Histol Histopathol (2002) 17: 973-1003

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Physiology and pathophysiology of nitric oxide in the nervous system, with special mention of the islands of Calleja and the circunventricular organs

J. Rodrigo, D. Alonso, M.L. Bentura, S. Castro-Blanco, J.M. Encinas, A.P. Fernández, P. Fernández-Vizarra, A. Richart, M. Santacana, J. Serrano and A. Martínez

Department of Neuroanatomy and Cell Biology, Instituto Cajal, CSIC, Madrid, Spain

Summary. Nitric oxide (NO) has been recognized as a key regulatory factor in many physiological processes, including central nervous system function, development, and phatophysiology. NO is produced by a class of enzymes known as NO synthases (NOS) and in normal adult animals only the neuronal isoform (nNOS) is detectable. During cortical development, nNOS was found at E14 in neuroblasts of the marginal zone and its expression raised to a zenith by P5, decreasing afterwards until reaching a steady level by P10. At that time, nNOS was found mainly in pyramidal neurons. Interestingly, the inducible isoform of the enzyme (iNOS) was also active from P3 to P7, but it disappeared almost completely by P20. The neurodegeneration observed during normal aging and following hypoxic accidents seems to be the result of cumulative free radical damage, and excessive production of NO may be at the basis of the cascade. After ischemic events we observed an elevation in the number of neurons expressing nNOS coincident with an elevation in Ca^{2+}-dependent NOS activity for up to 120 min. After this period, nNOS activity began to decrease but it was substituted by a rapid increase in Ca^{2+}-independent activity coincident with the histological appearance of previously undetectable iNOS-immunoreactive neurons. These increases in NO production were accompanied by specific patterns of protein nitration, a process that seems to result in loss of protein function. In particular, we observed a correlation between exposure to ischemia-reperfusion and nitration of cytochrome c. This process was coincident with the exit of the cytochrome from the mitochondria to the surrounding cytoplasm, an early event in neuronal apoptosis. Interestingly, most of the morphological and molecular changes associated with ischemic damage were prevented by treatment with inhibitors of NO production, indicating a clear path in the search for efficacious drugs in the battle against cerebrovascular accidents.

Key words: Brain development, Aging, Ischemia, Hypobaria, Protein nitration

Offprint requests to: Dr. José Rodrigo, Department of Neuroanatomy and Cell Biology, Instituto Cajal, CSIC, Avenida Doctor Arce 37, 28002 Madrid, Spain. Fax: 34 91 585 4754. e-mail: rodmart@cajal.csic.es

Introduction

Nitric oxide (NO) is an important intercellular messenger molecule (Garthwaite, 1991; Moncada et al., 1991; Bredt and Snyder, 1992), first identified by Furchgott and Zawadzki (1980) as the endothelium-dependent relaxation (EDRF) mediating arterial dilatation in response to acetylcholine. NO is synthetized from L-arginine (Knowles et al., 1989; Bredt et al., 1990, 1991) in certain vertebrate and invertebrate (Gelperin, 1994; Meyer, 1994; Martínez, 1995) tissues by the action of the nitric oxide synthases (NOS). L-citrulline is formed as a side product in equimolar amounts. NO is an unstable free radical gas, lipid-soluble and highly diffusible from its tissues of origin (Moncada et al., 1991; Snyder, 1992). It may also have a targeted intracellular role, mediated by specific interactions of an adaptor protein, CAPON, and the small monomeric G protein, dexras1. These molecules form a ternary complex with nNOS in neuronal cells that enhances NO production (Jaffrey et al., 1998; Fang et al., 2001). In the nervous system, NO is neither stored nor released like traditional neurotransmitters, but diffuses through cell membranes after synthesis without the aid of specific transporters. In addition to its immediate effects on target cells, NO participates actively in the development and plasticity of the central nervous system (Gally et al., 1990). In the enteric nervous system, NO participates in different nonadrenergic/noncholinergic (NANC) regulatory mechanisms in mammals and lower vertebrates (McKirdy et al., 1992; Murray et al., 1995; Postorino et al., 1995; Yano et al., 1995). NO also influences the tone

of vascular beds throughout the whole organism, including cerebral circulation (Faraci, 1990, 1992; Faraci and Brian, 1994).

There are three major isoforms of the enzyme NOS, two constitutive isoforms (cNOS) and an inducible isoform (iNOS) (Forstermann et al., 1991; Moncada et al., 1991; Salter et al., 1991; Griffith and Stuerh, 1995). One of the cNOS isoforms is chiefly present in vascular endothelial cells (Mitchell et al., 1991; Pollock et al., 1991, 1993) and is known as endothelial NOS (eNOS), also referred to as NOS-III, NOS 3, or ecNOS. The other constitutive isoform, chiefly present in neurons, is known as neuronal NOS (nNOS), also referred to as NOS-I, NOS-1, bNOS, or ncNOS. This has been extensively described in the mammalian central and peripheral nervous system (Knowles et al., 1989; Bredt and Snyder, 1990; Bredt et al., 1990, 1991; Forstermann et al., 1990; Moncada et al., 1991; Springall et al., 1992; Terenghi et al., 1993; Egberongbe et al., 1994; Rodrigo et al., 1994, 1998) and also in neural structures of invertebrates (Elphick et al., 1993; Martínez et al., 1994; Regulski and Tully, 1995).

Neural NOS was purified from rat brain and cerebellum (Bredt and Snyder, 1990; Knowles et al., 1990) and cloned from rat (Bredt et al., 1991) and human brains (Nakane et al., 1993). The human nNOS gene contains 29 exons encoding 1433 amino acids and is located on chromosome 12q24.2 (Kishimoto et al., 1992; Xu et al., 1993). This isoform has been described as a soluble homodimer of 155 kDa (Bredt and Snyder, 1990; Schmidt and Murad, 1991), with a sequence similarity to the carboxy-terminal end of cytochrome P-450 reductase (Bredt et al., 1991). The molecule has recognition sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and calmodulin (Bredt et al., 1991). This form of the enzyme is found mainly in the cytosol, although most of the activity is membrane-associated, and totally dependent on activation by Ca^{2+} and calmodulin, using L-arginine as substrate and NADPH as cofactor (Knowles et al., 1989; Bredt and Snyder, 1990, 1992). Biochemical measurements in different regions of the brain have shown that the highest concentration of nNOS is found in the cerebellum, followed by the hypothalamus, midbrain, striatum and hippocampus, with the lowest activity in the medulla oblongata (Forstermann et al., 1990).

The human eNOS has 25 exons encoding 1207 amino acids and is located on chromosome 7q35 (Marsden et al., 1993; Xu et al., 1994). Endothelial NOS shows 60% sequence homology with nNOS, but differs in having an amino-terminal myristoylation site which determines its membrane-bound subcellular localization (Busconi and Michel, 1993).

The inducible isoform of NOS, also referred to as NOS-II, NOS 2, macNOS, or hepNOS, was purified from the cytosol of activated murine macrophages (Hevel et al., 1991), and cloned from human chondrocytes (Charles et al., 1993) and macrophages (Lyons et al., 1992; Xie et al., 1992). This isoform is a calcium-independent enzyme with 135 kDa of molecular mass. It can be induced in macrophages, mast cells, lymphocytes, neutrophils, hepatocytes, vascular smooth muscle and mesangial, and endothelial cells by inflammatory stimuli (Wright et al., 1989; Gross et al., 1991; Moncada et al., 1991; Yui et al., 1991; Geller et al., 1993; Radomski et al., 1993; Morris and Billiar, 1994; Riveros-Moreno et al., 1996). Inducible NOS has also been found in the central nervous system, in astrocytes, and neurons (Minc-Golomb et al., 1994, 1996; Moro et al., 1998). The human iNOS gen has 26 exons encoding 1153 amino acids and is located in the pericentric region of chromosome 17 (Xu et al., 1994).

NOS activity can be demonstrated in tissue sections by NADPH diaphorase histochemistry (Hope et al, 1991; Vincent and Kimura, 1992). This activity is also shown by other enzymes, but a high specificity for NOS can be achieved by the relative resistance of the NADPH diaphorase activity of NOS to aldehyde fixation, which selectively inactivates other enzymes (Hope et al., 1991; Matsumoto et al., 1993; Tracey et al., 1993). In aldehyde-fixed brain sections, NADPH diaphorase staining correlates largely with nNOS immunohistochemical staining. Hope et al. (1991) showed that the brain enzyme purified by chasing NADPH diaphorase activity had properties identical with nNOS, which also reacted with antibodies raised against the purified NADPH diaphorase. Transfection of nNOS cDNA into human kidney cells allowed the detection of both NADPH diaphorase and NOS activities, providing further evidence that both enzymatic activities reside in the same protein (Dawson et al., 1991). However, discrepancies between NADPH diaphorase histochemistry and NOS immunohistochemistry may arise because of (i) inadequate fixation, (ii) different sensitivity of the two techniques, and (iii) the possibility that some NOS immunoreactive molecules may not have enzymatic activity.

The reactivity of NO as a free radical is not only responsible for its efficacy as a short-lived messenger molecule interacting with its target proteins, but is also responsible for potentially noxious effects, especially when it is produced in supraphysiological concentrations or over extended periods of time. In fact, massive outbursts of NO produced by iNOS are used by cells of the reticulo-endothelial system to kill microbial invaders (Hibbs et al., 1987, 1988). NO is also considered as one of the most important mediators of ischemic brain injury (Iadecola, 1997) and neurotoxic effects in many neuropathological disorders. The free radical superoxide (O_2^-) reacts with •NO faster than with the enzyme superoxide dismutase (SOD) to form the powerful oxidant peroxynitrite ($ONOO^-$). Peroxynitrite in turn reacts with transition metals, transition metal complexes, or metalloproteins, as well as with carbon dioxide, to form intermediates which nitrate tyrosyl residues in neighboring proteins with greater efficiency than

peroxynitrite alone (Lipton et al., 1993; Beckman et al., 1994; Beckman, 1996; Radi et al., 1999). Nitration of tissue proteins has functional implications for enzymatic activity and the assembly of cytoskeletal proteins, and has been interpreted as a pathological phenomenon leading to cell death. However, antibodies with high affinity for nitrotyrosine (Uttenthal et al., 1998) can demonstrate protein nitration under basal physiological conditions, and the discovery of a denitrase activity in certain tissues (Kamisaki et al., 1998) suggests that nitration and denitration of proteins may constitute a physiological mechanism whose importance has yet to be assessed. Whatever the physiological or pathological implications, the detection of nitrotyrosine in tissue proteins has been used extensively as a marker of cumulative recent exposure to NO and hence an indicator of the activation state of NOS enzymes.

In the following pages, we review our studies on the NO system in rat brain, first in relation to normal development, adult life, and aging, and then in the changes following exposure to ischemia or hypoxia.

NO and the embryonic development of the rat cerebral cortex

During embryonic development of the central nervous system (CNS), NO generated by neurons expressing nNOS plays a significant role in maturation processes (Bredt and Snyder, 1994; Giuili et al., 1994). As nNOS expression during embryonic development of the rat cerebral cortex had only been partially described, we undertook a more extensive study to follow the expression of this isoform in the rat cerebral cortex from embryonic day (E) 13 to postnatal day (P) 0 by immunohistochemistry with a specific antibody against rat brain nNOS (Santacana et al., 1998). Expression of nNOS was first seen on E14 in cells with Cajal-Retzius morphology located in the marginal zone (MZ). An important role of the Cajal-Retzius cells is to direct the migration of neurons to their appropriate position in the CP. Ogawa (1995) has recently shown that Cajal-Retzius cells express the protein reelin. This protein, absent in reeler mice, is crucial for the correct migration of cortical neurons and the establishment of appropriate cortical lamination. Considering the role of these neurons during development, the massive expression of nNOS elements of the MZ and its coincidence with the duration of the migration process, suggests that NO may be involved in directing ingrowing axons and the migration process in general. The first afferent connections to the cortex are those from the primordium of the nucleus basalis magnocellularis which at E15, according to Bayer (1985), "enter through the anterolateral edge of the cortex, and grow dorsally, medially and posteriorly to cover the entire cortex". Another set of fibers arriving at the telencephalic vesicle at this stage are the dopaminergic fibers originating from the substantia nigra and the ventral tegmental area (Kalsbeek et al., 1988). Their progress to the telencephalic vesicle is similar to that from the basal forebrain.

In our study we have observed many nNOS-positive fibers coming from the ganglionic eminence and entering the intermediate zone (IZ). At E17 we described that: i) the MZ is richly labeled; ii) many CP neurons express nNOS immunoreactivity; iii) the IZ contains many fibers from the ganglionic eminence, which enter the telencephalic vesicle anterolaterally; and iv) neurons migrating to the CP also express nNOS. The coincident expression of nNOS in the cells of the CP and the axons arriving there suggests an NO-mediated cross talk between the target region and the ingrowing axons. From E19 onwards, nNOS-positive cells with the morphological characteristics of migrating cells were observed in and near the subventricular zone (SVZ). Confocal analysis of double immunofluorescence for nNOS and either glial fibrillary acidic protein or nestin showed no coexpression of nNOS and glial markers in these cells, indicating that nNOS-positive cells leaving the SVZ were not of glial origin. Commissural, callosal and fimbrial fibers were seen to express nNOS on E18 and E19. This expression decreased from E20 and was very weak on E21 and P0. These observations suggest that NO is expressed during embryonic life in relation to maturational processes including the organization of cerebral lamination, and that it is involved in controlling migration processes and fiber ingrowth (Santacana et al., 1998).

NO and postnatal cortical development.

In the newborn rat, neurons migrating into the cerebral cortex first occupy the posterior and deepest part of layer VI (Angevine and Sidman, 1961; Hicks and D'Amato, 1968; Bayer and Altman, 1991; Bayer et al., 1991b; Ogawa, 1995), while the cells that originate later position themselves in more superficial levels (Hicks and D'Amato, 1968). On P0, the cerebral cortex is comprised of the subplate layer, that will develop into layers Vi and V, the cell-dense cortical plate, and the marginal zone. By P2, layer IV is identifiable and by P4 layer III can also be found. All cortical layers can be identified by P6, and the marginal zone is referred to as layer I (Ignacio et al., 1995).

The role of NO in the maturation process of the rat cerebral cortex during the postnatal period has been studied by our group. During this period, there is a significant contribution to NO production from both nNOS and iNOS, as demonstrated by the work of Keilhoff et al. (1996). We studied the expression and distribution of these two isoforms, as well as the formation of nitrotyrosine as a marker of NO/superoxide action, from P0 to P20 by bright field immunohistochemistry. Calcium-dependent and independent NOS activities were measured and protein bands were quantified by Western blotting. Expression of nNOS increased from P0 to P5 and then declined rapidly from P7 to P10 without further modification up

to P20. This pattern was assessed by morphological quantification, Western blotting, and determination of calcium-dependent NOS activity. Morphologically, nNOS immunoreactivity was observed mainly in cortical bipolar neurons, compatible with immature pyramidal cells, with apical processes branching at layer II. This layer was intensely stained. There were also nNOS-positive cells with Cajal-Retzius-like morphology in layer I and multipolar cells in the deep layers, perhaps destined to develop into the adult type I nNOS-positive interneurons. Inducible NOS showed less consistent changes, but there was evidence of a clear elevation from P3 to P7, decreasing to negligible levels at P20. This immunoreactivity was located in groups of bipolar cortical neurons similar to those that also expressed nNOS immunoreactivity.

Two nitrated proteins of 52 kDa and 38 kDa were identified by nitrotyrosine immunoreactivity. The 52 kDa protein was found from P0 to P10 and then decreased progressively to P20. The 38 kDa protein was only found in significant amounts from P10 to P20. The fact that the cortex of newborn rats shows a higher expression of nNOS and iNOS immunoreactivity than the adult animals, with more complex changes in protein nitration, suggests that the NO system plays an important role in neuronal maturation and cortical plasticity during normal postnatal development.

NO in the adult rat central nervous system

Early studies in the CNS localized discrete neuronal populations in the rat and human brain containing brain NOS immunoreactivity, later confirmed as nNOS (Bredt et al., 1990; Bredt and Snyder, 1990; Springall et al., 1992). Nevertheless, a detailed mapping of nNOS immunoreactivity in the rat brain was not available until the extensive description by Rodrigo et al. (1994). Prior studies were performed with NADPH diaphorase histochemistry, assuming a complete correspondence between nNOS and NADPH diaphorase activity (Dawson et al., 1991; Hope et al., 1991). However, as mentioned above, some discrepancies between nNOS immunoreactivity and NADPH diaphorase activity often occur in tissue preparations. Our immunohistochemical results, while showing widespread similarities with previous histochemical results (Vincent and Kimura, 1992), also showed some differences. These discrepancies were easily appreciated in the main olfactory bulb, where we found the large periglomerular cells of the glomerular layer to exhibit nNOS. In contrast, Vincent and Kimura (1992) showed that only a few of these neurons exhibited NADPH diaphorase activity. Differences also occurred in the accessory olfactory bulb, where mitral cells are characteristically nNOS-positive but NADPH diaphorase-negative. Other differences were found in the globus pallidus, substantia nigra, ventral pallidum (Fig. 1), entopeduncular nucleus, taenia tecta, and hippocampus. In all these regions, neurons exhibiting nNOS immunoreactivity were more numerous than those displaying NADPH diaphorase activity. Immunoreactivity for nNOS was also found in neurons of the nucleus circularis, the medial tuberal nucleus and arcuate nucleus of the hypothalamus, the parafascicular and medial habenular nuclei of the thalamus, the pars reticulata and lateralis of the substantia nigra, the locus coeruleus, the ventral tegmnental nucleus of the pons, and the area postrema of the medulla oblongata. In contrast, in all these areas, Vincent and Kimura (1992) found NADPH diaphorase activity but only in some nerve fibers.

Endogenous nNOS was located using a specific polyclonal antiserum, produced against affinity-purified nNOS from whole rat brain. The primary antibody was followed by the avidin-biotin peroxidase complex procedure. Immunoreactive cells bodies and processes showed a widespread distribution in the brain. In the telencephalon, immunoreactive structures were distributed in all areas of the cerebral cortex, the ventral endopiriform nucleus and claustrum, the main and accessory olfactory bulb, the anterior and posterior olfactory nuclei, the precommisural hippocampus, the taenia tecta, the nucleus accumbens, the stria terminalis, the caudate putamen, the olfactory tubercle, and the islands of Calleja, septum, globus pallidus and substantia innominata, hippocampus and amygdala. In the diencephalon, immunoreactive cell bodies were characteristically located in the perivascular neurosecretory system and mammillary bodies. In addition, immunoreactive nerve fibers were detected in the median eminence of the infundibular system. The telencephalon showed nNOS immunoreactivity in the ventral tegmental area, the interpeduncular nucleus, the rostral linear nucleus of the raphe, and the dorsal raphe nucleus. Immunoreactive structures were also found in the central gray, the peripeduncular nucleus and substantia nigra pars lateralis, the geniculate nucleus and in the superior and inferior colliculi. The pons displayed immunoreactive structures mainly in the pedunculopontine and laterodorsal tegmental nuclei, the ventral tegmental nucleus, the reticulo tegmental pontine nucleus, the parabrachial nucleus and locus coeruleus. In the medulla oblongata, immunoreactive neurons and processes were detected in the principal sensory trigeminal nucleus, the trapezoid body, the raphe magnus, the pontine reticular nuclei, the supragenual nucleus, the prepositus hypoglossal nucleus, the medial and spinal vestibular nuclei, the dorsal cochlear nucleus, the medullary reticular field, the nucleus of the solitary tract, the gracile and cuneate nuclei, the dorsal nucleus of the vagus nerve and in the oral, interpolar, and caudal parts of the spinal trigeminal nucleus. In the cerebellum, the stellate and basket cells showed immunoreactivity, which was also seen in the basket terminal fibers of the Purkinje cell layer. Isolated immunoreactive Purkinje cells were found in the vermis and paraflocculus regions of the cerebellum. In the granular layer of the cerebellum, the granular cells and glomeruli were also immunoreactive. Numerous positive varicose nerve

fibers and occasional neurons were also found in the lateral and interposed cerebellar nuclei.

Since NO is a potent vasodilator, we devoted special attention to the distribution of nNOS in certain regions of the brain related to vascular control such as the island of Calleja. We also studied the areas in charge of cerebrospinal fluid production, namely the ventricular system and circumventricular organs.

NO and the islands of Calleja

Morphological and histochemical data suggest that the islands of Calleja, embedded in the olfactory tubercle (Calleja, 1893), can be considered part of a ventral extension of the corpus striatum which participates in the ventral striatopallidal system (Heimer and Wilson, 1975; Heimer, 1978; Ribak and Fallon, 1982; Fallon et al., 1983; Haber et al., 1985). However, the role of their most characteristic cells, the granule cells, is not easily explained in such terms. These cells do not extend their processes beyond the limits of the island complex and their main afferent innervation, according to Millhouse (1987), is provided by thick axons of unknown origin that branch out within the granule cell clusters, resembling cholinergic afferents to layer II of the lateral olfactory tract. However, the thin cholinergic neuropile surrounding groups of granule cells, observed by Talbot et al. (1988) in feline islands of Calleja, was considered to be intrinsic, derived from isodendritic satellite cells.

The islands of Calleja, which are highly developed and discretely organized in the rat, consist of morphological units of granule cell clusters surrounded by a population of large and medium-sized associated (satellite) neurons. The granule cells forming these

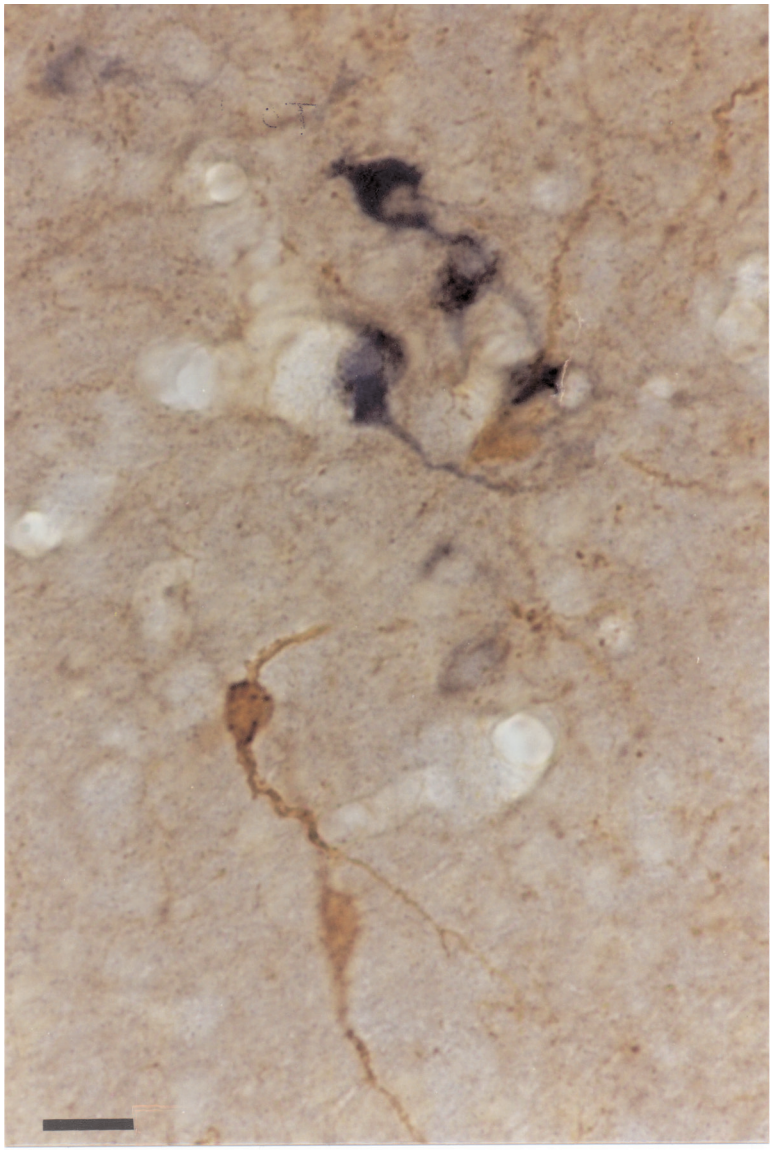


Fig. 1. Section of rat nucleus ventral pallidum showing a double staining for nNOS (brown color, immunohistochemistry) and NADPH-diaphorase (blue color, histochemistry). Two distinct subpopulations of neurons can be observed. Bar: 200 μm.

clusters are located between the polymorph and molecular cell layers of the olfactory tubercle, immediately below the ventral pallidum. The cell clusters of the insula magna are related medially to the medial septum and the vertical limb of the diagonal band of Broca, and laterally to the lateral septal nuclei, the nucleus accumbens and lateral ventricle. Non-immunoreactive associated (satellite) neurons of medium and large diameter were seen in the periphery of the granule cell clusters. Immunoreactive nerve fibers formed a dense plexus in the areas adjacent to the islands of Calleja. This plexus was more prominent in the hilar zones of the islands.

Abundant dopaminergic afferent fibers from the substantia nigra and ventral tegmental area and fewer noradrenergic fibers from the locus coeruleus reach the hilus and edge of each granule cell cluster, establishing synapses with dendrites of the more peripheral granule cells and medium-sized spiny neurons (Fallon et al., 1983). Similarly diffuse fibers containing enkephalins, luteinizing hormone releasing hormone (LHRH), or substance P occasionally penetrate within the granule cell clusters (Fallon et al., 1983). The efferent connections of the granule cells appear to be directed to the dendrites of the large and medium-sized satellite neurons, the large special hilar neurons described by Millhouse (1987), and neurons of the ventral pallidum (Switzer et al., 1982; Meyer and Wahle, 1986; Meyer et al., 1989). These neurons would seem to be responsible for the more far-reaching projections from the islands of Calleja to regions in the forebrain, diencephalon, and midbrain (Fallon et al., 1978; Fallon, 1983), and it is assumed that the nNOS-negative dendrites receiving synaptic contact from nNOS-immunoreactive terminal boutons derive from these satellite neurons.

In addition to the mentioned neurotransmitters and neuropeptides, the islands of Calleja receive part of the serotoninergic input to the olfactory tubercle (Moore et al., 1978; Fallon et al., 1983). Glutamate decarboxylase immunoreactivity has been demonstrated in fibers in the islands' core (Fuxe et al., 1978, 1979; Pérez de la Mora et al., 1981), and either the granule cells themselves (Mugnaini and Oertel, 1985) or cells just dorsal to them may be GABAergic (Fallon et al., 1983). These transmitters may be accompanied by a whole array of yet unidentified neuropeptides and their corresponding receptors, whose functional significance has to be evaluated.

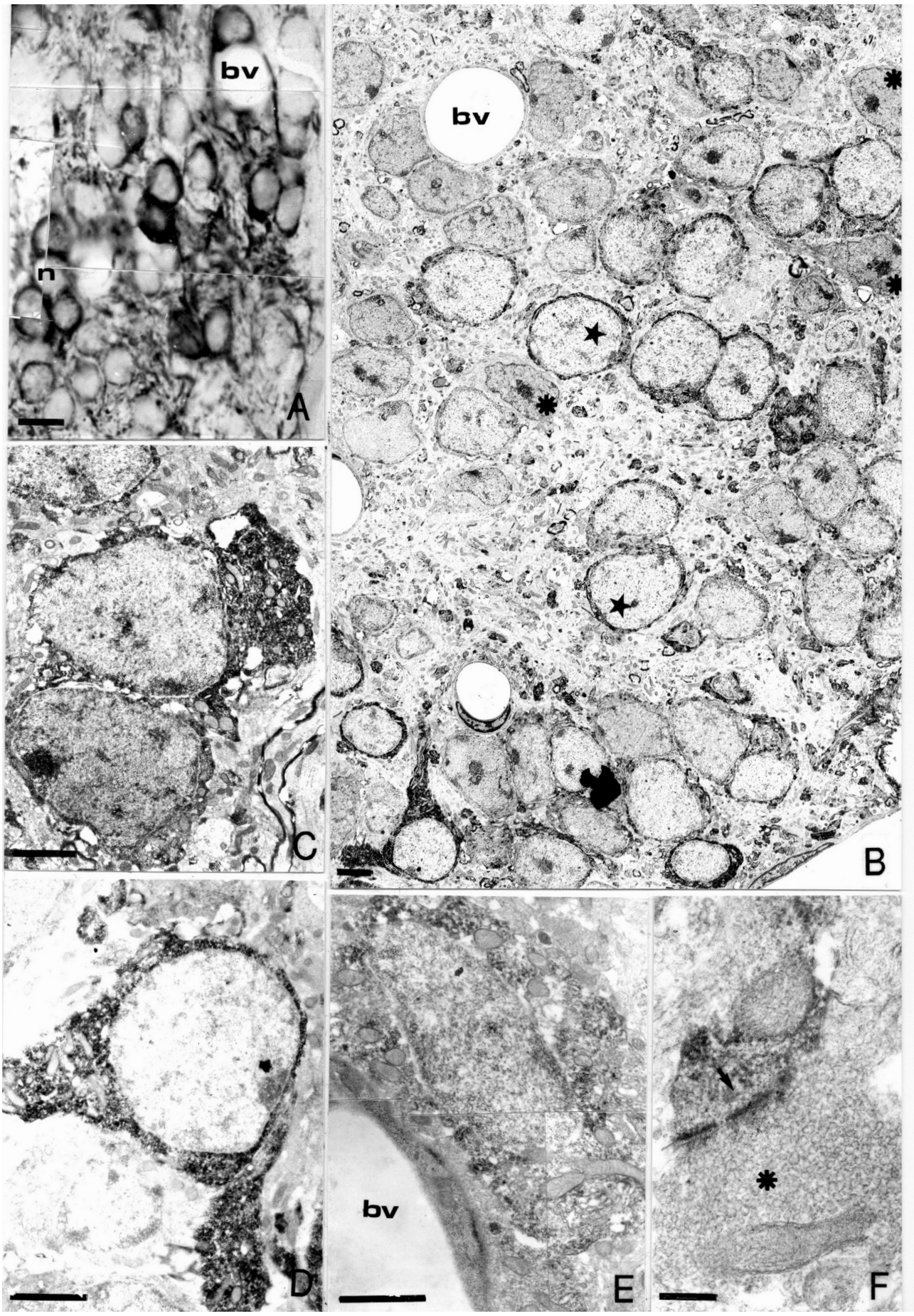
The distribution of nNOS immunoreactivity has been studied in these structures (Fig. 2). In the insula magna and the islands of the olfactory tubercle, all the granule cells showed nNOS immunoreactivity, the reaction product being homogeneously distributed throughout the cytoplasmic compartment, surrounding the unreactive nucleus as a thin ring (Fig. 2A). Granule cells were arranged in groups of four to six cells in direct apposition, the groups being separated by a dense neuropile of nerve fibers and an extensive network of blood vessels.

The islands of Calleja contain a large number of blood vessels (Fig. 2B) which arise from the anterior cerebral artery, the anterior communicating artery, and to a lesser extent from the middle cerebral artery. Each island appears to be supplied by one or more large arterioles that branch out within and around it (Fallon et al., 1983). Granule cell processes do not end on blood vessels, but our study showed groups of granule cells lying in close proximity to the vessels' wall (Fig. 2E).

Electron microscopy showed two types of granule cell, characterized by electron-dense and electron-clear nuclei, respectively (Fig. 2B). In all the nNOS-immunoreactive cells, the reaction product was associated to the smooth endoplasmic reticulum, ribosomes, outer mitochondrial and nuclear membranes, Golgi apparatus, and some cisternae (Fig. 2B-E). The subcellular localization of the reaction product suggests that a substantial portion of the enzyme may in fact be membrane-bound, mainly in association with the endoplasmic reticulum. Although nNOS is primarily regarded as a cytoplasmic enzyme, in contrast with the membrane-bound eNOS, Hecker et al. (1994) showed by subcellular fractionation that up to 60% of the total NOS activity in the cerebellum of the rat and rabbit was found in the particulate fraction, the highest specific activity occurring in the endoplasmic reticulum fraction.

The nitrergic granule cells received few synaptic contacts on the soma, but many contacts from non-immunoreactive terminal boutons on their dendrites (Fig. 2F). Adjacent granule cells were frequently linked by gap-like junctions, which were not specially associated with accumulations of reaction product. In the periphery and the hilus of the islands of Calleja, a large number of terminal boutons containing nNOS formed symmetric synaptic contacts with non-immunoreactive dendrites of large diameter. The relation of the immunoreactive granule cells to the walls of the blood vessels in the islands of Calleja is of special interest. The islands contain numerous blood vessels that pass through them in their way to the ventral pallidum. Immunoreactive granule cells and their sparse processes were found in close proximity to these blood vessels (Fig. 2E).

Fig. 2. Islands of Calleja of the rat brain stained with antibodies against nNOS. **A.** Light microscopical aspect of the insula magna showing immunoreactive granule cells in the proximity of blood vessels (bv). **B.** Low magnification electron micrograph of an island of Calleja showing immunopositive (stars) and immunonegative (asterisks) cells and the characteristic blood vessels (bv) of the organ. **C.** Detail of two characteristic granule cells immunoreactive for nNOS. **D.** Immunoreactive neuron in the periphery of the island projecting an immunoreactive proccess toward the inner regions of the nucleus (the same cell can be observed in the lower left corner of Fig. 2B). **E.** Granule cell immunoreactive for nNOS in close proximity to a blood vessel (bv). **F.** Negative axon (asterisk) establishing a synapsis with an immunopositive dendrite (arrow). Bars: A, 10 μm; B-E, 2 μm; F, 200 nm.


bv
n
A
bv
B
C
D
bv
E
F

The nitrergic granule cells of the islands of Calleja appear to be strategically arranged to exert an NO-mediated vasodilator influence on the blood vessels supplying the ventral pallidum, acting as functionally coordinated groups. This function may be regulated by changes in systemic blood pressure and by a variety of reported neuroendocrine mechanisms. In addition, at least some of the granule cells can be regarded as specialized interneurons making different kinds of synaptic contact with their afferent and efferent connections. One type of contact is formed by nNOS-immunoreactive terminal boutons originating from the short granule cell axons, establishing synapses with non-immunoreactive dendrites, probably originating from medium-sized or large satellite neurons situated around the islands or in the hila (Hosoya, 1973; Fallon et al., 1978; Ribak and Fallon, 1982), or from neurons of the ventral pallidum (Switzer et al., 1982; Meyer and Wahle, 1986; Meyer et al., 1989). The immunoreactive axon terminals cover nearly the entire surface of these dendrites where they commonly form symmetric synaptic junctions (Ribak and Fallon, 1982). Another type of synaptic contact is formed by non-immunoreactive terminal boutons on nNOS-immunoreactive dendrites originating from granule cells (Fig. 2F). Some of these synaptic junctions are also symmetric, as previously described by Hosoya (1973) and Ribak and Fallon (1982).

NO produced by the calcium-dependent activation of nNOS in neurons diffuses locally to exert its vasodilatory influence on adjacent small cerebral arterioles (Faraci, 1990; Faraci and Breeze, 1993; Iadecola, 1993). The diffusibility of NO means that actual neuronal contact is not necessary for this effect, and the fact that groups of granule cells seem to be electrically linked via their specialized gap-like (ephaptic) junctions points to the operation of a mass effect, rather than any precisely localized effect, of any substance released into their surroundings. The close proximity of nNOS-positive granule cells to blood vessels suggests that the produced NO may act directly on these vessels resulting in vasodilatation (Meyer et al., 1994). Virtually all the blood vessels that perfuse the ventral pallidum and end in the nucleus accumbens previously pass through NO-producing granule cell clusters in the islands of Calleja. The strategically placed islands may thus modulate the blood flow to the ventral pallidum, which may also regulate its own blood supply through the cholinergic input to the dendrites and perikarya of the nNOS-immunoreactive granule cells.

Recent evidence suggests that the islands of Calleja are in fact involved in cardiovascular regulation and are highly responsive to changes in systemic arterial blood pressure (Krukoff et al., 1992; McKitrick et al., 1992; Calaresu et al., 1994). In anesthetized rats, a significant proportion of single units recorded from the islands of Calleja responded with increased or decreased firing rates to falls or rises in systemic arterial pressure induced by intravenous injection of nitroprusside or phenylephrine, respectively. Microinjection of glutamate into the islands resulted in a decrease in arterial pressure and heart rate, which could be abolished by high cervical cord section or propranolol (Calaresu et al., 1994). The stimulation of granule cell discharge by falls in systemic blood pressure would provide a mechanism for maintaining blood flow to the ventral pallidum under these conditions. In addition, the islands of Calleja seem to have a predominantly inhibitory influence on the sympathetic outflow to the cardiovascular system in response to central baroreceptor inputs.

Other effects, including behavioral and emotional responses, may be mediated by the serotoninergic input to the islands of Calleja (Fallon et al., 1983) acting on their high levels of 5-HT2 and 5-HT4 receptors (Morilak et al., 1993; Waeber et al., 1994). The dopaminergic input and the high level of D3 dopamine receptors in the islands of Calleja (Bouthenet et al., 1991) have been related to a role in the behavioral changes of schizophrenia (Flores et al., 1996), while D2 dopamine receptors (Loopuijt, 1989; Yokoyama et al., 1994) may be involved in the limiting of limbic seizures (Alam and Starr, 1994). In addition, a syndrome of amnesia and personality changes appears to relate to ischemic lesions involving the islands of Calleja (Alexander and Freedman, 1984; Damasio et al., 1985; Phillips et al., 1987). In this regard, whereas NADPH diaphorase-positive neurons may be spared in hypoxia/ischemia (Ferreiro et al., 1988), the generation of NO appears to play a significant pathogenic role in the damage caused by focal brain ischemia (Huang et al., 1994). Preliminary observations on rat islands of Calleja after experimental global ischemia show a marked increase in the intensity of nNOS immunostaining in both granule cells and occasional satellite neurons, suggesting that expression of the enzyme is increased under these conditions.

The island of Calleja complex may also serve as a target for steroid sex hormones in both male and female rats (Pfaff and Keiner, 1973; Sar and Stumpf, 1975; Fallon et al., 1983). The large and medium-sized satellite neurons and some of the granule cells have the capacity to concentrate estradiol, and whereas only a few granule cells concentrate the hormone in the rhesus monkey (Pfaff et al., 1976), most of them do so in the rat (Pfaff and Keiner, 1973). The sex-hormone binding capacity of granule cells of the islands of Calleja in the rat suggests an important role in reproductive function. In normal Guinea pigs the administration of estradiol increased calcium-dependent NOS activity and NOS mRNA expression in the cerebellum, whereas the estrogen receptor antagonist tamoxifen reduced cerebellar calcium-dependent NOS activity in pregnant animals (Weiner et al., 1994). If similar mechanisms apply to the islands of Calleja, it would suggest that the nitrergic influence of the granule cells is augmented by estrogens, with a consequent potentiation of their influence on local blood vessels. The presence of LHRH-immunoreactive fibers to the more medial islands of Calleja in the rat, originating from neurons of the medial septum and the

nucleus of the diagonal band, has led to a suggestion that the islands are in some way involved in extrahypothalamic neuroendocrine control, including an influence on motor functions associated with mating behavior (Fallon et al., 1983). This, however, assumes that LHRH maintains a reproductive role in extrahypothalamic situations.

Ventricular system, circunventricular organs, and the hypothalamic nucleus circularis

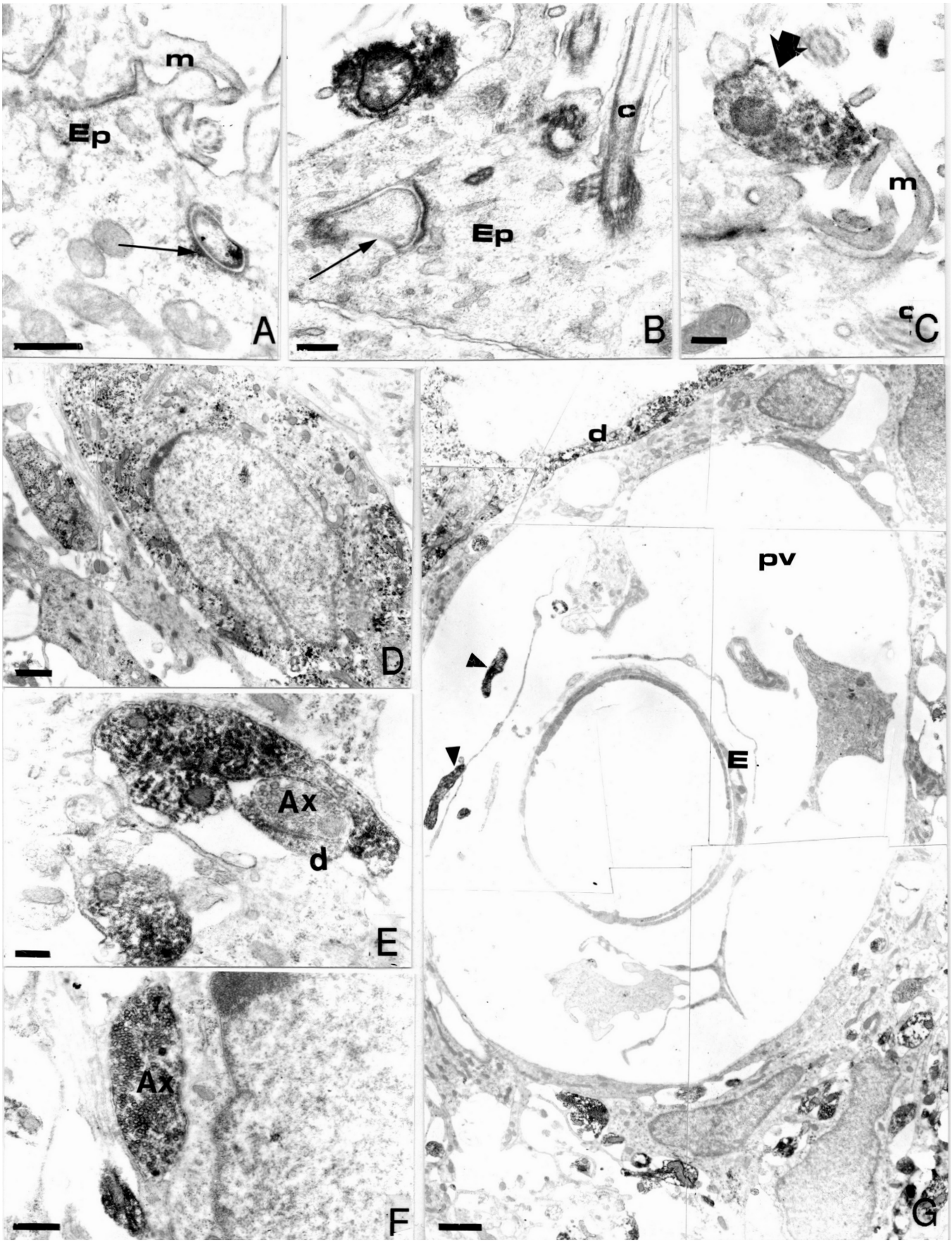
We have been particularly interested in the distribution of nNOS in the ependyma, and circumventricular organs such as the subfornical organ and area postrema. In the lateral ventricle of the rat brain, light microscopy showed nNOS-immunoreactive varicose nerve fibers and terminal boutons in supra- and subependymal areas. The spatial relationships among immunoreactive neuronal processes, the ependyma, and blood vessels were studied. Electron microscopy showed numerous nerve fibers in the wall of the lateral ventricle. Many of these fibers were nNOS immunoreactive and established very close contacts with ependymal cells (Fig. 3A-C). Immunoreactive neurons and processes were found in the subependymal plate of the ventricular wall, subfornical organ (Fig. 3D), area postrema and nucleus circularis of the hypothalamus. In the circumventricular organs and nucleus circularis, immunoreactive neurons were found close to the perivascular space of fenestrated and non-fenestrated blood vessels (Fig. 3G). The reaction product was localized next to the endoplasmic reticulum cisternae, ribosomes, neurotubules, and the inner face of the plasma membrane, where it formed clusters (Fig. 3D). The immunoreactive precipitate was also found in positive dendrites (Fig. 3E) and around the synaptic vesicles in positive terminal boutons (Fig. 3F). This distribution suggests that nNOS is a predominantly membrane-bound protein in these cells.

Nerve fibers have previously been observed in the supraependymal plexus (Richards et al., 1973; Lorez and Richards, 1973). Our study showed that this plexus is formed mostly by nitrergic fibers. We also observed, as previously reported by Noack et al. (1972), a close relationship between these supraependymal nerve fibers and the apical portion of the ependymal cells, where they ran among microvilli and cilia and occasionally formed structures redolent of zonulae adherens or synapses (Fig. 3A-C). NO may therefore be involved in the regulation of cerebrospinal fluid secretion and composition, and may also modulate the release of neurotransmitters and monoamines into the cerebrospinal fluid. As NO is a membrane-permeant gas, it can bypass normal signal transduction routes, which involve interactions with synaptic membrane receptors, and can react directly with post-synaptic proteins, such as soluble guanylate cyclase (Shuman and Madison, 1994).

In the subfornical organ, we found intense nNOS immunoreactivity in type I neurons (as classified by Dellman and Simpson, 1979) (Fig. 3D). The blood vessels did not show immunoreactive endothelial cells, but a few nNOS immunoreactive dendritic processes were observed in their perivascular space (Fig. 3G). Ultrastructural studies showed some nNOS immunoreactive terminals and synapses with the characteristic morphology described (Akert et al., 1967a,b; Rudert et al., 1968; Leonhard and Backhus-Roth, 1969). These immunoreactive terminals may originate from intrinsic or extrinsic neurons, as postulated by Andres (1965). The extrinsic connections of the subfornical organ include the triangular nucleus of the septum, the medial preoptic nucleus, and the vertical limb of the nucleus of the diagonal band of Broca (Hernesniemi et al., 1972; Broadwell and Brightman, 1976; Lind et al., 1987). Neurons in the diagonal band of Broca are immunoreactive to nNOS (Rodrigo et al., 1994). Many terminal axons in the subfornical organ make axosomatic and axodendritic contacts (Dellman and Simpson, 1979). Akert et al. (1967a) identified 83% of such synapses as axodendritic and 17% as axosomatic. They were described as synapses of Gray type I, although Gray type II synapses were also found (Akert et al., 1967b). Many of these terminals were immunoreactive for nNOS (Fig. 3E-F).

The subfornical organ is known to participate in the general regulation of fluid balance and blood pressure as well as in the production of hormones and neuropeptides (Kizer et al., 1974; Palkovits et al., 1976; Saavedra et al., 1976; Dellman and Simpson, 1979; Mangiapane et al., 1984; Schwartz et al., 1986). The subfornical organ also contains a large number of receptor sites for neuroactive substances (Iovino and Steardo, 1984; Lind et al., 1984; Saavedra et al., 1986; Azambuja et al., 1988; Koseki et al., 1989). The lack of a normal blood-brain barrier in many blood vessels of the subfornical organ (Dellman and Simpson, 1979; Shaver et al., 1990), and the dense localization of peptidergic receptors suggest a high degree of specialization, which might allow the subfornical organ to detect small changes in circulating hormone and peptide levels. We found a very close structural relationship between the different types of blood vessels in this area of the brain and nNOS-immunoreactive neurons, including dendrites and axons. The role of NO could be crucial in modulating the complex equilibrium of different hormones and neuropeptides, e.g. by altering the function of various proteins by modifying their release from synaptic vesicles (Shuman and Madison, 1994). The effects of NO may also be mediated through modulation of pre-synaptic Ca^{2+} influx (Lei et al., 1992).

The area postrema shows a great structural resemblance to the subfornical organ and is also a region with a modified blood-brain barrier function (Dempsey, 1973; Lucchi et al., 1989). Its blood vessels are surrounded by wide perivascular spaces (Dermietzel and Leibstein, 1978; Lucchi et al., 1989). These capillaries are fenestrated and the endothelial cells did not show immunoreactivity to nNOS, although some


m
Ep
A
Ep
c
B
m
c
C
D
d
pv
E
Ax
d
E
Ax
F
G

immunoreactive neural processes were found in the perivascular spaces. The area postrema has been demonstrated to have an intense neurotransmitter activity (Leslie and Osborne, 1984; Lanca and van der Kooy, 1985; Schwartz et al., 1986; Borison, 1989) and to be a chemoreceptive area or trigger zone involved in different vegetative functions, such as the neurosecretion, control of food and sodium intake, emetic response, cardiovascular and respiratory regulation, blood osmoreception, control of renal function and caloric homeostasis (Andrew and Taylor, 1958; Snyder and Sutin, 1961; Klara and Brizzee, 1975; Edwards and Ritter, 1981; Ossenkopp, 1983; Barnes et al., 1984; Gatti et al., 1985; Lucchi et al., 1989).

The widespread distribution of nNOS in the wall of the lateral ventricular and in neural structures of the subfornical organ and area postrema suggests that NO is involved in many neuroregulatory functions in addition to the regulation of vascular function and a possible role in cerebrospinal fluid homeostasis.

In the nucleus circularis, nNOS immunoreactive neurons surrounded the large perivascular spaces. NO may have a dual role in this area, on the one hand being involved in regulating the release of neuroactive substances from the hypothalamic-chiasmatic-perivascular-neurosecretory system, and on the other influencing local blood flow.

Our ultrastructural studies show that the nNOS immunoreaction product in these areas, as in the islands of Calleja, was always attached to cellular structures including endoplasmic reticulum, ribosomes, neurotubules, vesicle membranes, and the inner layer of the plasma membrane, but never evenly distributed in the cytoplasm. This suggests that the enzyme may be associated with membranes. This is in agreement with cell fractionation experiments that found nNOS in the particulate fraction (Hecker et al., 1994). Hendricks (1995), using a protein sequence database, found that nNOS has a peptide sequence at the amino terminal end that is usually involved in protein-protein interactions. This may explain the association of the reaction product to the cell membranes. Nevertheless, the fact that large amounts of enzyme activity are still found in the soluble fraction after homogenizing fresh tissue, suggests that the binding of nNOS to intracellular structures is relatively weak.

NO and cortical aging

Brain aging is a multifactorial process that finally leads to morphological and biochemical neurodegenerative changes (Amenta et al., 1994; Goldman et al., 1994). One prominent hypothesis on the possible mechanism of aging is the free radical theory, according to which free radicals produced by mitochondrial metabolism eventually result in abnormal cell function and death. One aspect of this mechanism would be neuronal production of the free radical NO, and its combination with superoxide to form the potent oxidant peroxynitrite. Over extended periods of time, peroxynitrite and maybe other highly reactive nitrogen species, may contribute to cumulative cell damage leading to neurodegeneration.

We therefore studied the cerebrocortical NO system in aging rats by light microscopic immunohistochemistry and Western blotting (Uttenthal et al., 1998), analyzing nNOS, iNOS, and protein nitration. In 26-month-old rats, layer I of the cerebral cortex was characterized by the occasional appearance of strongly labeled nNOS-positive cells with Cajal-Retzius-like morphology. The strongly nNOS-immunoreactive multipolar neurons found in layers II-VI of the cortex of aging rats (type I neurons, Yan et al., 1996; Yan and Garey, 1997; Judas et al., 1999) were seen in similar numbers to those found in young animals, but their processes had a varicose, vacuolated, and fragmented appearance, with an irregular outline and loss of spines. A large number of weakly nNOS-positive neurons, characterized by a ring of immunoreactive cytoplasm and not seen in young rats, were observed in layers II-VI of the aged rat cortex (type II neurons, Yan et al., 1996). These smaller neurons outnumbered the large type I neurons by about 50 to 1.

While no iNOS-immunopositive neurons were found in the cortex of young rats, a large number of such neurons appeared throughout the aged rat cortex, especially in pyramidal cells of layers IV-V. Nitrotyrosine-positive cells outnumbered total NOS-positive neurons in the cortex of young rats, but this relation was inverted in the aged rats because of the high increase in the number of NOS-immunoreactive cells, although the older animals had a slight increase in the number and staining intensity of nitrotyrosine-positive cells over the young ones. In parallel with the immunocytochemical results, Western blots of brain extracts showed a several-fold increase in both nNOS and iNOS immunoreactive bands in the aged rat, but a less marked increase in nitrotyrosine-containing proteins.

Both physiological and pathological aging are

Fig. 3. Electron microscopy of structures immunoreactive for nNOS in the ependymal wall of the third ventricle **(A-C)** and the subfornical organ **(D-G)**. Intracellular nerve fibers (arrows) and immunopositive terminals (thick arrows) can be seen in close proximity with the apical cytoplasm of ependymocytes (Ep), their cilia (c), and microvilli (m). **D.** Immunopositive neuron in the subfornical organ with abundant reaction product in its cytoplasm. **E.** Immunonegative axon (Ax) establishing a synapsis with a dendrite immunoreactive for nNOS. **F.** Axosomatic synapsis between a nNOS immunoreactive axon (Ax) and a negative neuron (N). **G.** Characteristic blood vessel of the subfornical organ. The endothelial cell (E) lacks immunoreactivity for nNOS but this immunoreactivity is clearly seen in nerve fibers (arrowheads) found in the perivascular space (pv) and in dendritic processes in the neighboring areas. Bars: A-C, E 500 nm; D, F, 200 nm; G, 1 μm.

associated with a variety of brain microanatomical changes, including loss of nerve cells, reduced extent of neural networks, decreased density of dendritic spines, hypertrophy and hyperplasia of astrocytes, neurofibrillary and granulovascular degeneration, lipofuscin accumulation, congophilic angiopathy and microvascular changes (Landfield et al., 1977; Brizzee and Ordy, 1979; Flood and Coleman, 1988). For many authors the frontal cortex is the main cortical area involved in the cognitive dysfunction associated with aging, but the changes induced by senescence affect the microanatomy of other cortical areas to varying degrees. Amenta et al. (1994) described a loss of nerve cells in the frontal cortex from adult to old age, and similar results have been described in the auditory, visual and pyriform cortices, in the hippocampus and subcortical regions (Flood and Coleman, 1988). However, other studies report no changes in neuronal density but increases in glial cell number in aged male Wistar rats (Peinado et al., 1993). Some of these discrepancies may be attributed to the different techniques employed.

The intensely nNOS-positive multipolar type I cells in the cortex of the aged rat showed a morphology that raises doubts about their functional integrity. The extent to which their intense expression of nNOS immunoreactivity results in actual NO production, or whether any NO production by these cells is functionally appropriate, is therefore questionable. Although a much larger number of weakly nNOS-immunoreactive type II cells appeared, the functional implication of this phenomenon in terms of NO production also remains to be elucidated. In further studies, we found that these cells had no NADPH diaphorase activity. In this regard, it may be of interest that Yamada et al. (1996) found a 50% reduction of NADPH diaphorase-positive neurons in the cerebral cortex of 30-month-old rats, while NOS activity in the cerebral cortex was not different from that in young rats. This might imply that for various reasons, much of the NOS present in cortical sections, also seen as increased intensity of the nNOS immunoreactive bands on Western blots, is not functional, while the overall capacity for NO production remains unchanged. Whether the substantially increased expression of nNOS seen in our study represents a compensatory mechanism for loss of function, or is induced by other age-related processes, is a subject for further study. The finding of a few nNOS-immunopositive Cajal-Retzius-like cells in layer I of the aged cortex may suggest an attempted remodeling mechanism, going back to the developmental stages of the cerebral cortex in which nNOS expression appears to play an important role, as shown by our embryological study (Santacana et al., 1998).

Whereas iNOS immunoreactivity is largely absent from cortical neurons in young rats, it appears in some cortical neurons, especially pyramidal cells of layers IV-V, after various insults or situations of stress. For example, iNOS immunoreactivity appeared in cortical neurons of rat forebrain slices 3 hours after being subjected to oxygen and glucose deprivation in vitro (Moro et al., 1998). The aged rat cortex showed the presence of iNOS immunoreactivity in a large number of cortical neurons, including pyramidal cells of layers IV-V. Inducible NOS can be expressed by cerebellar neurons following exposure to LPS and interferon-g (Minc-Golomb et al., 1994, 1996) and the iNOS gene promoter can be activated by a hypoxia-responsive element (Melillo et al., 1995) as well as by interferon regulatory factor-1 and NF-kB, which mediate the respective actions of interferon-g and LPS on the promoter. Whether relative hypoxia or any increased exposure to cytokines and endotoxin play a part in the neuronal expression of iNOS through aging remains to be determined.

The nitration of tyrosyl residues in tissue proteins is attributed chiefly to the action of peroxynitrite, formed by the combination of NO with superoxide (Beckman, 1996). The formation of nitrotyrosine-containing tissue proteins can therefore be seen as a complex function of local NO and superoxide concentrations, in conjunction with the exposure of protein tyrosyl residues, and the turnover rates of the tissue proteins concerned. Depending on the protein turnover rates, the concentrations of protein-bound nitrotyrosine will reflect overall NO and superoxide production over a period of time. While the slight increase in nitrotyrosine immunoreactivity demonstrated by immunocytochemistry and the 2-fold increase in a group of nitrotyrosine-containing proteins demonstrated by Western blotting may reflect an increased production of NO in the aging brain, this would require confirmation by other techniques. Other important factors that might contribute to the higher nitrated protein levels detected would be an increased production of superoxide and a reduced rate of turnover of the relevant tissue proteins with age. However, in relation to the number of nitrotyrosine-positive cells seen, their abundance when compared to the number of NOS-positive cells in the cortex of young rats implies that NO can diffuse far from its source and influence a large number of cell targets. The fact that there is only a slight further increase in the number of nitrotyrosine-positive cells in the cortex of aged rats may indicate that, whatever the cause of increased nitrotyrosine formation, the number of susceptible target cells may be limited. The fact that nNOS-positive and iNOS-positive neurons together outnumber nitrotyrosine-positive cells in the aged cortex is an indication that many of these cells may not be producing sufficient NO to overcome their intracellular protective factors and bring about a detectable nitration of their own cellular proteins.

The results of our study on the NO system in the aging rat cerebral cortex suggest that there is an activation of nNOS expression in type II neurons, but a significant fraction of these newly synthesized enzymes may be non-functional. At the same time, the intensely nNOS-positive type I neurons showed degenerative changes. Inducible NOS appeared in cortical pyramidal

neurons in a manner suggestive of some kind of stress. The fact that the increase in protein nitration was modest in comparison with the increase in nNOS and iNOS expression was compatible with doubts about the functionality of the newly synthesized NOS. The appearance of occasional Cajal-Retzius-like cells was suggestive of an attempted regenerative process that might be frustrated by the functional impairment of NOS.

NO and ischemia-hypoxia/reperfusion.

There is substantial evidence that the synthesis of NO, during certain brain injuries and specifically in hypoxia/ischemia, is often associated with rapid upregulation of nNOS expression (Zhang et al., 1994; Higuchi et al., 1996). This modification of nNOS, at least *in vivo*, depends on extracellular concentrations of the endogenous excitatory aminoacids, glutamate and aspartate (McDonald and Jonston, 1990; Andine, 1991), which stimulate the N-methyl-D-aspartate (NMDA) receptors (Garthwaite et al., 1988; Manzoni et al., 1992) leading to Ca^{+2} influx (Vannuci, 1990) and activation of nNOS to generate NO from L-arginine (Dawson et al., 1992).

The excessive extracellualr accumulation of these amino acids plays a key role in neuronal injury (Zhao et al., 1999; Choi and Rodman, 1990). After oxygen and glucose deprivation or global cerebral ischemia there is a large increase in the number of iNOS-positive neurons in the CNS (Moro et al., 1998; Rodrigo et al., 2001), indicating that overproduction of NO might participate in generating the intense tissue damage observed under these conditions. These effects of NO on the ischemic brain are thought to be dependent on the actual stage of the ischemic process and on the sources of its production (Iadecola, 1997). Many details of the involvement of NO in mechanisms of tissue damage and neuronal death after ischemia are unknown, although it is known that NO decreases energy production by inhibiting mitochondrial respiration and glycolysis, reduces intracellular glutathione levels, inhibits DNA synthesis, and damages the DNA structure.

The precise role of NO in the pathogenesis of ischemic brain damage has been a source of controversy (Iadecola, 1997; Buisson et al., 1993), considering that NO may play either a neuroprotective or neurotoxic role after cerebral ischemia. As a potentially protective agent, NO acts as a vasodilator, reducing blood pressure and vascular tone, and possibly this beneficial role of NO in the cortical areas of the ischemic brain may be related to the presence of NOS immunoreactive neurons placed close to cerebral arteries (Iadecola et al., 1993; Faraci and Brian, 1994). NO can also act as a superoxide radical scavenger, and may inhibit platelet aggregation and neutrophil adhesion.

The neurotoxic effect of NO in brain injury may be mediated by excesses of NO (Nagafuji et al., 1995; Hamada et al., 1994), but there is little information on the individual contribution of the three enzyme isoforms to NO production in brain injuries. Recently, in an ex vivo model of rat ischemic forebrain slices, it has been demonstrated that nNOS is upregulated in neurons and other cells of the CNS following deprivation of oxygen and glucose. This elevation of calcium-dependent NOS activity increases for 60 minutes after the ischemic insult and begins to decrease afterwards. Coincident with the zenith in nNOS activity, there is an increase in calcium-independent NOS activity that by 180 minutes would become more relevant than the calcium-dependent one (Moro et al., 1998; De Alba et al., 1999). It has been postulated that excessive production of NO during ischemia and reperfusion can injure adjacent tissues by its rapid combination with superoxide radicals (O^{2-}) to produce peroxynitrite (ONOO-) (Hibbs et al., 1988) and other free radicals (Nagafuji et al., 1995; Dawson and Dawson, 1996). These free radicals stimulate the synthesis and release of pro-inflammatory mediators such as cytokines (Gross et al., 1991; Geller et al., 1993; Radomski et al., 1993) and prostanoids, and by increasing microvascular permeability, thus promoting edema formation (Giraldelo et al., 1994). This is considered a potential major cytotoxic pathway leading to cell death in many systems (Gross et al., 1991; Moncada et al., 1991; Beckman, 1996).

The detailed mechanisms by which $ONOO^-$ and other downstream free radical species cause modification of biomolecules are not yet completely understood (Ischoripoulus et al., 1992; Pryor and Squadrito, 1995). Peroxynitrite has been involved in the pathology of a wide range of diseases, and the existence of ONOO- is related to the finding of 3-nitrotyrosine in injured tissues. Specifically, the cerebral hemispheres in ischemia-reperfusion models suffer increased production of reactive oxygen species and the production of peroxynitrite in direct relation with the availability of NO (Forman et al., 1998). Peroxynitrite undergoes protonation, isomerization, and decomposition at physiological pH to give nitrous products that deplete antioxidants, oxydizes and nitrates lipids, proteins, and DNA (Beckman, 1990; Beckman et al., 1991; Radi and Beckman, 1991; Darley-Usmar and Halliwel, 1995; Pryor and Squadrito, 1995). Recently, measured changes in NO metabolites in the brains of neonatal rats with hypoxic-ischemic damage have also been described (Higuchi et al., 1998).

Nitrotyrosine residues were demonstrated particularly in the placental vascular endothelium (Myatt et al., 1996), during experimental carbon monoxide (CO) poisoning in the rat brain (Ischiropoulos et al., 1992), in the inflamed colonic epithelium (Singer et al., 1996), and in the cortex of aging rats (Uttenthal et al., 1998). They were also found in blood vessels close to the lesions of the rat's somatosensory cortex and in the ipsilateral hippocampus and thalamus (Bidmon et al., 1998), suggesting the formation and action of peroxynitrite and nitration of cellular protein in these tissues, so that the nitrotyrosine produced over a period

of time is a marker for the production of reactive nitrogen species (Ischiropoulos et al., 1992).

Immunocytochemical studies have revealed that nitration of tyrosine residues takes place in various tissues and organs after ischemia and in other pathological conditions (Ischiropoulos et al., 1992; Uttenthal et al., 1998), including the brains of patients with Alzheimer's disease (Smith et al., 1997).

Various pharmacological agents can reduce NO production or prevent its biological effects by different mechanisms (Moncada et al., 1997), including the inhibition of L-arginine uptake into the cell, reducing the cellular availability of necessary cofactors by preventing their formation or promoting their breakdown, inhibiting the NOS enzymes, scavenging NO once formed, or inhibiting the cellular mechanisms leading to induction of a particular NOS isoform. Nw-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor, not only inhibits NO synthesis, but has also been reported to reduce glutamate efflux, therefore contributing to the reduction of infarct size after focal ischemia (Buisson et al., 1993; Hamada et al., 1994). However, in global forebrain ischemia the amounts of glutamate released in animals treated with L-NAME are not clear since there are reports supporting an increase (Kahn et al., 1987), decrease (Phills et al., 1994), or lack of variation (Ghribi et al., 1994) in comparison with controls. In support of the deleterious effect of NO in tissue survival, it has been reported that a reduction in NO synthesis, in mutant mice deficient in nNOS activity, decreases the volume of infarcted tissue area after hypoxic injury produced by occlusion of middle cerebral artery (Huang et al., 1994).

It has also been demonstrated in the adult rat brain that the NO produced by NOS is directly involved in cerebral ischemic neuronal lesions through rapid upregulation of nNOS mRNA (Zhang et al., 1994) and a subsequent elevation of NO production. In the cortical areas of the ischemic brain, the effect of NO may be related to the presence of immunoreactive neurons close to cerebral arteries (Faraci, 1991; Iadecola and Ross, 1997). The administration of N^G-nitro-L-arginine (L-NA), a competitive inhibitor of NOS, previous or simultaneous to the ischemic insult significantly reduces infarcted brain volume, whereas posthypoxic L-NA treatment does not have a neuroprotective effect.

NO and postnatal development of cortex and hippocampus of rats subjected to hypoxia during delivery

Cerebral hypoxia and ischemia suffered during the perinatal period often results in acute and chronic neurological morbidity such as encephalopathy, seizures, mental retardation, epilepsy, and cerebral palsy (Raichle, 1983; Vannucci, 1990; Volpe, 1995). There is increasing evidence that NO plays an important role in neuronal damage in neonatal hypoxic-ischemic encephalopathy (Trifiletti, 1992; Hamada et al., 1994; Higuchi et al., 1996).

The expression of nNOS and iNOS isoforms and the nitration marker, nitrotyrosine was studied in the postnatal development of cerebral cortex from P0 to P20 postnatal days of rats subjected to hypoxia during delivery by immunocytochemical procedures. The calcium-dependent and independent NOS activities demonstrated by biochemical techniques and Western blotting have been used to analyze and quantify these proteins.

Hypoxia-exposed newborn rats were obtained by decapitating the pregnant dams just before delivery and keeping the fetuses in the uterus for 25 to 30 minutes. The fetuses were then removed by hysterectomy and revived by thoracic stimulation and then studied on postnatal days P0, P2, P3, P5, P7, P10, P15 and P20.

The expression of the neuronal isoform in hypoxic animals increased during the first postnatal development days (P0 to P5), the immunoreactivity being very intense between P0 and P4 postnatal days, as compared to the control group, but the highest immunoreactivity was found in the cerebral cortex of rats in postnatal day P5. From this postnatal day on the expression of nNOS decreased slowly from P7 to P20. Immunoreactivity for iNOS was also more intense in the cortex of the hypoxic rats between P0 and P5, as compared to the control group. Nitrityrosine reaction identified two nitrated proteins of 52 kDa and 38 kDa. The levels of the 52 kDa protein increased from P0 to P10, and then decreased progressively. The 38 kDa band appeared at P10 and remained at a low level until P20. These observations suggest that the NO system influences the physiological mechanism of neuronal maturation and cortical plasticity along normal postnatal development. Also, the overexpression of these neuroactive substances in hypoxic newborn rats could increase blood flow and perfusion under these established hypoxic conditions.
Expression of nNOS in the hippocampus was found in Ammon's horn from P0 and in the dentate gyrus from P5. This immunoreactivity increased on subsequent postnatal days in both areas. Immunoreactive neurons were first found in the dentate gyrus in the ectal layer (P5-P7), appearing in the endal layer by P10. From P0 to P20, numerous immunoreactive neurons were found in Ammon's horn, these neurons being more abundant in CA3 than in the subiculum, parasubiculum, CA1 and CA2. In general, the intensity of immunoreaction and number of neurons containing nNOS were higher in the hippocampus of hypoxic rats than in the controls. The increased expression of nNOS in hypoxic rats could be considered to have a neuroprotective effect, but this point needs further investigation.

NO and ischemia/reperfusion in rat cerebral cortex

Increased NO production has been implicated in the toxic mechanism of many forms of cellular injury, including ischemia (Beckman, 1990; Nowicki et al., 1991; Beckman and Koppenol, 1996; Iadecola, 1997;

Strijbos, 1998). Transient cerebral ischemia is associated with NO release (Malinski et al., 1993; Forman, 1998). It has been suggested that the effect of NO varies depending on the stage of evolution along the ischemic process and on the cellular source of NO (Lipton 1993). The *in vivo* toxicity of NO has been attributed to its rapid combination with superoxide to form peroxynitrite, a potent oxidizing agent with cytotoxic actions (Beckman 1991; Radi et al., 1991; Szabó 1996). Interaction of peroxynitrite with proteins can nitrate tyrosine residues resulting in the formation of the stable end-product, 3-nitro-L-tyrosine (nitrotyrosine) (Beckman, 1996; Ye et al., 1996; Bidmon et al., 1998; Uttenthal et al., 1998). Brain ischemia triggers a cascade of events, eventually leading to neuronal death (Rehncrona, 1986; Cafe et al., 1993; Iadecola and Ross, 1997). The hypothesized sequence can be summarized as follows: depolarization, increase of extracellular glutamate concentration, overstimulation of NMDA receptors, increase in intracellular Ca^{2+} concentrations, activation of the calcium-dependent NOS isoforms nNOS and eNOS, release of NO which rapidly reacts with superoxide produced in excess during reperfusion, peroxynitrite formation, and finally nitrosylation and/or nitration of proteins. Furthermore, ischemia or reperfusion after ischemia eventually induces the expression of iNOS (Iadecola et al., 1995a,b; Moro et al., 1998; Forster et al., 1999). This isoform is not normally present in cerebral neurons in unaffected young animals but it can be detected in neurons after inflammatory, infectious, or ischemic damage, as well as in the normal aging brain (Uttenthal et al., 1998). It appears that activation of nNOS or induction of iNOS mediates ischemic brain damage, possibly by the action of NO on mitochondrial respiration resulting in energy depletion. In experimentally-induced cerebral lesions, nNOS (-/-) knockout mice show smaller lesions than those in wild-type animals (Huang, 1994; Panahian et al., 1996). On the other hand, eNOS is thought to act neuroprotectively by enhancing blood supply to the injured tissue (Huang, 1996; Lo et al., 1996).

Changes in the expression and distribution of the neuronal and inducible isoforms of NOS and in protein nitration in the rat cerebral cortex were investigated by immunohistochemistry and Western blotting. Calcium-dependent and independent NOS activities were measured by the rate of conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline (Radomski et al., 1993). Finally, diffusion magnetic resonance imaging (MRI) was used to evaluate the possible protective properties of L-NAME in our model (Rodrigo et al., 2001). Animals were perfused transcardially with an oxygenated plasma substitute and subjected to 30 min of oxygen and glucose deprivation (ischemia group), followed by reperfusion for up to 14 hours with oxygenated medium containing glucose (reperfusion group). Another group was perfused continuously without oxygen or glucose deprivation (sham group), and a final group was subjected to tissue collection without prior experimental perfusion (control group). Expression of nNOS showed an early increase up to 4 h after the ischemic period (Fig. 4A-B), followed by a progressive decrease afterwards. Inducible NOS was not seen in the control or ischemia groups, but appeared in pyramidal neurons of layers IV-V of the cerebral cortex after 2 h, and more clearly after 4 h of reperfusion. Immunoreactive nitrate proteins showed a widespread occurrence in cortical neurons in the ischemia and reperfusion groups, while weaker immunoreactivity was noted in the control and sham groups. Our diffusion MRI maps showed a decrease of the apparent diffusion coefficient (ADC) during the reperfusion period for rats exposed to ischemia. The ADC values at the beginning of the experiment were 4-6 10^{-6} cm^2/s and they decreased to less than 2 10^{-6} cm^2/s. This decrease was not observed in control rats (which suffered no ischemia) or in rats previously treated with L-NAME. Decreasing ADC has been related to ischemic injury (Zaharchuk et al., 1997), and our data indicate that the inhibition of NO production protects the brain against the injury caused by transient global ischemia. Our results also suggest that the early increase in expression of nNOS followed by the induction of iNOS in certain neurons is associated with a progressive nitration of tyrosine residues attributable to the action of peroxynitrite, which may contribute to cell damage.

Similar observations have been made in other areas of the brain, such as the caudate-putamen (Fig. 4C,D).

NO and hipobaric hypoxia

Few experimental studies on animals have been carried out to understand the neurobiological mechanims related to hypoxia caused by high altitude environments. Prolonged exposures to these environments can be debilitating to unacclimated individuals and can cause acute sickness (Carson et al., 1969; Hackett and Rennie, 1978; Hultgren, 1979). These disorders are characterized by symptoms such as headache, insomnia, irritability, depression, and thinking difficulties (Carson et al., 1969). Other neuropsychological impairments include vision, memory, and mood disorders which might be caused by altered function of the CNS. Forster et al. (1975) showed increases in cerebral electrical activity by electroencephalogram (EEG) and reduced EEG signal following visual evoked responses in hypobaria.

The severity and duration of these sicknesses vary depending on the individual conditions, levels of altitude, and rate of ascent (Hansen et al., 1967). These changes often persist after returning to lower altitudes (Rynn, 1971; Sharma et al., 1975; Sharma and Maljotra, 1976; West et al., 1983; West, 1986; Cavaletti et al., 1987, 1990; Oelz and Regard, 1988; Regard et al., 1989; Cavaletti and Tredici, 1992; Shukitt-Hale et al., 1994). Cavaletti et al. (1987) reported a decrease in memory performance 75 days after a climb to 7,075 m (23,211ft) without oxygen supplementation. These data were confirmed by Kassier and Such (1989), who reported how certain neurological signs (headache and taste

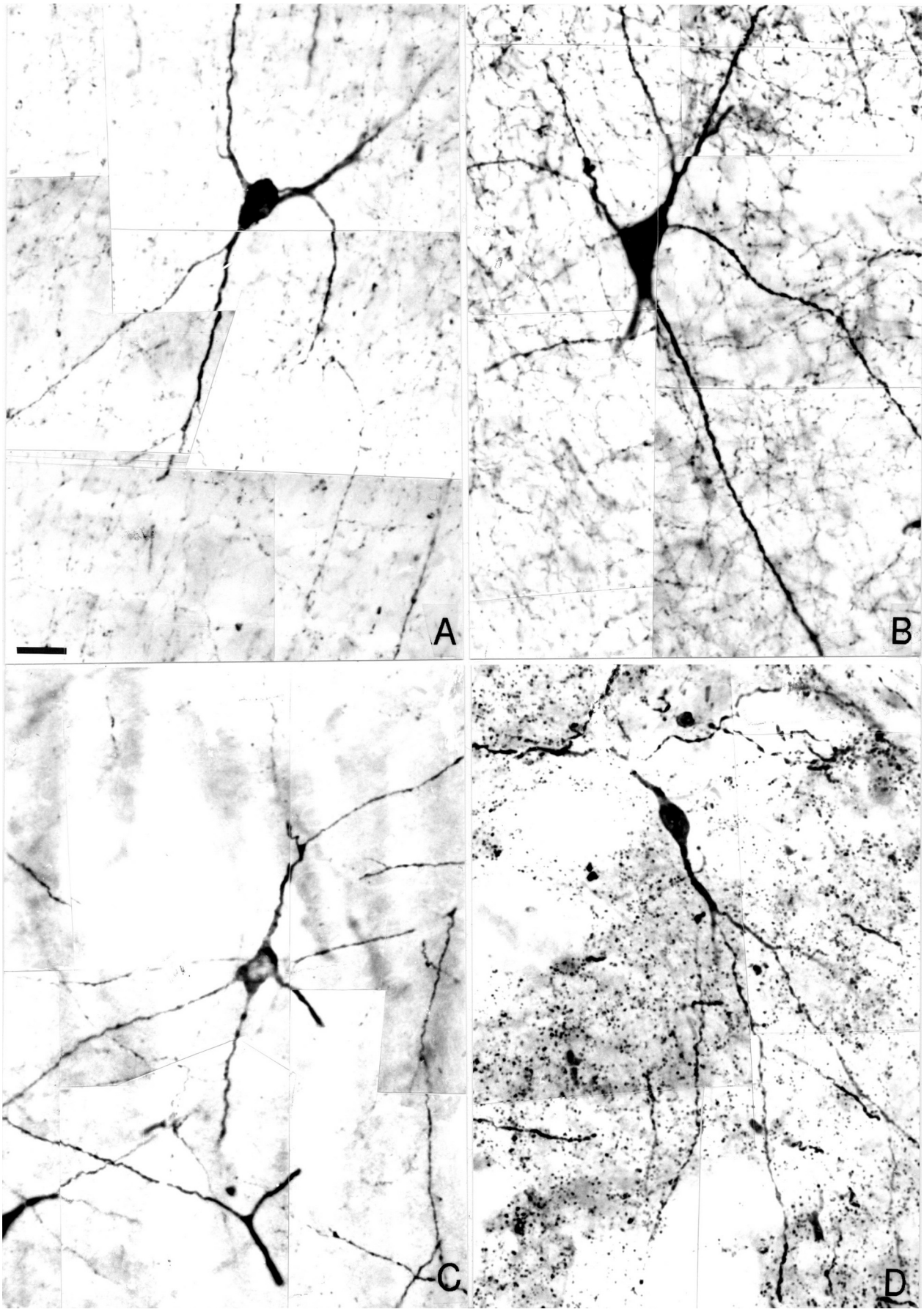


Fig. 4. Comparison of nNOS immunoreactive neurons in the cortex **(A,B)** and caudate-putamen **(C,D)** of the rat brain in control animals **(A,C)** and after 30 min of ischemia **(B,D)**. Note the high increase in the number of immunoreactive fibers after ischemia. Bar: 250 μm.

dysfunction) persisted 15 days after an accidental exposure to hypoxia in an experimental chamber, simulating an altitude of 7,620 m (25,000 ft). In the hippocampus, which is one of the cerebral areas most vulnerable to hypoxia (Arregui et al., 1991; Xun et al., 1992; Shukitt-Hale et al., 1996), some effects related to impairment of mental ability were also correlated with hypoxia (Haldane and Priestley, 1935; Cavaletti et al., 1990).

It is generally accepted that psychomotor performance is not impaired at altitudes below 3,048 m (1,000 ft) (Figarola and Billings, 1966; Pearson and Neal, 1970; Ersting, 1978), but certain alterations in cerebral function have been reported above 4,000 m (13,124 ft) (Fine and Kobrick, 1978). At pressures equivalent to an altitude of 2,440 m (8,000 ft), learning of complex mental tasks is slower than at sea level (Denison et al., 1986). These difficulties have been reported at altitudes as low as 1,829 to 2,438 m (6,000 to 8,000 ft) (McFarland, 1971). Thus it appears that even at the cabin pressure of today's commercial aircraft (equivalent to 2,500 m or 8,200 ft) sensitive psychometric tests can pick up minor degrees of impairment. After a prolonged stay at high altitude (3,500 m or 11,484 ft) certain changes in autonomic nervous system balance were reported by Malhotra and Mathew (1974), specifically those related to sympathetic and parasympathetic hyperactivity. The systolic and diastolic blood pressure and the mean pulse rate were also elevated at 4,200 m (13,796 ft) (Forster, 1985).

The changes in the CNS related to hypobaric hypoxia, as described above, may be mediated by endogenous NO, which as a chemical messenger and strong vasodilator is directly implicated in various pathophysiological mechanisms. The expression of nNOS, iNOS and nitrated proteins in the cerebral cortex, caudate putamen, islands of Calleja, hippocampus, superior colliculus, and cerebellum was analyzed in rat brains after acute hypobaric hypoxia for 15 minutes to 8 hours at a pressure (230.4 Torr) equivalent to 9,144 m (30,000 ft). This study was carried out by immunocytochemistry and Western Blotting, using specific polyclonal antibodies.

After 15 and 45 minutes of hypobaric hypoxia and 24 hours of recovery, the immunoreactivity to nNOS was indistinguishable from the levels found in control animals. The blood vessels were surrounded by numerous nNOS immunoreactive fibers, but no immunoreactivity was found in the endothelial cells.

A few iNOS neurons were found scattered throughout the cortical areas, hippocampus and cerebellum. The reaction product showed a granular pattern and was distributed throughout the cytoplasm of these neurons. Weak immunoreactivity was found in Purkinje cells of the cerebellar cortex. Immunoreactivity to nitrotyrosine was found in neurons of all areas studied, with the reaction product found mainly in the nuclei.

After 4 h of hypobaric hypoxia and 24 h of reoxygenation, immunoreactive nNOS neurons and varicose nerve fibres increased in all cortical areas and layers, forming a dense network, surrounding immunoreactive neurons and blood vessels. The blood vessels, specially those of small diameter, showed intense vasodilation, lending a spongy morphology to all cortical areas. Small immunoreactive neurons were also found distributed through the upper layer of all cortical regions.

In these animals, the caudate putamen, the islands of Calleja, the hippocampus, the superior colliculus, and cerebellum contained numerous nitrergic varicose nerve fibres and nNOS neurons were found distributed in all areas of the caudate putamen, in the molecular, pyramidal, and polymorph layers of the olfactory tubercle, and in all regions of the Ammon's horn, in the ectal and endal areas of the dentate gyrus and in the hiliar region of the hippocampus. The superior colliculus showed a characteristic spongy appearance as a consequence of intense vasodilatation of blood vessels which pushed and compressed the neural parenchyma.The surrounding parenchyma contained numerous nNOS-positive neurons that changed their morphology from multipolar to fusiform shapes. The cerebellum showed a more immunoreactive nitrergic plexus in the molecular layer, where some apical immunoreactive processes of Purkinje cells were found. The stellate, basket cells, and basket terminals that surrounded the Purkinje cells were also immunoreactive. The granular cells in the granular cell layer also showed increased immunoreactivity.

In the same animals, the immunoreactivity to iNOS was higher than in the controls in all cerebral areas studied. Numerous iNOS-immunoreactive neurons were found, in particular large pyramidal neurons in layers IV-V of the parietal and temporal cortices. In the hippocampus, immunoreactive pyramidal neurons were also found in the pyramidal layer of the subiculum and CA1, CA2 and CA3 regions of Ammon's horn. Immunoreactive neurons with multipolar shape were also found in the basal layer of the dentate gyrus, just underneath granular cells. The superior colliculus contained iNOS immunoreactive neurons in all layers. In the cerebellum some immunoreactive Purkinje cells were also found.

Numerous neurons immunoreactive to nitrotyrosine were found in the cortex, olfactory tubercle, superior colliculus and cerebellum, showing the reaction product in or around the nucleus, with some faint immunoreactivity in the cytoplasm. In the cerebellum, the stellate, basket, and Purkinje cells were immunoreactive.

After 8 h of hypobaric hypoxia and 24 h of reoxygenation, all cortical areas showed a spongy structure with blood vessels exhibiting a large diameter. This phenomenon confined the parenchyma into narrow and elongated cords. The low number of fusiform or elongated immunoreactive neurons to nNOS showed a low number of processes and collaterals, which formed a

dense network surrounding the dilated blood vessels.

The caudate putamen area showed a lower number of immunoreactive neurons and nerve fibers in the nitrergic plexus when compared to the previous time point. The islands of Calleja contained numerous intensely immunoreactive granular cells surrounding largely dilated blood vessels.

Few neurons positive for nNOS with short processes and collaterals were found in all hippocampal areas. Occasionally, some pyramidal cells with a well-stained cell body and processes were found in CA1. The blood vessels also showed an enlarged diameter, forming a dense vascular network that pervaded all the parenchyma of the hippocampus, being specially evident in the ectal and endal regions of the dentate gyrus.

Immunoreactivity to nNOS decreased in all layers of the superior colliculus where immunoreactive neurons and processes were found embedded in the compressed and narrow parenchyma originated by the dense network of vasodilated blood vessels.

A similar immunoreactive pattern was found in the cerebellum, where the blood vessels crossed the molecular and granular cell layers, showing a dramatic vasodilatation. In the molecular layer, some nNOS immunoreactive apical processes of the Purkinje cells were found as well as some Purkinje cell bodies, which were surrounded by nNOS-positive basket terminals.
Neurons immunoreactive to iNOS were found scattered through all cortical areas, but in layer IV-V of the parietal and temporal cortices these neurons had a clear pyramidal morphology. The caudate putamen and the hippocampus showed nNOS immunoreactive neurons with well-developed and stained processes, as well as the Purkinje cells in the cerebellum.

All regions studied contained neurons immunoreactive to nitrotyrosine and the Purkinje cells in the cerebellum were strongly stained . Nitrotyrosine immunoreactive neurons were also found in the cerebellar nuclei.

In general, the blood vessels of the cerebral cortex in animals treated for 4 and 8 h with hypobaric hypoxia followed by 24 h of reoxygenation showed intense vasodilation, originating narrow and elongated portions of neuronal parenchyma, pushing out the stained neurons and their processes, and suggesting that the blood vessels were surrounded by a dense nitrergic plexus.

The circularis nucleus, which is integrated in the magnocellular accessory group located in the anteromedial hypothalamic preoptic area, forming part of the hypothalamic chiasmatic-perivascular-neurosecretory system, showed a great number of nNOS immunoreactive neurons and processes surrounding the blood vessels after 4 hours of hypobaric hypoxia. After 8 h of hypobaric hypoxia and 24 h of reoxygenation, the vasodilatation of these blood vessels was the main characteristic. Apparently, the number of nNOS immunoreactive neurons that surround these blood vessels decreased when compared with previous time points. After 8 h of hypobaric hypoxia numerous blood vessels displayed endothelial cells immunoreactive to nitrotyrosine.

When the experimental animals were pretreated with L-NAME for five days before the treatment with hypobaric hypoxia, the immunoreactive pattern was undistinguisable from control animals.

Western blotting for nNOS, iNOS, and nitrated proteins was carried out in supernatants from control brains and from those subjected to 4 and 8 h of hypobaric hypoxia, followed by either 0 or 24 h of reoxygenation. The nNOS antibody revealed a band of 153 kDa. The iNOS antibody labeled a 135 kDa band, and the nitrotyrosine antiserum showed two or three bands with apparent molecular weights between 23 and 53 kDa. The nNOS protein band in the cortex decreased in animals treated with all protocols with respect to control animals. The nNOS immunoreactive band in the cerebellum, after 4 h of hypobaria followed by 0 h of reoxygenation, increased when compared to control animals, but after 4 h of hypobaric hypoxia and 24 h of reoxygenation no significant differences with control animals were observed. After 8 h of hypoxia and independently of the reoxygenation time period, the intensity of the nNOS-positive band in the cerebellum clearly decreased in relation to the band observed in control animals.

After 4/8 h of hypobaria and 0/24 h of reoxygenation, the iNOS-positive band increased in comparison with the control protein band. The cerebellum showed an increase in iNOS protein intensity after 4 h hypobaria and either 0 or 24 h reoxygenation, but this elevation was not so high after 8 h of hypobaric hypoxia. A small decrease in intensity of the iNOS protein band was found after 8 h of hypobaria and 24 h of reoxygenation.

The nitrotyrosine-positive bands in the cortex showed a slight decrease after 4/8 of hypobaric hypoxia and no reoxygenation, this decrease being more intense after 24 h of recovery. In the cerebellum, the nitrotyrosine-positive band increased after 4/8 h of hypobaria and 0/24 h reoxygenation. These results demonstrate that modifications in the expression of NOS isoforms and protein nitration were directly related to altitude, time of exposure to hypobaric hypoxia, and the post-hypoxic recovery period. After 8 h of hypobaric hypoxia and 24 h of recovery, an intense vasodilatation was also demonstrated. The animals treated with L-NAME did not show any immunocytochemical or biochemical modification of nNOS and iNOS expression, but induced a decrease in nitrotyrosine intensity. These results may be extrapolated to explain some psychological alterations found in humans after prolonged exposure to altitude.

NO and pathological disorders

NO and cell death

Alteration of mitochondrial function seriously

affects cell viability. One of the first signs of trouble is the release into the cytosol of various proteins, including cytochrome c, which are normally confined to the intermembrane space of mitochondria. This event can lead to necrosis through irreversible mitochondrial damage and collapse of the energetic capacity of the cell. Alternatively, it can induce apoptosis when proteins released from the mitochondria (i.e. the 14 kDa cytochrome c and the 50 kDa protein apoptosis-inducing factor, AIF) initiate caspase activation and the formation of a complex with apoptotic protease-activating factor (Apaf-1), procaspase 9, and ATP or dATP. These steps are followed by protein degradation and DNA cleavage. The release of cytochrome c from mitochondria during this is controlled by members of the Bcl-2 family (e.g. Bcl-2 and Bcl-x1) which inhibit cell death and prevent the release of cytochrome c. Other proteins such as Bax and bak promote cell death and induce cytochrome c release (Vander-Heiden et al., 1997). The rapid, complete, and kinetically invariant release of cytochrome c and AIF from the mitochondria to the cytosol has been recognized as a key event for commitment to apoptosis (Goldstein et al., 2000). Detection of cytochrome c in the cytosol preceded the changes of apoptosis, suggesting that NO-induced apoptosis is initiated by a NO-dependent modification of cytochrome c (Hortelano et al., 1999a; Kroemer, 1997).

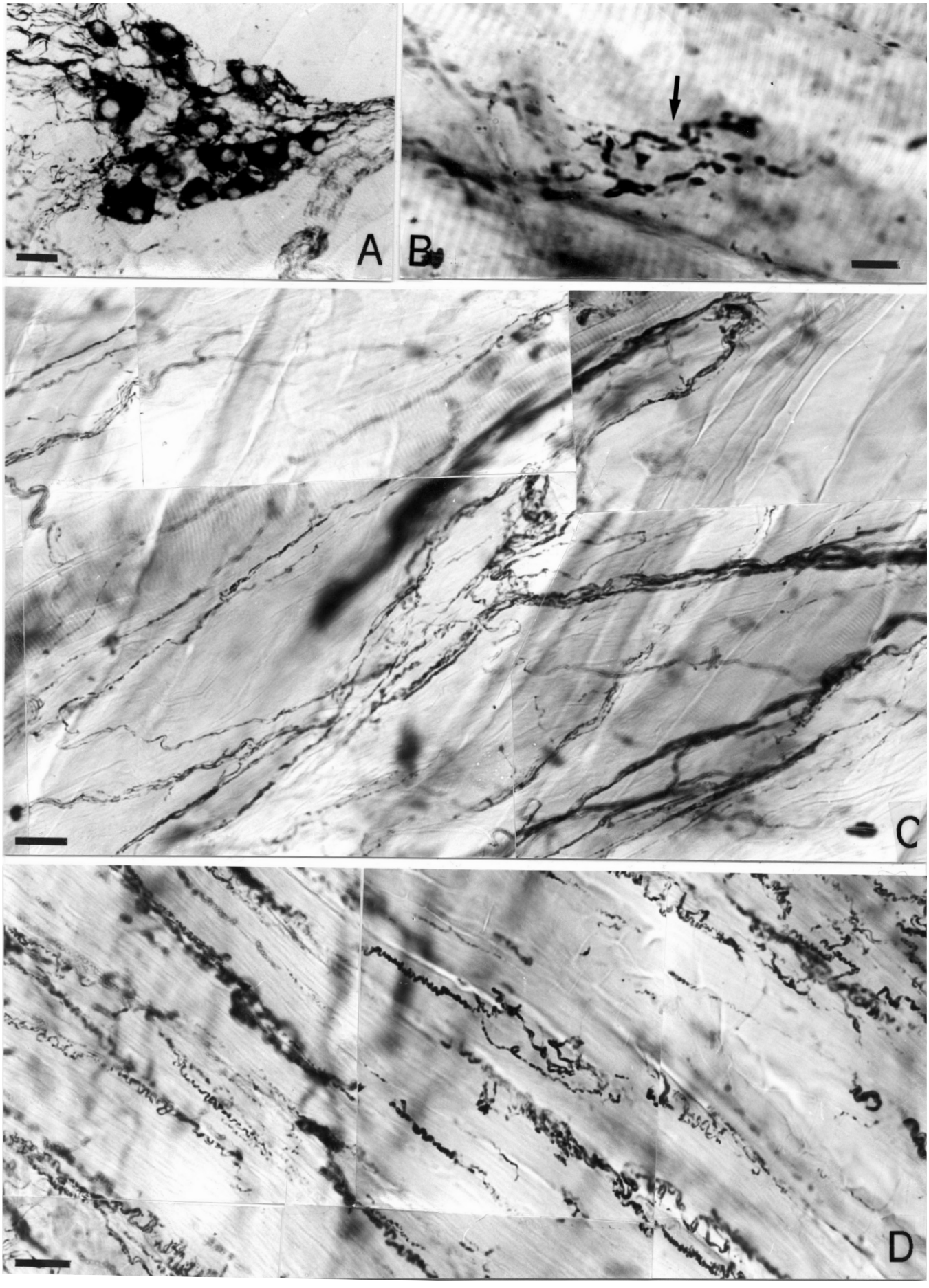
Recently, the existence of a Ca^{2+}-dependent mitochondrial NOS isoform (mtNOS) has been reported (Ghafourifart et al., 1999). This new isoform may be involved in apoptosis regulation. Ca^{2+} uptake by the mitochondria would trigger mtNOS activity and cause the release of cytochrome c and a parallel increase in lipid peroxidation. This has been observed in isolated mitochondria, being dependent on the Bcl-2 status (Ghafourifart et al., 1999).

Mitochondrial function is very sensitive to the presence of NO (Clementi et al., 1998), which is a potent inducer of mitochondrial permeability transition (MPT, Richter et al., 1994; Schweizer and Richter, 1994). The MPT reflects the formation of a proteinaceous pore in the mitochondrial membrane that allows free diffusion of all molecules smaller than 1.5 kDa. NO triggers a rapid modification of cytochrome c structure, probably via tyrosine nitration, which facilitates its release from the mitochondria to the cytosol (Cassina et al., 2000).

Finally, peroxynitrite ($ONOO^-$), the product of the reaction between superoxide (O_2^-) and NO, inhibits mitochondrial respiration and stimulates apoptosis. Cytochrome c is an important potential target of $ONOO^-$ during conditions involving accelerated generation rates of oxygen radicals and NO (Cassina et al., 2000). MPT induction might be inhibited by administration of specific drugs including bongkrekic acid, cyclosporine A (CsA), and non-immunosuppressive CsA derivatives (Hortelano et al., 1999b; Kristal and Dubinsky, 1997), thus providing interesting tools to study this phenomenon. Several studies have shown that ischemia or severe hypoxia result in release of cytochrome c from mitochondria. Thus, the mitochondrial swelling associated with ischemia or glutamate excitotoxicity (Mattson et al., 1993; Nakatsuka et al., 1999) could be explainable by induction of the MPT.

In a study of ischemia-reperfusion in rats, we studied the spatial relations between cytochrome c and nitrated proteins by immunohistochemistry (Alonso et al., 2002). Numerous cells containing nitrotyrosine immunoreactivity were found through all cortical layers of the different cortical regions studied in untreated animals. The reaction product was mainly found in the nuclear and perinuclear areas with a granular pattern and very seldom through the cytoplasm and the initial proximal portions of the main processes. Nucleoli were always unreactive. On the other hand, immunoreactivity to cytochrome c was always found in the cytoplasm and in the initial regions of proximal processes presenting a large granular pattern, redolent of mitochondrial structures. Nuclei were always negative for cytochrome c. Confocal microscopy confirmed the separation of these substances in different cell compartments. The nitrotyrosine reaction product was confined to the nuclei with unreactive nucleoli, whereas cytochrome c immunoreactivity was found in the cytoplasm. In untreated animals, electron microscopic observations showed that the reaction product for nitrotyrosine was restricted to the nuclear area. Occasionally, small amounts of reaction product for nitrotyrosine were found in the perikaryon in relation with cisternae of endoplasmic reticulum and membranes of the Golgi complex. No immunoreactivity was found in the mitochondrial intermembrane space. The reaction product for cytochrome c was always found in the mitochondrial membranes but absent from the intermembrane space.

After global ischemia and 4 h of reperfusion, the pattern of immunoreactivity distribution for nitrated proteins was modified. Immunoreactivity in the nuclear area decreased in intensity, whereas the reaction product in the perikaryon and initial portion of the processes was highly enhanced. At the same time, the immunoreactivity to cytochrome c was found in the cytoplasm and in all processes and collaterals, showing the reaction product as large granular deposits. Confocal microscopic observations demonstrated that the nitrotyrosine reaction product decreased in the nuclear area and increased in the cytoplasm and neuronal processes. In these locations it colocalized with cytochrome c immunoreactivity. Electron microscopy further confirmed that the reaction product for nitrotyrosine was increased in the perikaryon, specifically surrounding the mitochondria, and even in some regions of the mitochondrial intermembrane space. The reaction product for cytochrome c was increased as well, showing an intense reactivity between the outer and the inner mitochondrial membranes. Some immunoreactivity for cytochrome c was found in the cytoplasmic area in the vicinity of the mitochondrial membrane.


A
B
C
D

After 6 h of reperfusion, the pattern change observed at 4 h was more dramatic. Nitrotyrosine immunoreactivity was absent from the nuclei and concentrated in the cytoplasm, colocalizing with cytochrome c.

In animals treated with CsA, even after 6 hours of reperfusion, the pattern of immunoreactivities for both nitrotyrosine and cytochrome c were similar to the untreated control animals, indicating that the whole phenomenon was dependent on MPT function, and suggesting a protective role for CsA against neuronal cell death.

NO and peripheral nervous system

The role of NO in the gastrointestinal tract has been the subject of several studies focusing on individual organs such as the stomach (Barrachina et al., 1995), ileum (Costa et al., 1992), colon (McConalogue and Furness, 1993) and esophagus (Rodrigo et al., 1998, Cellek et al., 1999). In the esophagus, the presence of nitrergic nerve fibers has been studied in the human lower esophageal sphincter (McKirdy et al., 1992, Mearin et al., 1993, Singaram et al., 1995), where NO is a putative nonadrenergic-noncholinergic (NANC) inhibitory neurotransmitter (Mc Kirdy et al., 1992). In the opossum, NO mediates inhibitory potentials in the esophageal circular smooth muscle (Cayabyab and Daniel, 1995; Murray et al., 1995). NO is also involved, at least in part, in the relaxation of rat (Postorino et al., 1995), mouse (Yano et al., 1995), porcine (Sttebing et al., 1995), and human (McKirdy et al., 1992) gastrointestinal tract smooth muscle. Rodrigo et al. (1998) described the distribution of nitrergic neurons and processes in the esophagus of the cat and monkey using a specific antibody against purified rat brain NOS (Fig. 5). Immunoreactive nerve fibers were found pervading the myentric plexus (Fig. 5A) and the plexus of the muscularis mucosae. In the lower esophagus, a few immunoreactive fibres entered the epithelium as free nerve endings, some of which derived from the perivascular nerve plexus. In the upper esophagus, immunoreactive motor end-plates were found making contact with the striated muscle cells (Fig. 5B). Each rosette originated from a nerve fiber of smooth outline which gave off many collaterals in its distal portion, each ending in a particular motor end-plate. Although these motor end-plates were relatively scarce in the cat, they were more numerous than in the monkey esophagus. Their small number may correlate with the small number of nNOS-immunoreactive neurons in the nucleus ambiguous (Rodrigo et al., 1994), which is the source of the nerve fibers that give rise to the motor end-plates in the esophagus (Bieger and Hopkins, 1987; Lawn, 1964). The work of Bieger and Hopkins (1987) establishes, by retrograde and anterograde fluorescent tracer methodologies that the dorsal division of the nucleus ambiguous encompasses a compact rostral esophagomotor formation responsible for innervating the musculature of the upper alimentary tract. These findings were confirmed by Lee et al. (1992) and Collman et al. (1993). The motor nature of the end-plates has been demonstrated by different experimental procedures, including enucleation of the sensory neurons of the nodose ganglia, which did not cause degeneration of the esophageal end-plates (Rodrigo et al., 1985a), treatment with capsaicin, which produced irreversible degeneration of primary sensory neurons but did not affect the end-plates (Rodrigo et al., 1985a,b), and a combination of immunocytochemical techniques and fluorescent retrograde tracing (Lee et al., 1992). The role of nNOS immunoreactivity in these motor end-plates is yet unknown, but its presence points to a putative involvement of NO in some aspects of the end-plate function and hence in the control of esophageal striated muscle activity.

Between 30 and 45 % of neural cell bodies found in the intramural ganglia and along the course of nerve fiber bundles were immunoreactive for nNOS and were of the three morphological types previously described (Fig. 5C,D). In the intramural ganglia, immunoreactive nerve fibers formed a plexus in which varicose nerve terminals were in close relation with immunoreactive and non-immunoreactive neurons (Fig. 5A). The intramural blood vessels that crossed the different layers of the esophageal wall were surrounded by a paravascular and perivascular plexus containing nNOS immunoreactive nerve fibers (Fig. 6). These anatomical findings suggest that NO is involved in neural communication and in the control of peristalsis and vascular tone in the esophagus.

The function of NO in the small number of immunoreactive intraepithelial free nerve endings is also unknown. These only represent a small proportion of the intraepithelial nerve fibers identified in our previous studies of cat and monkey esophagus using other procedures (Robles-Chillida et al., 1981; Rodrigo et al., 1975, 1980, 1984, 1985a), and are also scarce in comparison with intrepithelial nerve fibers in other proximal areas of the alimentary tract, such as the palate (Rodrigo et al., 1985b), tongue, epiglottis, pharynx (Terenghi et al., 1986), and lung (Cadieux et al., 1986). The presence of nNOS in these fibers suggests a smooth muscle inhibitory function for these fibers earlier in their course, so that they represent "sensory-motor nerves" (Burnstock, 1990), providing the possibility of vasodilatation or smooth muscle relaxation in direct response to sensory stimulation of their intraepithelial

Fig. 5. nNOS immunoreactivity in neural structures of the cat esophagus. **A.** Immunoreactive ganglion in the myenteric plexus. **B.** nNOS-positive motor-plate on the surface of a striated muscle fiber. **C.** Nerve fibers of the myenteric plexus in the upper third of the esophagus. **D.** Nitrergic plexus in the lower third of the cat esophagus. Bar: A. C. D. 20 μm; B. 10 μm.

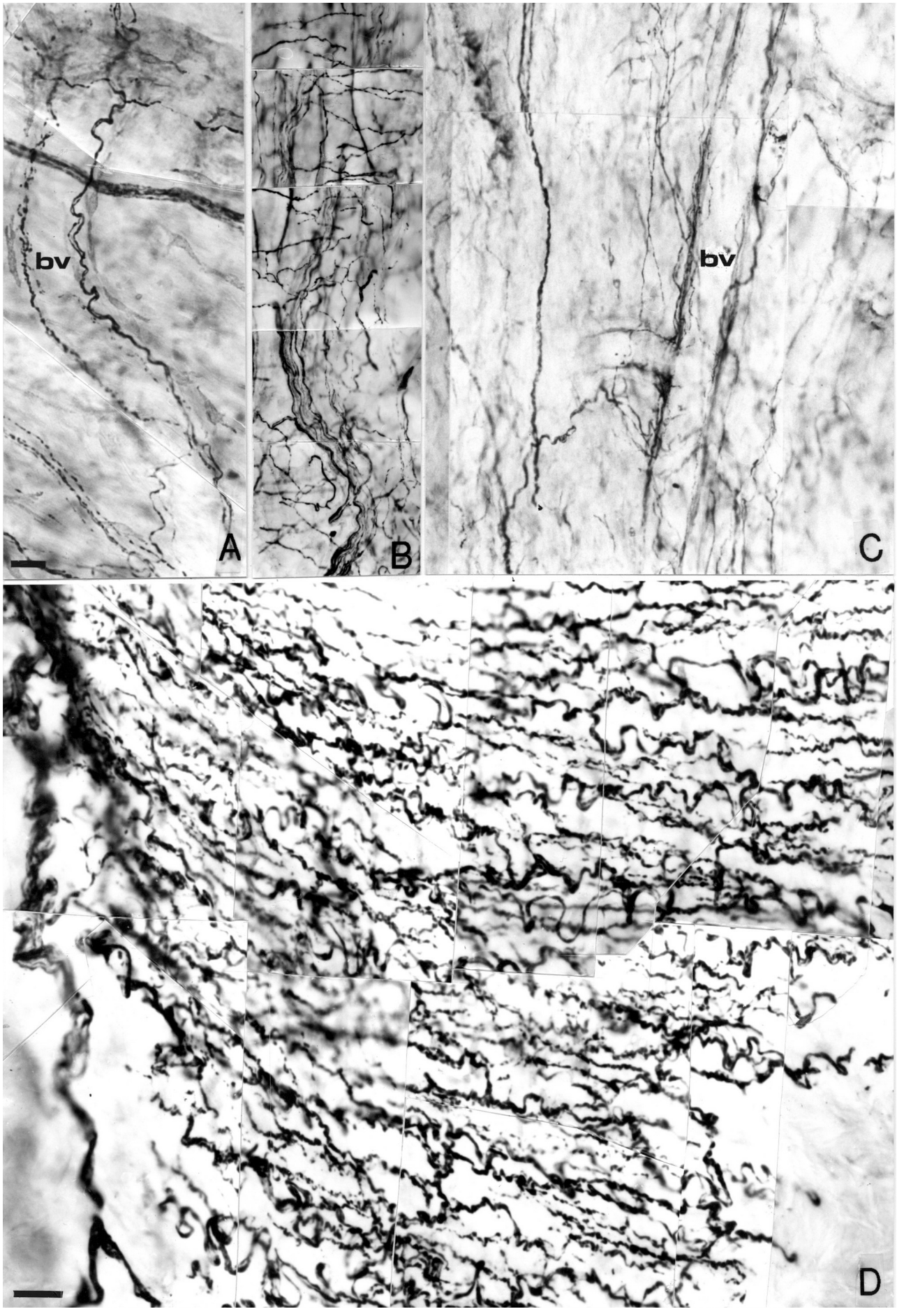

bv
A
B
bv
C
D

Fig. 6. Nitrergic innervation of peripheral blood vessels. Immunoreactive fibers in close relation to the wall of small vessels in the esophagus **(A)** and penis **(B)** of the cat. **C.** Dense nitrergic plexus in relation with a larger vessel in the cat esophagus. **D.** Numerous immunoreactive fibers in the wall of the dorsal artery of the penis. Bar: 20 μm.

terminations. Some of these intraepithelial nerve fibers were seen to originate as collaterals from the perivascular immunoreactive nerve fibers of blood vessels in the epithelial papillae.

References

Akert K., Pfenninger K. and Sandri C. (1967a). Crest synapses with subjunctional bodies in the subfornical organ. Brain Res. 5, 118-121.

Akert K., Pfenninger K. and Sandri C. (1967b). The fine structure in the subfornical organ of the cat. Z. Zellforsch. 81, 537-556.

Alexander M.P. and Freedman M. (1984). Amnesia after anterior communicating artery aneurysmrupture. Neurology 34, 52-757.

Alam A.M. and Starr MS. (1994). Effects of dopamine D3 receptor agonists on pilocarpine-induced limbic seizures in the rat. Neuroscience 60, 1039-1047.

Alonso D., Serrano J., Rodriguez I., Ruiz-Caballo J., Fernández A.P., Encinas J.M., Castro-Blanco S., Bentura M.L., Santacana M., Richart A., Fernández-Vizarra P., Uttenhal L.O. and Rodrigo J. (2002). Effects of oxygen and glucose deprivation on the expression and distribution of neuronal and inducible nitric oxide synthase and on protein nitration in rat cerebral cortex. J. Comp. Neurol. 443, 183-200.

Amenta F., Bograni S., Cadel S., Ferrante F., Valsecchi B. and Vega J.A. (1994). Microanatomical changes in the frontal cortex of aged rats: effect of L-deprenyl treatment. Brain Res. Bull. 34, 125-131.

Andine P., Sandberg M., Bagenholm R., Lehmann A. and Hagberg H. (1991). Intra- and extracellular changes of amino acids in the cerebral cortex of the neonatal rat during hypoxic-ischemia. Brain Res. Dev. Brain Res. 64, 115-120.

Andres K.H. (1965). Der Feinbaub des Subfornikalorganes von Hund. Z. Zerforsch. 68, 445-473.

Andrew J. and Taylor J. (1958). Effects of anisotonicity in the cisterna magna and 4th ventricle of cats. Am. J. Physiol. 193, 207-212.

Angevine J.B. and Sidman S.L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in mouse. Nature 192, 766-768.

Arregui A., Cabrera J., Leon-Velarde F., Paredes S. and Viscarra D. (1991). High prevalence of migraine in a high-altitude population. Neurology 41, 1668-1670.

Azambuja N.A.C.V., Achaval M., Morrone A.C.H. and Severino A.G. (1988). The human subfornical organ: an anatomical approach. Folia Morphol. 36, 6-13.

Barnes K.L., Ferrario C.M., Chernicky C.L. and Brosnihan K.B. (1984). Participation of the area postrema in cardiovascular control in the dog. Fed. Proc. 43, 2959-2962.

Barrachina D., Calatayud S., Espluges J., Whittle BRJ., Moncada S. and Esplujes J.V. (1995). Nitric oxide donors preferentially inhibit neuronally mediated rat gastric acid secretion. Eur. J. Pharmacol. 262, 181-183.

Bayer S.A, Altman J., Russo R.J., Dai X. and Simmons J.A. (1991a). Cell migration in the rat embryonic cortex. J. Comp. Neurol. 307, 499-516.

Bayer S.A., Altman J., Dai X.F and Humphreys L. (1991b). Planar differences in nuclear area and orientation in the subventricular and intermediate zones of the rat embryonic neocortex. J. Comp. Neurol. 307, 487-498.

Bayer S. (1985). Neurogenesis of the magnocellular basal telencephalic nuclei in the rat. Int. J. Dev. Neurosci. 5, 229-243.

Bayer S.A. and Altman J. (1991). Neocortical development. Raven Press, New York.

Beckman J S. (1990). Ischemic injury mediator. Nature 345, 27-28.

Beckman J.S. (1991). The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J. Dev.. Physiol. 15, 53-59.

Beckman J.S. (1996). Oxidative damage and tyrosine nitration from peroxynitrite. Chem. Res. Toxicol. 8, 836-844.

Beckman J S. and Koppenol W.H. (1996). Nitric oxide, superoxide and peroxynitrite: the good, the bad, and the ugly. Am. J. Physiol. 271, C1424-C1437.

Beckman J.S., Ischiropoulos H., Zhu L., Van der Woerd M., Smith C., Chen J., Harrison J., Martin J.C. and Tsai M. (1991). Kinetics of superoxide dismutase and iron-catalyzed nitration of phenolics by peroxynitrite. Arch. Biochem. Biophys. 298, 438-445.

Beckman J.S., Ye Z.Y., Anderson P.G., Chen J., Accaveti M.M., Tarpey C.R. and White J. (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunocytochemistry. Biol. Chem. 375, 81-88.

Bidmon H.J., Wu J., Buchkremer-Ratzmann I., Mayer B., Witte O.W. and Zilles K. (1998). Transient changes in the presence of nitric oxide synthases and nitrotyrosine immunoreactivity after focal cortical lesions. Neuroscience 82, 377-395.

Bieger D. and Hopkins D.A. (1987). Viscerotopic representation of the upper alimentary tract in the medulla oblogata of the rat: the nucleus ambiguous. J. Comp. Neurol. 262, 546-562.

Borison HL. (1989). Area postrema chemoreceptor cicumventricular organ of the medulla oblongata. Progr Neurobiol. 32, 351-390.

Bouthenet M.-L., Souil E., Martres M.P., Sokoloff B. and Schwartz J.C. (1991). Localization ofdopamine D_3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D_2 receptor mRNA. Brain Res. 564, 203-219.

Bredt D.S. and Snyder S.H. (1990). Isolation of nitric oxide synthase, a calmodulin requiring enzyme. Proc. Natl. Acad. Sci. USA 87, 682-685.

Bredt D.S. and Snyder S.H. (1992). Nitric oxide: a novel neuronal messenger. Neuron 8, 3-11.

Bredt D.S. and Snider S.H. (1994). Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia and olfactory epithelium. Neuron 13, 301-313.

Bredt D.S., Hwang P.M. and Snyder S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347,768-770.

Bredt D.S., Hwang P.M., Glatt C.E., Lowenstein C., Reed R.R. and Snyder S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450. Nature 351, 714-718.

Brizzee K.R. and Ordy J.M. (1979). Age pigments, cell loss and hippocampal functions. Mech. Ageing dev. 9, 143-162.

Broadwell R.D and Brightman M.W. (1976). Entry of peroxidase into neurons of the central and peripheral nervous systems from

extracerebral and cerebral blood. J. Comp. Neurol. 166, 257-284.

Buisson A., Margaill I., Callebert J., Plotkine M. and Boulu R.G. (1993). Mechanisms involved in the neuroprotective activity of a nitric oxide synthase inhibitor during focal cerebral ischemia J. Neurochem. 61, 690-696.

Burnstock G. (1990). Co-tranmission. The fith Heymans memorial lecture. Arch. Int. Pharmacol. Ther. 304, 7-33.

Busconi L. and Michell T. (1993). Endothelial nitric oxide synthase: -terminal myristoilation determines subcellular localization. J. Biol. Chem. 269, 8410-8413.

Cadieux A., Springall DR., Mulderry PK., Rodrigo J., Ghatei MA., Terenghi G., Bloom SR. and Polak JM. (1986). Occurence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of caosaicin treatment and surgical denervation. Neuroscience 19, 605-627.

Cafe C., Torri C. and Marzatico F. (1993). Cellular and molecular events of ischemic brain damage. Funct. Neurol. 8, 121-133.

Calaresu F.R., Zhang J., Chitravanshi V.C. and McKitrick D.J. (1994). Cardiovascular and single unit responses elicited by stimulation of the islands of Calleja and by changes in arterial pressure. Brain Res. 655, 45-50.

Calleja C. (1893). La región olfatoria del cerebro. Madrid: N. Moya.

Carson R.P., Evans W.O., Shields J.L. and Hannon J.P. (1969). Symptomatology, pathophysiology, and treatment of acute mountain sickness. Fed. Proc. 28, 1085-1091.

Cassina A.M., Hodara R., Souza J.M., Thomson L., Castro L., Ischiropoulos H., Freeman B.A. and Radi R. (2000). Cytochrome c nitration by peroxynitrite. J. Biol. Chem. 275, 21409-21415.

Cavaletti G., Moroni R., Garavaglia P. and Tredici G. (1987). Brain damage after high altitude without oxygen. Lancet 1, 101.

Cavaletti G., Garavaglia P., Arrigoni G and Tredici G. (1990). Persistent memory impairment after high altitude climbing. Int. J. Sports Med. 11, 176-178

Cavaletti G. and Tredici G. (1992). Effects of exposure to low oxygen pressure on the central nervous system. Sports Med. 13, 1-7.

Cayabyab F.S. and Daniel E.E. (1995). K^+ chanel opening mediates hyperpolarization by nitric oxide donors and IJPs in oposum esophagus. Am. J. Physiol. 268, G831-G842.

Cellek S., Rodrigo J., Lobos E., Fernández A.P., Serrano J. and Moncada S. (1999). Selective nitrergic neurodegeneration in diabetes mellitus–a nitric oxide- dependent phenomenon. Brit. J. Pharmacol. 128, 1804-1812.

Clementi E., Bronw G.C., Feelisch M. and Moncada S. (1998). Persistent inhibition of cell respiration by nitric oxide crucial brole of S-nitrosilation of mitochondrial complex I and protective action of glutatione. Proc. Natl. Acad. Sci. USA 95, 7631-7636.

Collman P.J., Tremblay L. and Diamant N.E. (1993). The central vagal efferent supply to the esophagus and lower esophageal sfinter of the cat. Gastroenterology 194, 1430-1438.

Costa M., Furness J.B., Pompolo S., Brookes S.J.H., Borndtein J.C., Bredt D.S. and Snyder S.H. (1992). Projection and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea.pig small intestine. Neurosci. Lett. 148, 121-125.

Charles I.G., Palmer R.M.J., Hickery M.S., Bayliss M.T., Chubb A.P., Hall V.S., Moss D.W. and Moncada S. (1993). Cloning, characterization and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. Proc. Natl. Acad. Sci. USA 90, 11419-11423.

Choi D.W. and Rothman S.M. (1990).The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu. Rev. Neurosci. 13, 171-182.

Damasio AR., Graff-Radford NF., Eslinger PJ., Damasio H. and Kassell N. (1985). Amnesia following basal forebrain lesions. Arch. Neurol. 42, 263-271.

Darley-Usmar V., Wiseman H. and Halliwell B. (1995). Nitric oxide and oxygen radicals: a question of balance. FEBS Lett. 369, 131-135.

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

Dawson V.I. and Dawson T.M. (1996). Nitric oxide neurotoxicity. J. Chem. Neuroanat. 10, 179-190.

Dawson T.M., Dawson V.I. and Snyder S.H. (1992). A novel neuronal messenger molecule in brain: the radical nitric oxide. Ann. Neurol. 32, 297-311.

De Alba J., Cardenas A., Moro M.A., Leza J.C., Lorenzo P. and Lizasoain I. (1999). Use of brain slices in the study of pathogenic role of inducible nitric oxide synthase in cerebral ischemia-reperfusion. Gen. Pharmacol. 32, 577-581.

Denison D.N., Ledwith F. and Poulton E.C. (1986). Complex reaction times at simulated cabin altitudes of 5000 feet and 8000 feet. Aerospace Med. 37, 1010-1013.

Dellmann HD. and Simpson JB. (1979). The subfornical organ. Int. Rew. Cytol. 58, 333-421.

Dempsey E.W. (1973). Neural and vascular ultrastructure of the area postrema in the rat. J. Comp. Neurol. 150, 177-200.

Dermietzel R. and Leibstein A.G. (1978). The microvascular pattern and perivascular linings of the area postrema. A combined freeze-etching and ultrathin section study. Cell Tiss. Res. 186, 97-110.

Edwards G.L. and Ritter R.C. (1981). Ablation of the area postrema causes exaggerated consumption of preferred foods in the rat. Brain Res. 216, 265-276.

Egberongbe Y.I., Gentleman S.M., Falkai P., Bogerts B., Polak J.M. and Roberts G.W. (1994). The distribution of nitric oxide synthase immunoreactivity in the human brain. Neuroscience 59, 561-578.

Elphick NA., Riveros-Moreno V., Moncada S. and O'Shea M. (1993). Identification of nitrergic neurons in invertebrates. Endothelium, 1 (Suppl), 57S, abstract 223.

Ernsting J. (1978). The 10th Ann. Harry G. Armstrong Lecture: Prevention of hypoxia - acceptable compromises. Aviat. Space Environ. Med. 49, 495-502

Fallon J.H. (1983). The islands of Calleja complex of rat basal forebrain. II. Connections of the medium and large size cells. Brain Res. Bull. 10, 775-793.

Fallon J.H., Riley J.N., Sipe J.C. and Moore R.Y. (1978). The islands of Calleja: organization and connections. J. Comp. Neurol. 181, 375-396.

Fallon J.H., Loughlin S.E. and Ribak C.E. (1983). The islands of Calleja complex of rat basal forebrain III. Histochemical evidence for a striatopallidal system. J. Comp. Neurol. 218, 91-120.

Fang M., Jaffrey S.R., Sava A., Ye K., Luo X. and Snyder S.H. (2001). Dexras 1: AG protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron 28, 183-193.

Faraci F.M. (1990). Role of nitric oxide in regulation of basilar artery tone in vivo. Am. J. Physiol. 259:H1216-H1221.

Faraci F.M. (1991). Role of endothelium-derived relaxing factor in cerebral circulation: large arteries and microcirculation. Am. J. Physiol. 261, H1038.-H1042.

Faraci F.M. and Breeze K.B. (1993). Nitric oxide mediates vasodilatation in response to activation of N-methyl-D-aspartate receptors in brain. Cir. Res. 72, 476-480.

Faraci F.M. and Brian J.E. (1994). Nitric oxide and cerebral circulation. Stroke 25, 692-703.

Ferreiro D.M., Arcaro L.J., Sagar S.M., McIntosh T.K. and Simon R.P. (1988). Selective sparing of NADPH-diaphorase neurons in neonatal hypoxia-ischemia. Ann. Neurol. 24, 670-676.

Figarola T.R. and Billings C.E. (1966). Effect of meprobamate and hypoxia on psychomotor performance. Aerospace Med. 37, 951-954.

Fine B.J. and Kobrick J.L. (1978). Effect of altitude and heat on complex cognitive tasks. Human Factors 20, 115-122.

Flood D.G. and Coleman P.D. (1988). Neurons number and sizes in aging brain; Comparison of human, monkey and rodent data. Neurobiol Aging 9, 453-463.

Flores G.D., Barbeau R.., Quirion D. and Srivastava L.K. (1996). Decreased binding of dopamine D_3 receptors in limbic subregions after neonatal bilateral lesion of rat hippocampus. J. Neurosci. 16, 2020-2026.

Forman L.J., Liu P., Nagele R.G., Yin K. and Wong P.Y. (1998). Augmentation of nitric oxide, superoxide, and peroxynitrite production during cerebral ischemia and reperfusion in the rat. Neurochem. Res. 23, 141-148.

Forster P.J.G. (1985). Effect of different ascent profiles on performance at 4,200 m elevation. Aviat. Space Environ. Med. 56, 758-764.

Forster H.V., Soto R.J., Dempsey J.A. and Hosko M.J. (1975). Effect of sojourn at 4,300 m altitude on electroencephalogram and visual evoked response. J. Appl. Physiol. 39, 109-113.

Forster C., Clark H.B., Ross M.E. and Iadecola C. (1999). Inducible nitric oxide synthase expression in human cerebral infarcts. Acta Neurol. Pathol. 97, 215-220.

Forstermann U., Gorsky L.D., Pollock J.S., Schmidt H.H.H.W., Heller M. and Murad F. (1990). Regional distribution of EDRF/NO-synthesizing enzyme(s) in rat brain. Biochem. Biophys. Res. Commun. 168, 727-732.

Forstermann U., Schmidt H.H.H.W., Pollock J.S., Sheng H., Mitchell J.A., Warner T.D., Nakane M. and Murad F. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. Biochem. Pharmacol., 42, 1849-1857.

Furchgott R.S. and Zawadki J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373-376.

Fuxe K., Hökfelt T., Agnati LF., Johansson O., Goldstein M., Pérez de la Mora M., Possani L., Tapia R., Teran L. and Palacios M. (1978). Mapping of central catecholamine neurons: Immunocytochemical studies on catecholamine synthesizing enzymes. In: Psychopharmacology: a generation of progress. Lipton M.A., Di Mascio A. and Killan K.F. (eds). New York: Raven Press, pp. 353-371.

Fuxe K., Anderson K., Schwarz R., Agnati L.F., Pérez de la Mora M., Hökfelt T., Goldstein M., Ferland M., Possani L. and Tapia R. (1979). Studies on different types of dopamine nerve terminals in the forebrain and their possible interactions with hormones and with neurons containing GABA, glutamate and opioid peptides. In: Advances in neurobiology. L.J. Poirier, T.L. Sourkes and P.J. Bedard (eds): Vol. 24. New York: Raven Press, pp. 199-215.

Gally J.A., Montague P.R., Reeke G.N. and Edelman G.M. (1990). The NO hydrophotesis. Possible effect of a shortlived, rapidly diffusible signal in the development and function of the nervous system. Proc. Natl. Acad. Sci. USA 87, 3547-3551.

Garthwaite J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. Trends. Neurosci. 14, 60-67.

Garthwaite J., Charles S.L. and Chess-Williams R. (1988). Endothelial-derived relaxing factor release on activation of NMDA receptors suggests role as intracellular messenger in the brain. Nature 336, 385-388.

Gatti P.J., Dias Souza J., Taveira Da Silva A.M., Quest J.A. and Gillis R.A. (1985). Chemical stimulation of the area postrema induces cardiorespiratory changes in the cat. Brain Res. 346, 115-123.

Geller D.A., Nussler A.K., Di Silvio M., Lowenstein C.J., Shapiro R.A., Wang S.C., Simmons R.L. and Billiar T.R. (1993). Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc. Natl. Acad. Sci. USA. 90, 522-526.

Gelperin A. (1994). Nitric oxide mediates networ oscilations of olfactori interneurons in a terrestrial molusc. Nature 369, 61-63.

Ghafourifar P., Schenk U., Klein S.D. and Richter Ch. (1999). Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated mitochondria. Am. Soc. Biochem. Mol. Biol. 274, 31185-31188.

Ghribi O., Callebert J., Plotkine M. and Boulu R.G. (1994). L-NAME modulates glutamate accumulation induced by K(+)-depolarization but not by forebrain ischaemia in the rat striatum. Neurosci. Lett. 17, 34-38

Giraldelo C.M., Zappellini A., Muscara M.N., De Luca I.M., Hyslop S., Cirino G., Zatz R., De Nucci G. and Antunes E. (1994). Effect of arginine analogues on rat hind paw oedema and mast cell activation in vitro. Eur. J. Pharmacol. 257, 87-93.

Giuili G., Luzi A., Poyard M. and Guellaen G. (1994). Expression of mouse brain soluble guanylyl cyclase and NO synthase during ontogeny Dev. Brain Res. 81, 269-283.

Goldman J.E., Calingasan N.Y and Gibson G.E. (1994). Aging and the brain. Curr. Opin. Neurol. 7, 287-293.

Goldstein J.C., Waterhouse N.J., Joit Ph., Evan G.I. and Green D.R. (2000). The coordinate release of cytochrom c during apoptosis is rapid, complete and kinetically invariant. Nature Cell Biol. 2, 156-162.

Griffith O.W. and Stuerh D.J. (1995). Nitric oxide synthases:properties and catalytic mechanisms. Annu. Rev. Physiol. 57, 707-736.

Gross B.B., Jaffi E.A., Levi R. and Kilbourn R.G. (1991). Cytokine activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin dependent, calmodulin-independent and inhibited by arginine analogs with a range-order of potency characteristic of activated macrophages. Biochem. Biophys. Res. Commun. 170, 823.829.

Hackett P.H. and Rennie D. (1978). The incidence, importance, and prophylaxis of acute mountain sickness. Lancet 2, 1149-1155.

Haber S.N., Groenewegen H.J., Grove E.A. and Nauta W.J.H. (1985). Efferent connections of the ventral pallidum: Evidence of a dual striato-pallidofugal pathway. J. Comp. Neurol. 235, 322-335.

Haldane J.S. and Priestley J.G. (1935). Respiration. 2nd ed. New Haven, Yale University Press. Hansen, JE., Harris C.W. and Evans W.G. (1967) Influence of elevation of origin, rate of ascent and a physical conditioning program on symptoms of acute mountain sickness. Mil. Med. 132, 585-582.

Hamada Y., Hayakawa T., Hattori H. and Mikawa H. (1994). Inhibitor of nitric oxide synthesis reduces hypoxic-ischemic brain damage in the

neonatal rat. Pediatr. Res. 35, 10-14.

Hansen J.E., Harris C.W. and Evans W.O. (1967). Influence of elevation of origin, rate of ascent and a physical conditioning program on symptoms of acute mountain sickness. Mil. Med. 132, 585-592

Hecker M., Mülsch A. and Busse R. (1994). Subcellular localization and characterization of neuronal nitric oxide synthase. J. Neurochem. 62,1524 -1529.

Heimer L. (1978). The olfactory cortex and the ventral striatum. In: Limbic mechanisms: the continuing evolution of the limbic system concept. Livingston K.E. and Hornykiewicz O. (eds). New York. Plenum. Press. New York pp 95-187.

Heimer L. and Wilson R..D. (1975). The subcortical projections of the allocortex: similarities in the neural associations of the hippocampus, the piriform cortex and the neocortex. In: Golgi centennial symposium. Santini M. (ed). New York. Raven Press, pp 177-193.

Hendricks W. (1995). Neuronal nitric oxide synthase contains a discs-large homologous region (DHR) sequence motif. Biochem. J. 305, 687-688.

Hernesniemi J., Kawana E., Bruppacher H. and Sandri C. (1972). Afferent connections of the subfornical organ and of the supraoptic crest. Acta Anat. 81, 321-336.

Hevel J.M., White K.A and Marletta M.A. (1991). Purification of inducible murine macrophage nitric oxide synthase. J. Biol. Chem. 266, 22789-22791.

Hibbs JB. Jr., Taintor R.R. and Vavrin Z. (1987). Macrophage cytotoxicity : role for L-arginine deiminase and amino nitrogen oxidation to nitrite. Science 235, 473-476.

Hibbs J.B. Jr., Taintor RR., Vavrin Z. and Rachlin E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. 157, 87-94.

Hicks S.P. and D'Amato C.J. (1968). Cell migration in the isocortex in the rat. Anat. Rec. 160, 619-634.

Higuchi Y, Hattori H, Hattori R. and Furusho K. (1996). Increased neurons containing neuronal nitric oxide synthase in the brain of a hypoxic-ischemic neonatal rat model. Brain Dev. 18, 369-375.

Higuchi Y., Hattori H., Kune T., Masahiro T., Akaike A. and Furusko K. (1998). Increase in nitric oxide in the hypoxia ischemia neonatal rat brain and supression by 7-nitroindazole and aminoguanidine. European. J. Pharm. 342, 47-49.

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

Hortelano S, Alvarez AM. and Bosca L. (1999a). Nitric oxide induces tyrosine nitration and release of cytochrome c preceding an increase of mitochondrial transmembrane potential in macrophages. FASEB J. 13, 2311-2317.

Hortelano S., Lopez-Collazo E. and Bosca L. (1999b). Protective effect of cyclosporin A and FK506 from nitric oxide-dependent apoptosis in activated macrophages. Br. J. Pharmacol. 126, 1139-1146.

Hosoya Y. (1973). Electron microscopic observations of the granule cells (Calleja's island) in the olfactory tubercle of rats. Brain Res. 54, 330-334.

Huang Z., Huang P.L., Ma J., Meng W., Ayata C., Fishman M.C.and Moskowitz M.A. (1996). Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J. Cereb. Blood Flow Metab. 16, 981-987.

Huang Z., Huang PL., Panahian N., Dalkara T., Fishman M.C. and Moskowitz M.A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265, 1883-1885.

Hultgren H.N. (1979). High altitude medical problems. West. J. Med. 131, 8-23.

Iadecola C. (1993). Regulation of the cerebral microcirculation during neural activity: is nitric oxide the missing link? Trends Neurosci. 16, 206-214.

Iadecola C. (1997). Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci. 20, 132-139.

Iadecola C. and Ross ME. (1997). Molecular pathology of cerebral ischemia: delayed gene expression and strategies for neuroprotection. Ann. NY Acad. Sci. 835, 203-217.

Iadecola C., Zhang F., Xu S., Casey R. and Ross M.E. (1995a). Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. J. Cerebral Blood Flow Metab. 15, 378-384.

Iadecola C., Xu X., Zhang F., Fakahany E.E. and Ross ME. (1995b). Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia. J. Cerebral Blood Flow Metab. 15, 52-59.

Ignacio M.P.D., Kimm E.J., Kajeyama G.H., Yu J. and Robertson R.T. (1995). Postnatal migration of neurons and formation of laminae in rat cerebral cortex. Anat. Embryol. 191, 89-100.

Iovino M. and Steardo L. (1984). Vasopressin release to central and peripheral angiotensin II in rats with lesions of the subfornical organ. Brain Res. 322, 365-368.

Ischitropoulos H., Zhu L., Chen J., Tsai M., Martin J.C., Smith C.D. and Beckman J.S. (1992). Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch. Biochem. Biophys. 298, 431-437.

Jaffrey S.R., Snowman A.M., Bliasson M.J.L., Cohem N.A. and Snyder S.H. (1998). CAPON AG protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. Neuron 20, 115-124.

Judas M., Sestan N. and Kostovic I. (1999). Nitrinergic neurons in the developing and adult human telencephalon: transient and permanent patterns of expression in comparison to other mammals. Microsc. Res. Tech. 45, 401-419.

Kahn M. and Furchgott R.F. (1987). Aditional evidence that endothelium-derived-relaxing factor is nitric oxide. In: Pharmacology. Rand M.L. and Raper J. (eds). Elsevier, Amsterdam. pp 341-344.

Kalsbeek A., Voorn P., Buijs R.M., Pool C.W. and Uylings H.B.M. (1988). Development of monoaminergic innervation in the prefrontal cortex of the rat J. Comp. Neurol. 269, 58-72.

Kamisaki Y., Wada K., Bian K., Balabanli B., Davis K., Martin E., Behbod F., Lee Y.C. and Murad F. (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. Proc. Natl. Acad. Sci. USA 95, 11584-11589.

Kassirer M.R. and Such R.V.P. (1989). Persistent high-altitude headache and aguesia without anosmia. Arch. Neurol. 46, 340-341.

Keilhoff G., Seidel B., Noack H., Tischmeyer W., Stanek D. and Wolf G. (1996). Patterns of nitric oxide synthase at the messenger RNA and protein levels during early rat brain development. Neuroscience 75, 1193-1201.

Kishimoto J., Spurr N., Liao L., Emson P. and Xu W. (1992). Localization of brain oxide synthase (NOS) to human chromosome 12. Genomics 14, 802-804.

Kizer J.Z., Palkovits M. and Brownstein M.J. (1974). Releasing factors in the circumventricular organs of the rat brain. Endocrinology 98, 311-317.

Klara P.M. and Brizzee K.R. (1975). The ultrastructural morphology of the squirrel monkey area postrema. Cell Tissue Res. 160, 315-326.

Koseki C., Imai M., Hirata Y., Yanagisawa M. and Masaki T. (1989). Autoradiographic distribution in the rat tissues of bindings sites for endothelin, a neuropeptide. Am. J. Physiol. 256, R858-R866.

Knowles R.G., Palacios M., Palmer R.M.J. and Moncada S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc. Natl. Acad. Sci. USA 86, 5159-5162.

Knowles R.G., Palacios M., Palmer R.M.J. and Moncada S. (1990). Kinetic characteristics of nitric oxide synthase from rat brain. Biochem. J., 269, 207-210.

Kristal S.B. and Dubinsky J.M. (1997). Mitochondrial permeability trasition in the central nervous system: induction by calcium cyclic-dependent and independent pathway. J. Neurochem. 69, 524-538.

Kroemer G. (1997). Nitric oxide induces apoptosis vis triggering mitochondrial permeability transition. FEBSS Lett. 419, 373-377.

Krukoff T.L., Morton T.L., Harris K.H. and Ihamandas J.H. (1992). Expression of c-fos protein in the rat brain elicited by electrical stimulation of the pontine parabrachial nucleus. J. Neurosci. 12, 3582-3590.

Lanca A.J. and Van der Kooy D. (1985). A serotonin-containing pathway from the area postrema to the parabrachial nucleus in the rat. Neuroscience 14, 1117-1126.

Landfield P.W., Rose G., Sandles L., Wohlstadter T.C. and Lynch G. (1977). Patterns of astroglial hypertrophy and neuronal degeneration in the hippocampus of aged, memory-deficient rats. J. Gerontol. 12, 3-12.

Lawn A.M. (1964). The localization by means of elstrical stimulation of the origins and path in the medulla oblongata of the motor nerve fibres of the rabbit esophagus. J. Physiol. 174, 232-244.

Lee B.H., Lynn R.B., Lee H.S., Miselois R.R. and Altschler S.M. (1992). Calcitoning gene related peptide in nucleus ambiguous motoneurons in rat: viscerotopic organization. J. Comp. Neurol. 320, 531-543.

Lei S.Z., Pan Z.H., Aggarwal S.K., Chen H.S., Hartman J., Sucher N.J. and Lipton S.A. (1992). Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. Neuron 8, 1087-1099.

Leonhard H. and Backhus-Roth A. (1969). Synapsenartige Kontakte zwischen intraventrikularen Axonendigungen und freien Oberflachen von Ependymzellen des Kaninchengehirns. Z. Zelforsch. 97, 369-376.

Leslie R.A. and Osborne N.N. (1984). Amines and other transmitter-like compounds in the bovine area postrema. Brain Res. Bull. 13, 357-362.

Lipton S.A., Chol Y.B., Pan Z.H., Lei S.Z., Chen H.S.V., Sucher N.J., Loscalzo J., Singel D.J. and Stamber J.S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364, 626-632.

Lind R.W., Van Hoesen G.W. and Johnson A.K. (1987). An HRP study of the connections of the subfornical organ of the rat. J. Comp. Neurol. 216, 265-277.

Lind R.W., Swanson L.W. and Ganten D. (1984). Angiotensin II immunoreactivity in the neuronal afferents and efferents of the subfornical organ of the rat. Brain Res. 321, 209-215.

Lo E.H., Hara H., Rogowska J., Trocha M., Pierce A.R., Huang P.L., Fishman M.C., Wolf G.L. and Moskowitz M.A. (1996). Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene expression. Stroke 27, 1381-5.

Loopuijt L.D. (1989). Distribution of dopamine D-2 receptors in the rat striatal complex and its comparison with acetylcholinesterase. Brain Res. Bull. 22, 805-817.

Lorez H.P. and Richards J.G. (1973). Distribution of indolealkylamine nerve terminals in the ventricles of the rat brain. Z. Zellforsch. 144, 511-522.

Lucchi M.L., Costerbosa G.L., Barazzoni A.M., Faccioli G., Petrosino G. and Bortolami R. (1989). The fine structure of the area postrema of the sheep. Arch. Ital. Biol. 127, 37-61.

Lyons C.R., Orloff G.J. and Cunningham J.M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. J. Biol. Chem. 267, 6370-6374.

Malhotra M.S. and Mathew L. (1974). Effect of prolonged stay at altitude (4000 m) on autonomic balance. Aerosp. Med. 45, 869-872.

Malinski T., Bailey F., Zhang Z.G. and Chopp M. (1993). Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J. Cerebral Blood Flow Metab.13, 355-358.

Mangiapane M.L., Thrasher T.N., Keil L.C., Simpson J.B. and Ganong W.F. (1984). Role of the subfornical organ in vasopressin release. Brain Res. Bull. 13, 43-47.

Manzoni O., Prezeau L., Marin P., Deshanger S., Bockaer J. and Pagy L. (1992). Nitric oxide induced blockade of NMDA receptors. Neuron 8, 375-397.

Marsden P.A., Heng H.H., Scherer S.W., Stewart R.J., Hall A.V., Shi X.M., Tsui L.C. and Schappert K.T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J. Biol. Chem. 268, 17478-17488.

Martínez A. (1995). Nitric oxide in invertebrates. Histochem. J. 27, 770-776.

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

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

Mattson A.P., Zhang Y. and Bose S. (1993). Growth factor prevent mitochondrial dysfunction, loss of calcium homeoestasis, and cell injury, but not ATP depletion in hippocampal neurons deprived of glucose. Exper. Neurol. 121, 844-847.

McCanalogue K. and Furness J.B. (1993). Projections of nitric oxide synthesising neurons in the guinea-pig colon. Cell Tissue Res. 271, 545-553.

McDonald T.M. and Johnston M.V. (1990). Physiological and pathophysiological roles of excitatory aminoacids during central nervous system development. Brain Res. Rev. 15, 41-70.

McFarland RA. (1971). Human factors in relation to the development of pressurized cabins. Aerosp. Med. 12, 1303- 1318.

McKirdy H.C., McKindy M.L., Levis M.J. and Marshall R.W. (1992). Evidence for involvement of nitric oxide in the non adrenergic non cholinergic (NANC) relaxation of human lower esophageal sphincter muscle strips. Exp. Physiol. 77, 509-511.

McKitrick D.J., Krukoff T.L. and Calaresu F.R. (1992). Expression of c-fos protein in the rat brain after electrical stimulation of the aortic depressor nerve. Brain Res. 599, 215-222.

Mearin F., Mourelle M., Guarner F., Salas A., Riveros-Moreno V., Moncada S. and Malagelada J.R. (1993). Patiens wich lack nitric oxide synthase in the gastro-esophageal junction. Eur. J. Clin.Invest.

23, 724-728.

Melillo G., Musso T., Sica A., Taylor L.S., Cox G.W. and Varesio L. (1995). A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J. Exp. Med. 182, 1683-1693.

Meyer W. (1994). NADPH diaphorase (nitric oxide synthase) in the central nervous system of spiders (Arachnida: Araneida). Neurosci. Lett. 165, 105-108.

Meyer G. and Wahle P. (1986). The olfactory tubercle in the cat. I. Morphological components. Exp. Brain Res. 62, 515-527.

Meyer G., González-Hernández T., Carrillo-Padilla F. and Ferres-Torres R. (1989). Aggregations of granule cells in the basal forebrain (islands of Calleja): Golgi and cytoarchitectonic study in different mammals, including man. J. Comp. Neurol. 284, 405-428.

Meyer G., González-Hernández T., Galindo-Mireles D., Carrillo-Parrilla F. and Ferres-Torres R. (1994). NADPH-d activity in the island of Calleja: a regulatory system of blood flow to the ventral striatum/pallidum? NeuroReport 5, 1281-1284.

Millhouse O.E. (1987). Granule cells of the olfactory tubercle and the question of the islands of Calleja. J. Comp. Neurol. 265, 1-24.

Minc-Golomb D., Tsarfaty I. and Schwartz J.P. (1994). Expression of inducible nitric oxide synthase by neurons following exposure to endotoxin and cytokine. Br. J. Pharmacol. 112, 720-722.

Minc-Golomb D., Yadid G., Tsarfaty I., Resau J.H. and Schwartz J.P. (1996). In vivo expression of inducible nitric oxide synthase in cerebellar neurons. J. Neurochem. 66, 1504-1509.

Mitchell JA., Forstermann U., Warner T.D., Pollock JS., Schmidt H.H.H.W., Heller M. and Murad F. (1991). Endothelial cells have a particular enzyme system responsible for EDRF formation: measurement by vascular relaxation. Biochem. Biophys. Res. Commun. 176, 1417-1423.

Moncada S., Palmer R.M.J. and Higss E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43, 109-142.

Moncada S., Higgs A.E. and Furschgot R. (1997). XIV International Union of pharmacology nomenclature in nitric oxide research. Am. Soc. Pharmacol. Exp. Ther. 49, 137-142.

Moore R.Y., Halaris A.E. and Jones B.E. (1978). Serotonin neurons of the midbrain raphe: ascending projections. J. Comp. Neurol. 180, 417-438.

Morilak D.A., Garlow S.J. and Ciaranello R.D. (1993). Immunocytochemical localization and description of neurons expressing serotonin2 receptors in the rat brain. Neuroscience 54, 701-717.

Morris S.M. Jr and Billiar T.R. (1994). New insights into the regulation of inducible nitric oxide synthesis. Am. J. Physiol. 266, E829-E839.

Moro M.A., De Alba J., Leza J.C., Lorenzo P., Fernández A.P., Bentura M.L., Boscá L., Rodrigo J. and Lizasoain I. (1998). Neuronal expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. Eur. J. Neurosci. 10, 445-456.

Mugnaini E. and Oertel W.H. (1985). An atlas of the distribution of GABAergic neurons and terminals in the rat CNS as revealed by GAD immunohistochemistry. In: Handbook of chemical neuroanatomy, GABA and neuropeptides. Part I. New York. Vol 4. Björklund A. and Hökfelt T. (eds). Elsevier Science Publishers, pp. 436-608.

Murray J., Du C., Ledlow A., Bates J.N. and Conklin J.L. (1995). Nitric oxide: mediator of nonadrenergic noncholinergic responses of aposum esophageal muscle. Am. J. Physiol. 261, G401-G406.

Myatt L., Rosenfield R.B., Eis A.L., Brockman D.E., Greer I. and Lyall F. (1996). Nitrotyrosine residues in placenta. Evidence of peroxynitrite formation and action. Hypertension 28, 488-493.

Nagafuji T., Suguyama M., Muti A., Makino T., Miyuachi T. and Nabata H. (1995). The neuroprotective effect of a potent selective inhibitor of type INOS (L-MIN) in a rat model of focal cerebral ischemia. Neuroreport 6, 1541-1545.

Nakane M., Schmidt H.H.H.W., Pollock JS., Förstermann U. and Murad F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett. 316, 175-180.

Nakatsuka H., Ohta S., Tanaka J., Toku K., Kumon Y., Maeda N., Sakanaka M. and Sakaki S. (1999). Release of cytochrome c from mitochondria to cytosol in gerbil hippocampal CA1 neurons after transient forebrain ischemia. Brain Res. 849, 216-219.

Noack W., Dumitrescu L. and Schweichel J.U. (1972). Scanning and electron microscopical investigations of the surface structures of the lateral ventricles in the cat. Brain Res. 46, 121-129.

Nowicki J.P., Duval D., Poignet H. and Scatton B. (1991). Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. Eur. J. Pharmacol. 204, 339-340.

Oelz O. and Regard M. (1988). Physiological and neurophysiological characteristics of world-class extreme-altitude climbers. Am. Alpine J. 12, 83-86.

Ogawa M. (1995). The reeler gene-asociated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron 14, 899-912.

Ossenkopp K.P. (1983). Taste aversions conditioned with gamma radiation: attenuation by area postrema lesions in rats. Behav. Brain Res. 7, 297-305.

Palkovits M., Brownstein M.J., Arimura A., Sato H., Schally A.V. and Kizer J.S. (1976). Somatostatin content of the hypothalamic ventromedial and arcuate nuclei and the circumventricular organs in the rat. Brain Res. 109, 430-434.

Panahian N., Yoshida T., Huang P.L., Hedley-Whyte E.T., Dalkara T., Fishman M.C. and Moskowitz, M.A. (1996). Attenuated hippocampal damage after global cerebral ischemia in mice mutant in neuronal nitric oxide synthase. Neuroscience 72, 343-354.

Pearson R.G. and Neal G.L. (1970). Operator performance as a function of drug, hypoxia, individual and task factors. Aerosp. Med. 41, 154-158.

Peinado M.A., Martínez M., Pedrosa J.A., Quesada A. and Peinado J.M. (1993). Quantitative morphological changes in neurons and glia in the frontal lobe of the aging rat. Anat. Rec., 237, 104-108.

Pérez de la Mora M., Possani L.D., Tapia R., Teran L., Palacios R., Fuxe K., Hökfelt T and Ljungdahl, A. (1981). Demonstration of central γ-aminobutyrate-containing nerve terminals by means of antibodies against glutamate decarboxylase. Neuroscience 6, 875-895.

Pfaff D., Gerlach J., McEwen B., Ferin M., Carmel P. and Zimmerman E.A. (1976). Autoradiographic localization of hormone-concentrating cells in the brain of the female rhesus monkey. J. Comp. Neurol. 17O, 279-293.

Pfaff D. and Keiner M. (1973). Atlas of estradiol-concentrating cells in the central nervous system of the female rat. J. Comp. Neurol. 151, 121-158.

Phillips S., Saugalang V. and Sterns G. (1987). Basal forebrain infarction. A clinicopathologic correlation. Arch. Neurol. 44, 1134-1138.

Phillips J.W., Smith-Barbour M., Perkins L.M. and O'Regan M.H. (1994). Characterization of glutamate, aspartate, and GABA release from ischemic rat cerebral cortex. Brain. Res. Bull. 34, 457-466

Pollock J.S. Forstermann U., Mitchell J.A., Warner T.D, Schmidt H.H.H.W. and Nakane M. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelium cells. Proc. Natl. Acad. Sci. USA 88, 10480-19485.

Pollock J.S., Nakane M., Buttery L.D.K., Martínez A., Springall D.R., Polak J.M., Förstermann U. and Murad F. (1993). Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. Am. J. Physiol. 265, C1379-C1387.

Postorino A., Serio R. and Mulè F. (1995). Nitric oxide is involved in non-adrenergic non-cholinergic inhibitory neurotranmision in rat duodenum. J. Auton. Pharmacol. 119, 1105-1111.

Pryor W.A. and Squadrito G.L. (1995). The chemistry of peroxynitrite, a product from the reaction of nitric oxide with superoxide. Am. J. Physiol. 268, L699-L722.

Radi R., Beckman J.S., Bush K.M. and Freeman B.A. (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. 288, 481-7.

Radi R., Denicola A. and Freeman B.A. (1999). Peroxynitrite reactions with carbon dioxide-bicarbonate.Methods. Enzymol. 301, 353-367.

Radomski M.W., Vallance P.J., Whitely G., Foxwell N. and Moncada S. (1993). Modulation of platelet adhesion to human vascular endothelium by the constitutive and cytokine-inducible nitric oxide synthases. Cardiovasc. Res. 27, 1380-1382.

Raichle M.E. (1983). The pathophysiology of brain ischemia. Ann Neurol 13, 2-10.

Regard M., Oelz O., Brugger P. and Landis T. (1989). Persistent cognitive impairment in climbers after repeated exposure to extreme altitude. Neurology 39, 210-213.

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

Rehncrona S. (1986). Molecular mechanisms for ischemic brain damage and aspects on protection. 36, 125-128.

Ribak C.E. and Fallon J.H. (1982). The island of Calleja complex of rat basal forebrain. I. Light and electron microscopic observations. J. Comp. Neurol. 205, 207-218.

Richards J.G., Lorez H.P. and Tranzer J.P. (1973). Indolealkylamine nerve terminals in cerebral ventricles: Identification by electron microscopy and fluorescence histochemistry. Brain Res. 57, 277-288.

Richter C., Gogvadze V., Schlapbach R., Schweizer M. and Schlegel J. (1994). Nitric oxide kills hepatocytes by mobilizing cellular calcium. Biochem. Biophys. Res. Commun. 205, 1143-1150.

Riveros-Moreno V., Palacios M., Rodrigo J., Martinez-Murillo R. and Moncada S. (1996). Characterization of inducible nitric oxide synthase in human monocytes/macrophages after ligation of CD23 receptor. In: The biology of nitric oxide. Part 5. Moncada S., Stamber J., Gross S. and Higgs E.A. (eds), Portland Press. London. pp 160.

Robles-Chillida E.M., Rodrigo J., Mayo I., Arnedo A. and Gómez A. (1981). Ultrastructure of free-endings nerve fibres in eosophageal epithelium. J. Anat. 133, 227-233.

Rodrigo J., Hernández C.J., Vidal M.A. and Pedrosa J.A. (1975). Vegetative innervation of the esophagus. III. Intraepithelial endings. Acta Anat. 92, 242-258.

Rodrigo J., Robles-Chillida E.M., Espinosa F., De Felipe J., Hernández C.J., Arnedo A. and Mayo I. (1980). New contribution on the oesophageal mucous innervation in certain monkeys (Cercopithecidae). Acta Anat. 108, 510-520.

Rodrigo J., Pedrosa J.A., Alvarez F.J., Bentura M.L., Uttenthal L.O., Martínez-Murillo R. and Polak J.M. (1984). Presence of calcitonin gene related peptide in intraepithelial nerve fibres and motor end-plates of cat esophagus: a light and electroin microscopic study. J. Auton. Nervous. Syst. 49, 21-31.

Rodrigo J., Alonso D., Fernández A.P., Serrano J., Richart A., López J.C., Santacana M., Martínez-Murillo R., Bentura M.L., Ghiglione M. and Uttenthal L.O. (2001). Neuronal and inducible nitric oxide synthase expression and protein nitration in rat cerebellum after oxygen and glucose deprivation. Brain Res. 909, 20-45.

Rodrigo J., Polak J.M., Fernández L., Ghatei M.A., Mulderry P. and Bloom S.R. (1985a). Calcitonin gene related peptide (CGRP) immunoreactive sensory and motor nerves in the rat, cat and momkey esophagus. Gastroenterology 88, 444-451.

Rodrigo J., Polak J.M., Terenghi G., Cervantes C., Ghatei M.A., Mulrerry P.K. and Bloom S.R. (1985b). Calcitonin gene related peptide (CGRP) immunoreactive sensory and motor nerves of the mammalian palate. Histochemistry 82, 67-74.

Rodrigo J., Springall D.R., Uttenthal O., Bentura M., Abadia-Molina F., Riveros-Moreno V., Martinez-Murillo R., Polak J.M. and Moncada S. (1994). Localization of nitric oxide synthase in adult rat brain. Phil. Trans. R. Soc. Lond. B. 345, 175-221.

Rodrigo J., Uttenthal L.O., Peinado M.A., Esteban F.J., Fernández A.P., Serrano J., Martínez de Velasco J., Santacana M., Bentura M.L., Martínez-Murillo R. and Pedrosa, J.A. (1998). Distribution of nitric oxide synthase in the esophagus of the cat and monkey. J. Auton. Nervous Syst. 79, 164-179.

Rudert H., Schwink A. and Wetzstein R. (1968). Die Feinstruktur des Subfornikalorgans beim Kaninchen. II. Das neuronale und gliale Gewebe. Z. Zellforsch. 88, 145-179.

Ryn Z. (1971). Psychopathology in alpinism. Acta Med. Pol. 12, 453-467.

Saavedra J.M., Brownstein M.J., Kizer J.S. and Palkovits M. (1976). Biogenic amines and related enzymes in the circumventricular organs of the rat. Brain Res. 107, 412-417.

Saavedra J.M., Correa F.M.A., Plunkett L.M., Kurihara M. and Shigematsu K. (1986). Binding of angiotensin and atrial natriuretic peptide in brain of hypertensive rats. Nature. 320, 758-760.

Salter M., Knowles R.G. and Moncada S. (1991). Widespread tissue distribution, species distribution and changes in activity of $Ca(^{2+})$-dependent and $Ca(^{2+})$-independent nitric oxide synthases. FEBS Lett 291, 145-149.

Santacana M., Uttenthal L.O., Bentura M.L., Fernández A.P., Serrano J., Martínez de Velasco J., Alonso D., Martínez-Murillo R. and Rodrigo J. (1998). Expression of neuronal nitric oxide synthase during embryonic development of the rat cerebral cortex. Dev. Brain Res. 111, 205-222.

Sar M. and Stumpf W.E. (1975). Distribution of androgen-concentrating neurons in rat brain. In: Anatomical neuroendocrinology. Stumpf W.E. and Grant L.D. (eds). Anatomical Neuroendocrinology. New York: S. Karger, pp. 120-133.

Schmidt H.H.H.W. and Murad F. (1991). Purification and characterization of a human NO synthase. Biochem. Biophys. Res. Commun. 181, 1372-1377.

Schwartz J.C., Agid Y., Bouthenet M.L., Javoy-Agid F., Llorens-Cortes C., Martres M.P., Poallard H., Sales N. and Taqued H. (1986). Neurochemical investigations into the human area postrema. In: Nausea and vomiting: Mechanisms and treatment. Davis C.J., Lake-Bakaar G.V. and Grahame-Smith V. (eds). Springer-Verlag. Berlin, pp. 18-30.

Schweizer M. and Richter C. (1994). Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. Biochem. Biophys. Res. Commun. 204, 169-174.

Sharma Y.M. and Malhotra M.S. (1976). Ethnic variations in psychological performance under altitude stress. Aviat. Space Environ. Med. 47, 248-251.

Sharma Y.M., Malhotra M.S. and Baskaran A.S. (1975). Variation in psychomotor proficiency during prolonged stay at high altitude. Ergonomics 18, 511-516.

Shaver S.W., Sposito N.M. and Gross P.M. (1990). Quantitative fine structure of capillaries in subregions of the subfornical organ. J. Comp. Neurol. 294, 145-152.

Shukitt-Hale B., Stillman M.J., Welch DI., Levy A., Devine J.A. and Lieberman H.R. (1994). Hypobaric hypoxia impairs spatial memory in an elevation-dependent fashion. Behav. Neural Biol. 62, 244-252.

Shukitt-Hale B., Kadar T., Marlowe B.E., Stillman M.J., Galli R.L., Levy A., Devine J.A. and Lieberman H.R. (1996). Morphological alterations in the hippocampus following hypobaric hypoxia. Hum. Exp. Toxicol. 15: 312-319..

Shuman E.M. and Madison D.V. (1994). Nitric oxide and synaptic function. Annu. Rev. Neurosci. 17, 153-183.

Singaram C., Sweet M.A., Gaumnitz E.A., Cameron A.J. and Camilleri M. (1995). Peptidergic and nitrergic denervation in congenital esophageal stenosis. Gastroenterology 109, 275-281.

Singer I.I., Kawka D.W., Scott S., Weidner J.R., Mumford R.A., Riehl T.E. and Stenson W.F. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 111, 871-885.

Smith M.A, Richey Harris P.L., Sayre L.M., Beckman J.S. and Perry G. (1997). Widespread peroxynitrite-mediated damage in Alzheimer's disease. J. Neurosci. 17, 2653-2657.

Snyder S.H. (1992). Nitric oxide: first in a new class of neurotransmitters. Science 257, 494-498.

Snyder R.L and Sutin J. (1961). Effects of lesions of the medulla oblongata on electrolyte and water metabolism in the rat. Exp. Neurol. 4, 424-435.

Springall D.R., Riveros-Moreno V., Buttery L., Suburo A., Bishop A.E., Merret M., Moncada S. and Polak J.M. (1992). Immunological detection of nitric oxide synthase in human tissues using heterologous antibodies suggesting different isoforms. Histochemistry 98, 259-266.

Stebbing J.F., Brading A.F. and Mortensen N.J.M. (1995). Nitrergic inhibitory innervation of porcine rectal circular smooth muscle. Br. J. Srg. 82, 1183-1187.

Strijbos P.J. (1998). Nitric oxide in cerebral ischemic neurodegeneration and excitotoxicity. Crit. Rev Neurobiol. 12, 223-243.

Switzer R.C.III., Hill J. and Heimer L. (1982). The globus pallidus and its rostroventral extension into the olfactory tubercle of the rat: a cyto- and chemoarchitectural study. Neuroscience 7, 1891-1904.

Szabó C. (1996). The pathophysiological role of peroxynitrite in shock, inflammation and ischemia-reperfusion injury. Shock 6, 74-88.

Talbot K., Woolf N.J. and Butcher L.L. (1988). Feline islands of Calleja complex: II. Cholinergic and cholinesterasic features. J. Comp. Neurol. 275, 580-603.

Terenghi G., Polak J.M., Rodrigo J., Mulderry P.K. and Bloom S.R. (1986). Calcitonin gene related peptide immunoreactive nerve in the tongue, epiglotis and pharynx of the rat: occurence, distribution and origin. Brain Res. 365, 1-14.

Terenghi G., Riveros-Moreno V., Hudson L.D., Ibrahim N.B.N. and Polak J.M. (1993) Immunohistochemistry of nitric oxide synthase demonstrates immunoreactive neurons in spinal cord and dorsal root ganglia of man and rat. J. Neurol. Sci. 118, 34-37.

Tracey W.R., Nakane M., Pollock J.S. and Forstermann U. (1993). Nitric oxide synthases in neuronal cells, macrophages and endothelium are NADPH diaphorases, but represent only a fraction of total cellular NADPH diaphorase activity. Biochem. Biophys. Res. Commun. 195, 1035-1040.

Trifiletti R.R. (1992). Neuroprotective effects of NG-nitro.L-arginine in focal stroke in the 7-day old rat. Eur. J. Pharmacol. 218, 197-198.

Uttenthal L.O., Alonso D., Fernández A.P., Campbell R.O., Moro M.A., Leza J.C., Liozasoain I., Esteban F.J., Barroso J.B., Valderrama R., Pedrosa J.A., Peinado M.A., Serrano J., Richart A., Bentura ML., Santacana M., Martínez-Murillo R. and Rodrigo J. (1998). Neuronal and inducible nitric axide synthase and nitrotyrosine immunoreactivities in the cerebral cortex of the aging rat. Micros. Res. Tech. 43, 75-88.

Vander-Heiden M.G., Chandel N.S., Williamson E.K., Schumaker P.T. and Thompson C.B. (1997). BCL-x2 regulates the membrane potential and volume homeostasis of mitochondria. Cell 91, 627-637.

Vannuci R.C. (1990). Experimental biology of cerebral hypoxia-ischemia: Relation to perinatal brain damage. Pediatr. Res. 27, 17-26.

Vincent S.R. and Kimura H. (1992). Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46, 755-784.

Volpe J.J. (1995). Neurology of the newborn. 3rd ed. Philadelphia: WB Saunders. Philadelphia. Von Economo C. and Koskinas G.N. (1925) Die Cytoarchitecktonik der hirninde, Springer Verlag. Berlin.

Waeber C., Sebben M., Nieoullon A., Bockaert J. and Dumuis A. (1994). Regional distribution and ontogeny of 5-HT4 binding sites in rodent brain. Neuropharmacology 33, 527-541.

Weiner C.P., Lizasoain I., Baylis S.A., Knowles R.G., Charles I.G. and Moncada S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc. Natl. Acad. Sci. USA 91, 5212-5216.

West J.B. (1986). Do climbs to extreme altitude cause brain damage? Lancet 2, 387-388.

West J.B., Lahiri S., Maret K.H., Peters R.M. and Pizzo C.J. (1983). Barometric pressures at extreme altitudes on Mt. Everest. J. Appl. Physiol. 54, 1188-1194.

Wright C.D., Mulsch A., Busse R. and Oswald H. (1989). Generation of nitric oxide by human neutrophils. Biochem. Biophys. Res. Commun. 27, 154-158.

Xie Q.-W., Cho H.J., Calaycay J., Mumford R.A., Swidderek K.M., Lee T.D., Ding A., Troso T. and Nathan C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256, 225-228.

Xu W., Gorman P., Sheer D., Bates G., Kishimoto J., Liu L and Emson P. (1993) Regional localization of the gene coding for human brain nitric oxide synthase (NOS 1) to 12q24.2-24.31 by fluorescent in situ hibridization. Cytogene Cell Genetic 64, 62-63.

Xu W., Charles I.G., Moncada S., Gorman P., Sheer D., Liu L. and Emson P. (1994). Mapping of the genes encoding human inducible and endothelial nitric oxide synthase (NOS2 and NOS3) to the pericentric region of chromosome 17 and to chromosome 7, respectively. Genomics 21, 419-422.

Xun W., Yin-Shan C., Liang-Gui G. and Lan-Hua S. (1992). EEG characteristics of healthy adults living at higher elevation. Clin. Electroencephalog. 23, 52-57.

Yamada K., Noda Y., Komori Y., Sugihara H., Hasegawa T. and Nabeshima T. (1996). Reduction in the number of NADPH-diaphorase-positive cells in the cerebral cortex and striatum in aged rats. Neurosci. Res. 24, 393-402.

Yan X.X., and Garey L.J. (1997). Morphological diversity of nitric oxide synthesising neurons in mammalian cerebral cortex. J. Hirnforsch. 38, 165-172.

Yan X.X., Jen L.S. and Garey L.J. (1996). NADPH-diaphorase-positive neurons in primate cerebral cortex colocalize with GABA and calcium-binding protein. Cerebral Cortex 6, 524-529.

Yano S., Kiyota Y., Yamamoto M. and Watanabe K. (1995). Pharmacological features of non-adrenergic non-cholinergic (NANC) relaxation induced by electrical vagal stimulation in isolated mouse stomach. Jpn. J. Pharmacol. 69, 9-15.

Ye Y.Z., Strong M., Huang Z.Q. and Beckman J.S. (1996). Antibodies that recognize nitrotyrosine. Methods Enzymol. 269, 201-209.

Yokoyama C., Okamura H., Nakajima T., Taguchi J.I. and Ibata Y. (1994). Autoradiographic distribution of [3H]YM-09151-2, a high-affinity and selective antagonist ligand for the dopamine D2 receptor group, in the rat brain and spinal cord. J. Comp. Neurol. 344, 121-136.

Yui Y. , Hattori R., Kosuga K., Eizawa H., Hiki K. and Kawai C. (1991). Purification of nitric oxide synthase from rat macrophages. J. Biol. Chem. 266, 12544-12547.

Zaharchuk G., Hara H., Huang P.L., Fishman M.C., Moskowitz M.A., Jenkins B.G. and Rosen B.R. (1997). Neuronal nitric oxide synthase mutant mice show smaller infarcts and attenuated apparent diffusion coefficient changes in the peri-infarct zone during focal cerebral ischemia. Magnetic Resonance Med. 37, 170-5.

Zhang Z.G., Chopp M., Zaloga R.L., Schmidt H.H.H.W., Pollock J.S. and Forstermann U. (1994). Upregulation of neuronal nitric oxide synthase and mRNA and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rats. Brain Res. 654, 85-95.

Zhao H., Assai A. and Ishikawa K. (1999). Neither L-NAME nor L-arginine changes extracellular glutamate elevation and anoxic depolarization during global ischemia and reperfusion in rat. Neuroreport 12, 313-318.

Accepted April 23, 2002