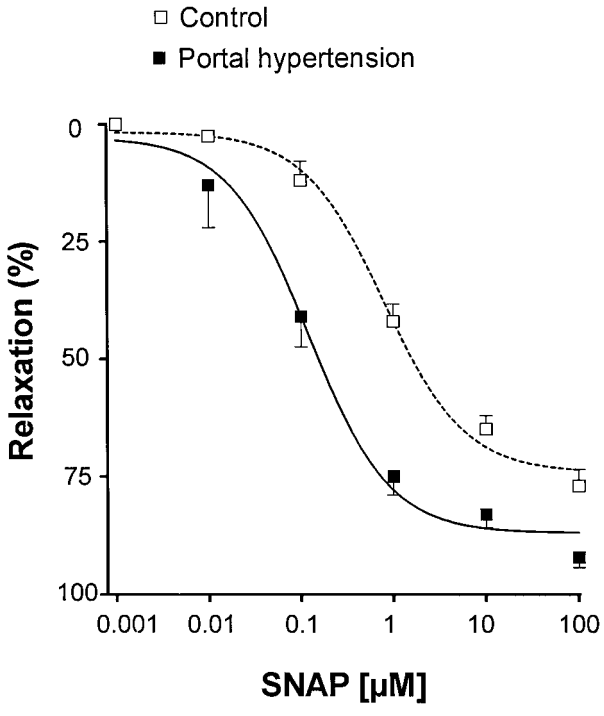

Figure 5 Selective destruction of nitrergic nerves in a model of portal hypertension results in supersensitivity of vascular tissue to the effects of exogenously administered NO. The graph shows cumulative concentration-response to SNAP in isolated mesenteric veins from control and portal hypertensive rats. Relaxations induced by SNAP are expressed as % of decrease induced by KCL (30 mM), and each point is the mean ± s.e.mean of at least five experiments.

Urogenital system nNOS is most prominent in the parasympathetic postganglionic innervation of the urethra. Likewise, stimulation of bladder afferent nerves leads to the release of NO and chronic irritation of the bladder augments nNOS expression in dorsal root ganglion cells. Finally, bladder hyperactivity provoked by intravesical irritants can be moderated by inhibition of NO synthesis, thus suggesting a role for spinal cord NO in the micturition reflex pathway (de Groat & Yoshimura, 2001).

Recent years have seen a major focus on the pharmacological modulation of the NO released by the endothelium and nitrergic nerves and which is involved in penile erection. nNOS neurones innervate the corpus cavernosum and blood vessels of the penis and nerve stimulation leads to erection, which involves sGC stimulation and is blocked by NOS inhibitors (Burnett *et al*., 1992; Holmquist *et al*., 1992; Rajfer *et al*., 1992). Nitrergic neurones are also implicated in the effects of sexual hormones. For instance, nNOS levels in the penis decrease substantially after castration but return to normal levels following testosterone replacement (Penson *et al*., 1996). Levels of nNOS diminish with age, and this decrease correlates with impaired erectile responses (Carrier *et al*., 1997). Similarly, impotence occurring with diabetes mellitus, spinal cord injury and treatments for prostate cancer is now related to damage of the nitrergic structures controlling erection (Goldstein *et al*., 1998). Phosphodiesterase-5 is the isoenzyme predominantly responsible for cyclic GMP hydrolysis in the corpus cavernosum, and recently, different isoforms of this isozyme have been described (Lin *et al*., 2000). Selective inhibition with drugs such as zaprinast or sildenafil restores erectile responses, which are linked to prolongation of the NO/sGC/cyclic GMP signalling pathway (Bivalacqua *et al*., 2000; Gibson, 2001; Saenz de Tejada *et al*., 1989). However, this mechanism of action is dependent on a level of integrity of the nitrergic nerves and a pre-activated endogenous NO-cyclic GMP system. This explains the clinical observation that sildenafil does not aid erection in patients with complete loss of sacral nerve activity nor where there is an absence of sexual arousal (Maytom *et al*., 1999).

Nitrergic structures also innervate smooth muscle structures in the female urogenital tract, and are particularly abundant in the clitoral corpus cavernosum (Burnett *et al*., 1997; Papka *et al*., 1995) where they appear to be responsible for the NANC erectile response of the clitoris (Cellek & Moncada, 1998). There have been few studies of female sexual dysfunction, but existing results suggest that inhibitors of phosphodiesterase-5 may be effective in specific cases, particularly those associated with the use of anti-depressant,

anti-psychotic and anti-anxiety agents (Shen *et al*., 1999). Encouraging results have also been obtained with sildenafil in other cases of sexual dysfunction (Kaplan *et al*., 1999; Sipski *et al*., 2000), but these need confirmation. The use of sildenafil to aid *in vitro* fertilization is also a possibility since its application in the vagina increases both uterine blood flow and the thickness of the endometrium (Chwalisz & Garfield, 2000). Finally, NO appears to be responsible for a tonic inhibition of spontaneous contractile activity in the uterus while there is evidence of increased biosynthesis of NO during pregnancy and a rapid drop in NOS activity preceding delivery. This points to the involvement of nitrergic mechanisms during pregnancy, which promote a relaxed state in the uterus, whereas a decrease in responsiveness to NO would appear to be involved in the initiation of labour (Chwalisz & Garfield, 2000; Weiner & Thompson, 1997).

Skeletal muscle The high levels of nNOS expressed in skeletal muscle, particularly the muscle-specific splice variant nNOSμ, tend to be located beneath the sarcolemma of fast twitch fibres, emphasizing the role of NO as a modulator of contractile force (Kobzik *et al*., 1994; Nakane *et al*., 1993). NO derived from sarcolemmal nNOS is also implicated in various other physiological functions occurring near the muscle membrane. Myocytes fuse to form muscle myotubes during muscle development, and this process is prevented by inhibition of NO (Lee *et al*., 1994). In myocyte/motor neuron co-cultures, NO produced at the postsynaptic muscle membrane functions as a retrograde messenger, regulating myotube innervation (Wang *et al*., 1995). In mature muscle fibres, NOS modulates glucose uptake across the sarcolemma. Although glucose uptake in skeletal muscle is regulated by both rigorous exercise and insulin, inhibition of NO synthesis has a selective action on glucose uptake in the former (Roberts *et al*., 1997). Interestingly, regular exercise increases nNOS protein expression in the muscle and this has long-lasting enhancing effects on glucose transport in the muscle (Roberts *et al*., 1997). Finally, both eNOS and iNOS isoforms are also present in the skeletal muscle, the former mostly related with the control of skeletal blood flow and the latter with inflammatory conditions and responses elicited by cytokines or lipopolysaccharides (LPS) (Stamler & Meissner, 2001).

Several muscular diseases have been linked to a dystrophin deficiency, and although the specific cause is unconfirmed, perturbed NO signalling would seem to be responsible (Brenman *et al*., 1995). A mutation in the rod-like domain of dystrophin causes Becker's dystrophy and results in a loss of sarcolemmal nNOS, while other components of the dystrophin complex are preserved (Chao *et al*., 1996). Similarly, patients with Duchenne muscular dystrophy and *mdx* mice that lack dystrophin both exhibit a reduction of nNOS in the sarcolemma (Brenman *et al*., 1995; Chao *et al*., 1996). This diminishing of dystrophin disrupts the normal link between the extracellular matrix and myofiber cytoskeleton (Campbell, 1995), which results in sarcolemmal damage and myofiber necrosis. Sarcolemmal instability in Duchenne dystrophy leads to a repeated cycle of myofibre degeneration and subsequent regeneration. Redistribution of nNOS from sarcolemma to cytosol is thought to be involved in myofibre necrosis, whereas the involvement of NO in myofibre differentiation suggests that an altered sarcolemmal nNOS signalling contributes to failed muscle regeneration in Duchenne dystrophy.

Concluding remarks

The last few years have seen the publication of a plethora of information concerning the multiple roles played by NO in the nervous system. Although we now know that NO is involved in many aspects of CNS function, we are still far away from the pharmacological breakthrough which could have a clinical impact in CNS related diseases other than stroke. This is not the case in the peripheral nervous system where the introduction of sildenafil has represented a major breakthrough in a condition for which, until now, there existed complicated and only partially effective drug treatment or simply a psychological approach. However, one should bear in mind the many advances in our knowledge in this field, and how our insight has improved regarding diseases about which we knew relatively little 10 years ago. The field continues to grow and recent important findings include the role of NO in mitochondrial function, clarification of signalling pathways, targeting of diseases associated with organ-specific changes in nitrergic activity, development of more selective NOS inhibitors and the identification of different isoforms of phosphodiesterase-5. These discoveries raise the prospect of future therapeutic leads, thus achieving the pharmacological goal of linking physiological knowledge with drug development.

The author would like to thank Prof Salvador Moncada for his insight on the topics discussed. The help of Annie Higgs, Prof Arnold Herman, Selim Cellek, María Dolores Barrachina, Dora Martí and Brian Normanly is also very much appreciated. The writing of this manuscript has been supported by CICYT (Comisión Interministerial de Ciencia y Tecnología) grants IFD97-1029, SAF98-0118 and SAF2001-0763.

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(Received September 25, 2001
Revised December 19, 2001
Accepted December 20, 2001)