# NITRIC OXIDE AND THE CENTRAL NERVOUS SYSTEM

# STIKSTOFOXYDE EN HET CENTRAAL ZENUWSTELSEL

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Nitric oxide and the central nervous system /Eleonora Dzoljic
Thesis Erasmus University Rotterdam. - With references and summary in Dutch
Subject headings: central nervous system / convulsion / nitric oxide / locomotion / vigilance

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## PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof. Dr. P.W.C. Akkermans, MA

En volgens besluit van het college voor promoties

De openbare verdediging zal plaats vinden op
woensdag, 6 mei 1998 om 13:45 uur

door

**ELEONORA DZOLJIC**
Geboren te Belgrado, Joegoslavië

**PROMOTIECOMMISSIE:**

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| CO-PROMOTOR: | Dr. M.R. Dzoljic |

To my teachers

# CONTENTS

# PREFACE

During the last ten years, several investigators have reported that biological effects of endothelium-derived relaxing factor (EDRF; Furchgott and Zawadzki, 1980) are actually activities of a signal molecule, *nitric oxide* (NO; Moncada *et al.*, 1988). This molecule is synthesised by endothelial vascular cells (Palmer *et al.*, 1987, 1988; Ignarro, 1990).

Endogenous NO is involved in many biological processes. The horseshoe crab *(Limulus polypherus)*, that exists for 500 million years, synthesises NO from L-arginine to avoid aggregation of its circulating haemocytes (Radomski *et al.*, 1991), while the blood sucking insect *Rhodnius prolixus* uses NO in its prey for vasodilatation and platelet anti-aggregation (Ribeiro *et al.*, 1993). The goldfish *Carassius auratus* (Bruning *et al.*, 1995) and the frog *Xenopus laevis* (Bruning and Mayer, 1996) use NO as a molecule for neuronal signalling.

Evidence has accumulated that endogenous NO regulates not only mammalian blood vessels but many other systems (Moncada and Higgs, 1991). Almost every mammalian cell/system is under influence of NO, involving endothelium-dependent relaxation (Furchgott and Zawadzki, 1980), neurotransmission (Garthwaite *et al.*, 1988; Gillespie *et al.*, 1989) and cell-mediated immune response (Nathan and Hibbs, 1991). Appropriately, NO was proclaimed as a *Molecule of the Year* for 1992 by the journal *Science* (Koshland, 1992).

In addition, the beneficial effects of glyceryl trinitrate in coronary heart disease, known since 1867, have been recently explained through NO (Änggård, 1994). Alfred Nobel, who invented nitroglycerine, used the drug himself to relieve his coronary heart problems (Snyder and Bredt, 1992; Hölscher *et al.*, 1995).

Recently, it has been found that NO can exert not only cytoprotective but also cytotoxic effects in mammalian cells (Snyder and Bredt, 1992; Kröncke *et al.*, 1997). Moreover, it has been demonstrated that clarification of the dual effect of NO might have implications for clinical medicine with therapeutic opportunities (Snyder, 1993; Schmidt and Walter, 1994; Vallance and Moncada, 1994). Thus, the main goal of this thesis is to highlight the importance of this molecule, particularly in the neuropharmacology.

**References**


Änggård E. Nitric oxide: mediator, murderer and medicine. *Lancet* 343:1199-1206, 1994.

Bruning G, Katzbach R, Mayer B. Histochemical and immunocytochemical localization of nitric oxide synthase in the central nervous system of the goldfish, carassius auratus. *J Comp Neurol* 358:353-382, 1995.

Bruning G, Mayer B. Localization of nitric oxide synthase in the brain of the frog, Xenopus Laevis. *Brain Res* 741:331-341, 1996.

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376, 1980.

Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor released on activation of NMDA receptors suggest a role as intracellular messenger in the brain. *Nature* 336:385-388, 1988.

Gillespie JS, Lui X, Martin W. The effects of L-arginine and $N^G$-monomethyl-L-arginine on the response of the rat anococcygeus to NANC nerve stimulation. *Br J Pharmacol* 98:1080-1982, 1989.

Hölscher C, Doyle CA, McGlinchey L, Anwyl R, Rowan MR. A selective neuronal nitric oxide synthase inhibitor impairs spatial learning in the rat. Abstract, *Society for Neuroscience*, San Diego, USA, November 1995.

Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Ann Rev Pharmacol Toxicol* 30:535-560, 1990.

Kröncke KD, Fehsel K, Kolb-Bachofen V. Nitric oxide: cytotoxicity versus cytoprotection: why, when, and where? *Nitric oxide* 1:107-120, 1997.

Koshland DE. The molecule of the year. *Science* 258:17861, 1992.

Moncada S, Radomski MW, Palmer RMJ. Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* 37:2495-2501, 1988.

Moncada S, Higgs EA. Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur J Clin Invest* 21:361-374, 1991.

Nathan C, Hibbs JB. Role of nitric oxide synthase in macrophage antimicrobial activity. *Curr Opin Immunol* 3:65-70, 1991.

Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526, 1987.

Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesise nitric oxide from L-arginine. *Nature* 333:664-666, 1988.

Radomski MW, Martin JF, Moncada S. Synthesis of nitric oxide by the haemocytes of the American horseshoe crab (Limulus polypherus). *Philos Trans R Soc*, Lond 334:129-133, 1991.

Ribeiro JMC, Hazzard JMH, Nussenzveig RH, Champagne DE, Walker FA. Reversible binding of nitric oxide by a salivary haem protein from a blood-sucking insect. *Science* 260:539-541, 1993.

Schmidt HHHW, Walter U. NO at work. *Cell* 78:919-925, 1994.

Snyder SH, Bredt DS. Biological roles of nitric oxide. *Scientific American* 5:28-35, 1992.

Snyder SH. Janus faces of nitric oxide. *Nature* 364:577, 1993.

Vallance P, Moncada S. Nitric oxide-from mediator to medicines. *J Royal Coll Phys*, London 28:209-219, 1994.

# PART I

# NITRIC OXIDE
## An Overview

# CHAPTER 1

## NITRIC OXIDE: SYNTHESIS, MECHANISM OF ACTION AND DISTRIBUTION

### 1.1 Nitric Oxide Synthase Isoenzymes

The nitric oxide (NO) gas is a reactive and membrane permeable signal molecule, with an average life of about 15 s (Snyder and Bredt, 1992). The unconventional transmitter NO does not seem to be prestored or packed in vesicles, but is synthesised on demand with quick diffusion from its site of production in random direction (Lundberg, 1996). Therefore, much of the understanding of its functions is derived from the characterization of the synthesising enzyme nitric oxide synthase (NOS).

So far three structurally distinct NOS isoenzymes have been purified and biochemically characterised. Distinction can be made on the basis of the sensitivity to $Ca^{2+}$ stimulation, the distinct genes that have been cloned/sequenced and on cell/organ localization (Bredt *et al.*, 1991a,b; Nathan and Hibbs, 1991; Lowenstein *et al.*, 1994). Neuronal NOS (n-NOS) or brain (b-NOS), denoted as NOS-1 (Knowles *et al.*, 1989; Bredt *et al.*, 1990) and endothelial NOS (e-NOS), sometimes denoted as NOS-3 (Knowles and Moncada, 1992; Marsden *et al.*, 1992) are constitutively expressed (constitutive NOS; c-NOS) and regulated by intracellular concentration of free $Ca^{2+}$. The third isoenzyme is $Ca^{2+}$-independent and cytokine-inducible (inducible NOS; i-NOS; NOS-2; Hibbs *et al.*, 1988; Moncada *et al.*, 1988). However, recent results have found that both c-NOSs could also be upregulated (induced) during focal brain ischaemia; it is then followed by a delayed induction of i-NOS (Iadecola, 1997). The three distinct NOS isoenzymes (n-NOS, e-NOS, and i-NOS) are derived from three different genes, found on human chromosomes 12, 7 and 17, respectively (Wang and Marsed, 1995). A comparison of the NOSs shows great similarity in structure, with overall homology around 50%, except the amino terminal, which is unique for each isoenzyme (Vallance and Moncada, 1994). Unlike n-NOS and i-NOS, which are largely cytosolic, e-NOS is predominately (>90%) membrane bound (Snyder and Bredt, 1992).

#### *1.1.1 Constitutive Nitric Oxide Synthases: n-NOS and e-NOS*

e-NOS and n-NOS are regarded as c-NOSs because they are present as normal constituents of healthy cells and synthesise NO on demand in short time periods (second-minutes) after enzyme activation by calmodulin upon elevations of the intracellular $Ca^{2+}$ concentrations (Moncada *et al.*, 1991). c-NOS makes relatively small amounts (picomols) of NO, which produces changes in the target cells by activating soluble guanylyl cyclase (Kiechle and Malinski, 1993; Presta *et al.*, 1997).

*1.1.1.1 n-NOS.* n-NOS has been found in various neuronal, epithelial and skeletal muscle cells (Bredt *et al.*, 1991c; Schmidt *et al.*, 1992; Nakane *et al.*, 1993). Within the central nervous system (CNS), n-NOS is distributed in many brain areas (see details in Chapter 1.5, Bredt *et al.*, 1991c) and spinal cord (Dawson *et al.*, 1991). The highest expression is found in the cerebellum (Bredt *et al.*, 1991). Distribution in the brain is not exclusively neuronal, because recently n-NOS has been found in astrocytes as well (Murphy *et al.*, 1995). During embryonic development, major presence of n-NOS occurs in a developing neuronal system, from sensory ganglia, olfactory neurons, to the thalamus and cerebral cortex (Bredt and Snyder, 1994).

Within the autonomic and peripheral nervous system (PNS), n-NOS is localized in non-adrenergic non-cholinergic (NANC, nitrergic) neurons (Rand *et al.*, 1995), in the retina (Bredt *et al.*, 1990), cardiovascular system (Forsterman *et al.*, 1993), arteriovenous anastomoses (Funk *et al.*, 1994), lung (Belvisi *et al.*, 1992), gastrointestinal system (Mearin *et al.*, 1993), genitourinary tract (Leone *et al.*, 1994) and sensory system (Vincent and Hope, 1992). Additionally, the cerebral vasculature is densely innervated with NANC neurons found in the adventitial layers (Bredt *et al.*, 1990; Faraci *et al.*, 1994). In the peripheral vasculature, NANC innervation of the human corpus cavernosum has been demonstrated as an important factor in the mechanism of penile erection (Leone *et al.*, 1994).

Outside the nervous system, n-NOS is localized in the anterior pituitary, adrenal medulla, macula densa of the kidney (Snyder and Bredt, 1991), in the epithelium of the respiratory tract (Kobzik *et al.*, 1993), fast-twitch fibres at the sarcolemma (Nakane *et al.*,; 1993, Kobzbik *et al.*, 1994) and neuromuscular junction (Chao *et al.*, 1997) of striated somatic and visceral muscle (Grozdanovic *et al.*, 1995a), including myocardium (Silvagno *et al.*, 1996). Expression of n-NOS in the sarcolemma region of visceral and somatic striated muscle has been species-independent (Grozdanovic *et al.*, 1995 b). n-NOS in striated muscle, unlike other n-NOS, is slightly more massive and concentrated in the membrane (not in cytosol like other n-NOSs). This novel n-NOS isoenzyme, called *n-NOS-μ*, has a catalytic activity similar to n-NOS expressed in cerebellum, but with a function exclusively in skeletal muscle and heart (Silvagno *et al.*, 1996).

Stimulation of excitatory N-methyl-D-aspartate (NMDA) receptor by glutamate (GLU) leads to the opening of $Ca^{2+}$ ion channels, increasing intracellular $Ca^{2+}$ concentration and activation of n-NOS (Garthwaite *et al.*, 1988). Recently, it has been found that the increase in NO production in focal brain ischaemia is accompanied by upregulation (induction) of both n-NOS activity and n-NOS gene expression. The increase in NOS activity corresponds to an increase of both n-NOS mRNA and a number of n-NOS-immunoreactive neurons (Iadecola, 1997). Additionally, after injury in CNS or PNS, high levels of n-NOS are found in neurons that normally do not express n-NOS (Snyder, 1994).

*1.1.1.2 e-NOS.* e-NOS is a constitutively expressed enzyme, found in the endothelium of human arteries (Yang *et al.*, 1991), arterioles (Woolfson *et al.*, 1990), veins (Vallance *et al.*, 1989; Janssens *et al.*, 1992), platelets (Radomski *et al.*, 1990), endocardium (Finkel *et al.*, 1992; Henderson *et al.*, 1992). However, recent results have shown that e-NOS, in addition to the blood vessels, is also present in the various neuronal structures of the brain, including the hippocampus. This supports the idea that NO may have a role in memory processes (Snyder, 1994).

The activators of e-NOS are substances released from nerves, such as acetylcholine (Ach), bradykinin, substance P and those released from platelets (e.g. thrombin and serotonin [5-HT]). They increase intracellular free $Ca^{2+}$ and thereby cause activation of e-NOS. e-NOS can also be upregulated (induced). It has been shown that chronic exercise in dogs is a stimulus for endothelial cell e-NOS gene expression, which can explain the beneficial effects of physical exercise (Sessa *et al.*, 1994).

### *1.1.2 Inducible Nitric Oxide Synthase*

i-NOS is $Ca^{2+}$-independent as it contains tightly bound calmodulin (Cho *et al.*, 1992). It is induced by products of infection, including bacterial endotoxins (Hibbs *et al.*, 1988) and

exotoxins (Zembowicz *et al.*, 1992), as well as inflammatory cytokines, such as tumour necrosis factor, interferon gamma and interleukins 1 and 2 (Vallance and Moncada, 1993). i-NOS is not a normal constituent of cells but is induced in immune cells (macrophages, neutrophils, monocytes; Vallance and Moncada, 1993, 1994; Snyder, 1994) and in many other cells, such as those from the vascular endothelium, smooth muscle, myocardium, lung and liver cells. i-NOS can also be expressed in brain astrocytes as well as glial cells in the retina (Murphy *et al.*, 1995). The high concentrations of NO produced by i-NOS have cytostatic effects on parasitic microorganisms and tumour cells. Once expressed, the enzyme continuously generates large amounts (nanomols) of NO, over periods of many hours or days. However, the released NO in higher quantities produced by i-NOS can have additional toxic effects. It interacts with iron-sulphur centred enzymes, impairs mitochondrial respiration (Hibbs *et al.*, 1988; Stadler *et al.*, 1991) and can damage DNA (Nguyen *et al.*, 1992). Furthermore, continuous release of high amounts of NO acts with superoxide anion, also secreted from activated immune cells, causing oxidative injury and cell death. Reaction of NO with superoxide may lead to the formation of other more stable toxic reactive oxygen intermediates, including peroxynitrate and hydroxyl anion (Beckmen *et al.*, 1990). Conceivably, constant unregulated NO synthesis and cytotoxicity usually correlate with i-NOS activity and not with the regulated NO production by the two c-NOSs (with possible exceptions in brain injury; Kröncke *et al.*, 1997).

## 1.2 Biosynthesis of Nitric Oxide

NOS catalyses oxidation of the semi-essential amino acid L-arginine (L-Arg) into equal amounts of NO and L-citrulline (Mayer *et al.*, 1989; Palmer and Moncada, 1989; Moncada *et al.*, 1991). Substrate oxidation occurs at a haem iron and requires nicotine amide dinucleotide phosphate (NADPH, Pollock *et al.*, 1991). The byproduct L-citrulline is recycled back to L-Arg (Hecker *et al.*, 1990); this process can be blocked by L-glutamine (Lee *et al.*, 1996). This cycle has two functions: an excretory role to eliminate excess nitrogen created by the cell metabolism and a secretory role to regenerate L-Arg for NO synthesis (Änggård, 1994).

### *1.2.1 $Ca^{2+}$and Biosynthesis of Nitric Oxide*

In the brain, $Ca^{2+}$ influx through GLU NMDA receptor channels stimulates calmodulin thereby activating n-NOS. This mode of action explains the ability of GLU or neuronal depolarization to stimulate NO formation in a matter of seconds. In endothelial cells, biogenic substances such as Ach and bradykinin acting at the receptor sites mobilize intracellular $Ca^{2+}$, which activates e-NOS. Thus, $Ca^{2+}$-regulated c-NOSs releases NO, which mediates rapid events such as neurotransmission and blood vessel dilatation. The $Ca^{2+}$ requirement for NOS activity reflects the affinity of $Ca^{2+}$ for NOS cofactor calmodulin (Abu-Soud and Stuehr, 1993). L-Arg binding, however, is unaffected by $Ca^{2+}$ or calmodulin (White and Marletta, 1992). In contrast, i-NOS generates NO at resting intracellular $Ca^{2+}$ levels (Mayer, 1995).

### *1.2.2 L-Arginine as Substrate of Nitric Oxide Synthase*

Generation of NO by $Ca^{2+}$/calmodulin-activated NOS is further controlled by intracellular availability of substrate and cofactors. Normally, levels of L-Arg are sufficient for

continuous NO biosynthesis. The content of free L-Arg in total brain is about 10 µmol per 100 g wet weight (Mayer, 1995), a concentration sufficient for saturation of NOSs. Nevertheless, several *in vivo* and *in vitro* studies have demonstrated biological effects of administration of L-Arg. L-Arg-stimulated NO formation indicates that NOS might not be substrate saturated in certain brain areas (Buchmann *et al.*, 1996). Moreover, neurons can be dependent on transport of the NOS substrate from L-Arg pools. As observed with cultured endothelial cells, macrophages and neuronal cells (Schmidt *et al.*, 1993, 1994), a specific transport system is responsible for the uptake of L-Arg. This transport system is also important for several basic amino acids which may competitively reduce intracellular availability of L-Arg. It has been reported that L-Arg deficiency would not only decrease rates of NO formation but may induce generation of superoxide anions and hydrogen peroxide, which can participate in neurotoxicity. Thus, NO toxicity seems to be concurrent with the production of superoxide, precisely the NOS product under reduced L-Arg availability (Mayer, 1995).

### *1.2.3 Cofactors of Nitric Oxide Synthase*

All NOS isoenzymes are haemoproteins (White and Marletta, 1992) and require cofactors for their full activities: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH and tetrahydrobiopterin (H4B, Moncada *et al.*, 1991; Klatt *et al.*, 1966). The intracellular concentration of NOS is regulated by the availability of H4B (Brand *et al.*, 1994).

## 1.3 Nitric Oxide Synthase Inhibitors

NOS-blocking drugs have been essential for evaluating the role of NO in physiological and pathophysiological processes. Therefore they can have particular importance in medicine with possible therapeutic implications.

### *1.3.1 'Selective' n-NOS Inhibitors*

*1.3.1.1 Indazole derivatives.* Indazole derivatives are relatively selective n-NOS inhibitors. Recently, 7-nitro indazole (7-NI) has been reported as a NOS-blocking drug with *in vitro* as well *in vivo* selectivity for n-NOS (Babbedge *et al.*, 1993; Moore *et al.*, 1993a,b). This rather simple compound does not exhibit structural similarities with L-Arg or any of the known NOS cofactors (Fig. 1.1). Inhibition of n-NOS by 7-NI was shown to be reversible (Wolff and Gribin, 1994), competitive with L-Arg (Babbedge *et al.*, 1993) and also with H4B (Klatt *et al.*, 1994b). These data suggest that binding of the drug occurs at a distinct site which interacts with the binding domains for both L-Arg and H4B. It has been proposed that the inhibitory effect of 7-NI is a consequence of its binding to the haem group of NOS, resulting in decreased affinities of the enzyme for L-Arg and H4B, accounting for selectivity for n-NOS (Klatt *et al.*, 1994b). Additionally, it has been reported that 7-NI blocks formation of hydrogen peroxide, that is catalyzed by NOS in decreased L-Arg availability. Thus, 7-NI resembles imidazole, a known haem-site NOS inhibitor (Mayer *et al.*, 1994, 1995). Inhibition of n-NOS by 7-NI (maximal effect: 0.5-1 h after i.p., injection) is relatively short-lasting with complete recovery at either 4 h (oral administration) or 24 h (i.p. injection) in many brain regions (Babbedge *et al.*, 1993; MacKenzie *et al.*, 1994).

The availability of structurally distinct inhibitors selective for the n-NOS allows the possibility of dissociating effects of n-NOS from e-NOS and i-NOS. It has been reported that potent and selective inhibition of n-NOS compared to e-NOS and i-NOS may be useful to treat cerebral ischaemia (stroke) and other neurodegenerative diseases (Furfine *et al.*, 1994). 7-NI may be a valuable starting point for the development of selective, centrally acting n-NOS inhibitors devoid of cardiovascular side effects, serving as tools for studying the central pharmacological effects of NO (Mayer, 1995).

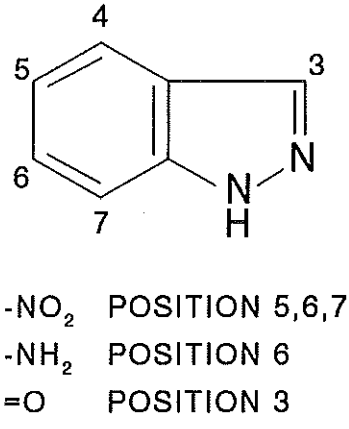


*Figure 1.1 Structure of indazole derivatives showing points of substitution.*

It has been recently shown that the other indazole derived substance, 3-bromo-7-nitro-indazole (3-Br-7-NI; Fig. 1.2) is approximately 4-fold more potent than 7-NI in inhibiting n-NOS (Bland-Ward and Moore, 1995). However, unlike 7-NI, which does not affect blood pressure (Moore *et al.*, 1993a,b), the cardiovascular effects of 3-Br-7-NI are still unknown (Bland-Ward and Moore, 1995).

*1.3.1.2 Imidazole derivative.* They also show some selectivity towards n-NOS. Like 7-NI, imidazole and its derivative 1-(2-trifluoromethylphenyl)imidazole (TRIM, Fig. 1.3) are haem-site NOS inhibitors (Mayer *et al.*, 1994; 1995). In addition to 7-NI and 3-Br-7-NI (Fig. 1.1, 1.2; Bland-Ward and Moore, 1995), TRIM interferes with the binding of both L-Arg and H4B to NOS, which underlines the selectivity for n-NOS (Handy and Moore, 1997). Its effects are also competitively reversible by L-Arg (Handy *et al.*, 1995; Handy and Moore, 1997). Like 7-NI, which inhibits n-NOS in a selective with a little effect on e-NOS *in vivo* (Babbedge *et al.*, 1993; Moore *et al.*, 1993a), TRIM is also without cardiovascular effects *in vivo* (Handy *et al.*, 1995).

Concerning new and selective n-NOS inhibitors it has been reported that delta-(S-methylisothioureido)-L-norvalin exhibits a more than 40-fold weaker inhibitory effect on other NOS isoenzymes and reduces the size of focal cerebral ischaemia (Nagafuji *et al.*, 1995).

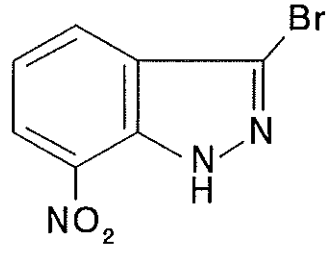


*Figure 1.2 Structural formula of 3-bromo 7-nitro indazole (3-Br-7-NI). Mw:242.*

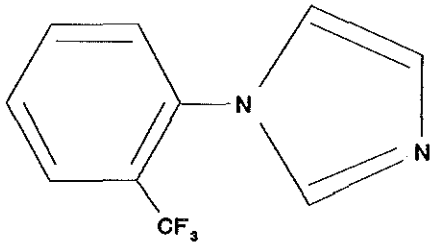


*Figure 1.3 Structural formula of 1-(2-trifluoro methylphenyl)imidazole (TRIM). Mw:212.*

*1.3.2 'Selective' i-NOS Inhibitors*

It is of considerable interest to find inhibitors which would be more selective for i-NOS, because then a hyperactive NO system could be damped down without influencing any constitutive NO release. So far, there has been only a limited success. Unlike most L-Arg derived NOS inhibitors, some guanidino and S-alkyl-isothioureas, particularly aminoethyl-isothiourea (Southan *et al.*, 1996) and S-ethyl-isothiourea, show selectivity towards i-NOS over n-NOS and e-NOS, with several potential therapeutic applications, such as treatment of sepsis and autoimmune diseases (Tracey *et al.*, 1995). However, i-NOS inhibitors of even greater selectivity may need to be developed for therapeutic applications. Aminoguanidine is a also weak but selective inhibitor of i-NOS and is undergoing clinical exploration (Misko *et al.*, 1993).

*1.3.3 Non-Specific NOS Inhibitors*

*1.3.3.1 Citrulline Derivatives.* It has been reported that S-methyl-L-thiocitrulline (S-Me-TC, Fig. 1.4) is the most potent n-NOS and i-NOS inhibitor described to date (Narayanan *et al.*, 1994), although this compound is about 15-fold more selective for n-NOS than for e-NOS (Furfine *et al.*, 1994). Inhibition is reversible, stereoselective and competitive with L-Arg. In contrast to indazole and imidazole derivatives, S-Me-TC has a strong pressor activity that reverses hypotension in the septic shock (Narayanan *et al.*, 1995; Joly *et al.*, 1995). Compared to L-Arg-based NOS blocking drugs, S-Me-TC is not metabolised to citrulline, which can be converted back to L-Arg *in vivo*, thus sustaining overproduction of NO (Hattori *et al.*, 1994).

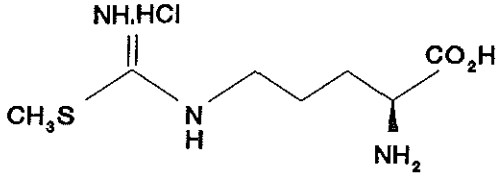


*Figure 1.4 Structural formula of S-methyl-L-thiocitrulline (S-Me-TC). Mw:266.*

*1.3.3.2 L-Arginine Derivatives.* Analogues of L-Arg act by competing with L-Arg at the active site of the NOS and their action can be reversed by adding higher amounts of L-Arg (Mayer, 1995). Additionally, activation of c-NOS or induction of i-NOS increase the rate of L-Arg transport into endothelial and neuronal cells or macrophages, respectively. Competitive inhibition of this L-Arg transport mechanism by L-Arg analogues may result in apparent decreased NOS activity secondary to substrate reduction (Kiechle and Malinski, 1993). Methylated L-Arg analogues such as L-$N^G$-mono-methyl-L-arginine (L-NMMA) and asymmetric $N^G$-dimethylarginine (ADMA) have been found endogenously and observed to accumulate in renal failure. Perhaps endogenous methylarginines provide control over NO synthesis *in vivo* (Vallance *et al.*, 1992a,b). Because NO cannot be stored in vesicles like

other neurotransmitters, its release is regulated by NOS activity and modulated by NOS inhibitors. However, care must be taken in the experiments estimating biological roles of NO using L-Arg analogues as they can block not only NOS but they may interfere with other iron-dependent systems and inhibit mitochondrial electron transport (Peterson *et al.*, 1992). Therefore, studies that use L-Arg analogues to modulate biologic effects of NO can not be conclusive since the obtained results are only due to the inhibition of NOS (Kiechle and Malinski, 1993).

NOS isoenzymes vary in their susceptibility to inhibition by different L-Arg-analogues. For example, $N^G$-nitro-L-arginine (L-NOARG) preferentially blocks n-NOS and e-NOS (Lambert *et al.*, 1991), while L-NMMA selectively inhibits n-NOS and i-NOS (Feldman *et al.*, 1993; Klatt *et al.*, 1994 a).

*1.3.3.3 Other NOS inhibitors.* NOSs are inhibited by a wide variety of drugs which interfere with one of the multiple catalytic functions of the enzyme, such as: calmodulin antagonist, trifluoroperazine, preventing $Ca^{2+}$-calmodulin binding (Bredt and Snyder,1990), cyanide and carbon monoxide (CO) blocking function of the haem (Marletta, 1993) or nitro blue tetrazolium inhibiting activity of NADPH (Klatt *et al.*, 1992).

Recent results have brought into light new NOS blocking drugs such as selenium-containing antioxidant, ebselen, which inhibits both c-NOS and i-NOS (Änggård, 1994).

### *1.3.4 NO as NOS Inhibitor*

It has been shown that NOS can be inhibited by its reaction product NO (Rogers and Ignarro, 1992; Assreuy *et al.*, 1993; Buga *et al.*, 1993; Griscavage *et al.*, 1993; Rengasamy and Johns, 1993). This feed-back blockade on NOS catalytic activity by NO (but not oxidation products such as nitrite, nitrate, peroxynitrate) modulates NO production in the cell of origin and in near neighbour cells. Conceivably, the possible therapeutic potential of exogenous NO administration (Ahler *et al.*, 1991; Geggel *et al.*, 1993; Rossaint, 1994; Rossaint *et al.*, 1996) should consider the feed-back inhibition of NOS as clinically relevant.

## 1.4 Mechanisms of NO Actions

While the number of newly discovered potential targets of NO continually increase, under physiological conditions the major effects of NO still appear to be mediated primarily by the activation of the intracellular NO receptor soluble guanylyl cyclase, leading to the increase of cyclic guanosine 3',5'-monophosphate (cGMP, Garbers, 1992). Activation of soluble guanylyl cyclase is the main mechanism of action of NO in both the vascular and the nervous system (Arnold *et al.*, 1977, Miki *et al.*, 1977; Vincent and Hope, 1992). A growing family of guanylyl cyclase has been discovered but only the soluble form serves as the NO receptor (Ignarro, 1990; Mayer, 1994). The purified guanylyl cyclase contains haem, to which NO binds in a manner similar to its interactions with other haemoproteins, including haemoglobin. Precisely, NO binds with very high affinity to $Fe^{2+}$ in the haem-active centre of the soluble guanylyl cyclase, causing the enzyme activation with resulting rises in cGMP levels (Snyder and Bredt, 1992). The cGMP formed may then regulate protein kinase, phosphodiesterases and ion channels. The intracellular effects of cGMP are achieved by the regulation of cGMP-regulated cyclic nucleotide phosphodiesterases and each of these cGMP targets represents the regulatory proteins (Kiechel and Malinski, 1993; Schmidt *et al.*, 1993).

Thus, the effects of NO on individual target cells may be determined by each cell's preprogrammed characteristic response to NO as a mediator molecule (Lancaster, 1997).

However, under some pathophysiological conditions NO can act in a cGMP-independent way. For example, the target of NO as a toxic agent is different than as a messenger molecule. NO binds avidly to $Fe^{2+}$-sulphur centres of enzymes, including those involved in the mitochondrial electron transport chain and the DNA synthesis (Hibbs *et al.*, 1988). The cytotoxic effect of macrophages-derived NO on parasites or tumour cells is the result of NO reaction with $Fe^{2+}$-containing enzymes, leading to massive oxidative- and DNA injury (Lundberg, 1996). The reaction of NO with superoxide anions, another product of activated immune cells, leads to the formation of a cytotoxic oxidant, peroxynitrate, which can induce tissue damage and other pathophysiological processes, including inflammation and ischaemia-reperfusion injury (Änggård, 1994; Iadecola, 1997). A similar mechanism is probably active in brain ischaemia-reperfusion damage accompanied with overstimulation of GLU NMDA receptors. Accordingly, recent findings have implicated that NOS inhibitors, particularly selective ones, may have a therapeutic benefit in endotoxic shock (Narayanan *et al.*, 1995), stroke and neurological damage associated with excess GLU release (Snyder and Bredt, 1992; Iadecola, 1997).

While small amounts of NO from c-NOS bind to haem-containing enzymes such as soluble guanylyl cyclase, excess NO from i-NOS may bind to thiols. It has been suggested that NO circulates in mammalian plasma bound to thiol-group of albumin, serving as a store which prolongs the actions of released NO (Änggård, 1994).

Interestingly, CO, as a normal body compound, also appears to be able to activate soluble guanylyl cyclase via a similar mechanism as NO, exhibiting many of the physiological effects ascribed to NO. However, these activities are distinguishable by NOS inhibitors (Snyder and Bredt, 1992).

NO diffuses within quite large spatial limits of about 0.3-0.4 mm which makes actions of NO significantly long range compared to other neurotransmitters (Lancaster, 1997). Nevertheless, NO can be rapidly inactivated by superoxide, $Fe^{2+}$, $Fe^{3+}$ and $O_2$, scavenged by haemoglobin or quickly converted by oxygen and water into nitrates and nitrites. All those substances are found in great abundance in biological systems, making NO a short lived molecule (Snyder and Bredt, 1992; Kiechle and Malinski, 1993; Änggård, 1994).

### 1.5 Distribution of NOS in the Nervous System

There is a rather detailed understanding of the distribution of the neurons producing NO throughout the nervous system (Vincent, 1994). This is due to the identification of NOS as the enzyme responsible for the neuronal NADPH diaphorase (NADPH-d) histochemical reaction (Hope *et al.*, 1991), together with the development of antibodies to the purified NOS (Bredt *et al.*, 1990). There is an association between neurons expressing NOS mRNA or NOS immunoreactivity and those expressing NADPH-d (Dawson *et al.*, 1991; Bredt *et al.*, 1991c). Recently, it has been demonstrated that mice lacking a n-NOS gene, show a loss of NADPH-d staining in the nervous system (Huang *et al.*, 1993). Therefore, neurons that express NOS can be identified either by NADPH-d histochemistry or by NOS immunocytochemistry. In the nervous tissue, histochemical NADPH-d reaction is considered a suitable marker for NOS activity (Hope *et al.*, 1991; Dawson *et al.*, 1991; Grozdanovic *et al.*, 1995a). Accordingly, these results made possible the anatomical localization of the

sites of synthesis of the short-lived molecule NO (Snyder and Bredt, 1992). The expression of NOS in multiple regions of the nervous system supports a role of NO as a signalling molecule in these areas (Endoh *et al.*, 1994).

It has been found that the pyramidal cells of the cerebral cortex do not contain NOS (Vincent and Kimura, 1992), but can be stained with antibodies to soluble guanylyl cyclase (Ariano *et al.*, 1982; Nakane *et al.*, 1983). NOS is found in cortical cerebral neurons scattered in layers II-VI and in the subcortical white matter (Leigh *et al.*, 1990; Vincent and Kimura, 1992). Similar cell staining has been reported in human cortex (Springall *et al.*, 1992). In humans only a few neurons in the subcortical white matter contain NADPH-d (Meyer *et al.*, 1992). Since these cells are the oldest neurons in the cerebral cortex it may suggest a possible role for NO in cortical development (Vincent and Hope, 1992). Additionally, the striatum contains the highest activities of soluble guanylyl cyclase and cGMP phosphodiesterase in the brain (Greenberg *et al.*, 1978). The results implicate that in the striatum NOS expressed neurons may regulate the response of the output cells to cortical, nigral and local inputs (Vincent and Hope, 1992). NOS is present in various populations of hypothalamic neurons and NO appears to play a major role in regulation of hypothalamic functions. NADPH-d has also been described in supraoptic and paraventricular nuclei in humans (Leigh *et al.*, 1990; Sangruchi and Kowall, 1991; Vincent and Kimura, 1992). In addition to being present in hypothalamic neurosecretory neurons, there is recent evidence for NOS immunoreactivity and NOS mRNA expression in the anterior pituitary (Ceccatelli *et al.*, 1993). NADPH-d histochemistry indicates that the NO system is present throughout the visual pathway (Vincent and Hope, 1992). In the human eye, cone photoreceptors appear to display NADPH-d activity (Provis and Mittrofanis, 1990). Neurons in midbrain, pons, medulla and ascending reticular system in the human brain also appear to express NOS (Kowall and Mueller, 1988; Nakamura *et al.*, 1988; Vincent and Hope, 1992). NADPH-d positive neurons have been described in the nuclei of the solitary tract, vagus and hypoglossus (Kowal and Muller, 1988). NOS activity (Fortstermann *et al.*, 1990) and cGMP (Greenberg *et al.*, 1978) have been found in the cerebellum at the highest levels within the brain (Springall *et al.*, 1992). In the cerebellar cortex NADPH-d histochemistry and immunohistochemistry for NOS indicate that the NOS is present in basket and granule cells, but not in Purkinje cells, which showed immunohistochemical stained for soluble guanylyl cyclase (Vincent and Hope, 1992). NOS immunoreactivity has been seen in human spinal cord, particularly in dorsal root ganglia neurons (Springal *et al.*, 1992; Terenghi *et al.*, 1993), although some ventral horn motoneurons also appear to contain NOS (Springall *et al.*, 1992; Terenghi *et al.*, 1993). Since some of the ependymal cells are NADPH-d positive, it is suggested that they may be involved in the modulation of NO levels in the cerebrospinal fluid (CSF, Tang *et al.*, 1995). Histological studies have shown that NANC neurons in the cardiovascular, respiratory, gastrointestinal and urogenital system are stained by NADPH-d (Vincent and Hope, 1992). Concerning cerebral vasculature NANC nerves are shown by NADPH-d histochemistry and immunocytochemistry, indicating a crucial role of NO and soluble guanylyl cyclase in the cerebral circulation and permeability of the blood-brain barrier (Joo *et al.*, 1983; Vincent and Hope, 1992).

## 1.6 References

Abu-Soud HM, Stuehr DJ. Nitric oxide synthase reveal a role for calmodulin in controlling electron transfer. *Proc Natl Acad Sci USA* 90:10769-10772, **1993.**

Ahlner J, Ljusegren ME, Grundstrom N, Axelsson KL. Role of nitric oxide and cyclic GMP as mediators of endothelium-independent neurogenic relaxation in bovine mesenteric artery. *Circ Res* 68:756-762, **1991.**

Änggård E. Nitric oxide: mediator, murderer and medicine. *Lancet* 343:1199-1206, **1994.**

Ariano MA, Lewicki JA, Brandwein HJ, Murad F. Immunohistochemical localization of guanylate cyclase within neurons of rat brain. *Proc Natl Acad Sci USA* 79:1316-1320, **1982.**

Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide guanulate cyclase and increase guanosine 3'5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci USA* 74:3203-3207, **1977.**

Assreuy J, Cunha FQ, Liewm FY, Moncada S. Feed back inhibition of nitric oxide synthase activity by nitric oxide. *Br J Pharmacol* 108:833-837, **1993.**

Babbedge RC, Bland-Ward PA, Hart SL, Moore PK. Inhibition of rat cerebellar nitric oxide synthase by 7-NI indazole and related indazoles. *Br J Pharmacol* 110:225-228, **1993.**

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrate: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87:1620-1624, **1990.**

Belvisi MG, Stretton CD, Mivra M, Verleden MG, Tajdakarimi S, Yacoub MH, Barnes PJ. Inhibitory NANC nerves in human tracheal smooth muscle: a quest for the neurotransmitter. *J Appl Physiol* 73:2505-2510, **1992.**

Bland-Ward PA, Moore PK. 7-Nitro indazole derivatives are potent inhibitors of brain, endothelium and inducible isoforms of nitric oxide synthase. *Life Sci* 57: PL131-135, **1995.**

Bredt DS, Snyder SH. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* 87:682-685, **1990.**

Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neuronal role for nitric oxide. *Nature* 347:768-770, **1990.**

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351:714-718, **1991a.**

Bredt DS, Ferris CD, Snyder SH. Nitric oxide synthase regulatory sites: phosphorylation by cyclic AMP dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase: identification of flavin and calmodulin binding sites. *J Biol Chem* 267:10976-10981, **1991b.**

Bredt D, Glatt C, Hwang P, Fotuhi M, Dawson T, Snyder S. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* 7:615-624, **1991c.**

Bredt DS, Snyder SH. Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* 13:301-313, **1994.**

Bredt DS. Molecular characterization of nitric oxide synthase. In: *Nitric oxide in the nervous system.* Ed: Vincent SR, Academic Press, New York pp.1-21, **1995.**

Buchmann I, Milakofski L, Harris N, Hofford JM, Vogel WH. Effect of arginine administration on plasma levels of arginine and various related amino compounds in the rat. *Pharmacol* 53:133-142, **1996.**

Buga GM, Grisscavage JM, Rogers NE, Ignarro Lj. Negative feedback regulation of endothelial cell function by nitric oxide. *Circ Res* 73:808-812, **1993.**

Ceccatelli S, Erikson M. The effect of lactation on nitric oxide gene expression. *Brain Res* 625:177-179, **1993.**

Chao DS, Silvagno F, Xia H, Cornwell TL, Lincoln TM, Bredt DS. Nitric oxide synthase and cyclic GMP-dependent protein kinase concentrated at the neuromuscular endplate. *Neurosci* 76:665-672, 1997.

Cho HJ, Xie QW, Calaycay J, Mumfored RA, Swiderek KM, Lee TD, Nathan C. Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med* 176:599-604, 1992.

Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc Natl Acad Sci USA* 88:7797-7801, 1991.

Endoh M, Maiese K, Wagner JA. Expression of the neuronal form of nitric oxide synthase by CA1 hippocampal neurons and other nervous system neurons. *Neurosci* 63:679-689, 1994.

Faraci FM, Brain JE Jr. Nitric oxide and cerebral circulation. *Stroke* 5:692-706, 1994.

Feldman PL, Griffith OW, Hong H, Stuher DJ. Irreversible inactivation of macrophage and brain nitric oxide synthase by L-$N^G$-methyl-arginine requires NADPH-dependent hydroxilation. *J Med Chem* 36:491-496, 1993.

Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hathler BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 257:387-389, 1992.

Forstermann U, Gorsky LD, Pollock JS, Schmidt HH. Heller M, Murad F. Regional distribution of EDRF/NO-synthesizing enzyme(s) in rat brain. *Biochem Biphys Res Commun* 168:727-732, 1990.

Forstermann U, Nakane M, Tracey WR, Pollock JS. Isoforms of nitric oxide synthase: functions in the cardiovascular system. *Eur Heart J* 14: Suppl I:10-15, 1993.

Funk RH, Mayer B, Worl J. Nitrergic innervation and nitrergic cells in arteriovenous anastomoses. *Cell Tissue Res* 277:477-484, 1994.

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376, 1980.

Furfine ES, Farmon MF, Paith JE, Knowles RG, Salter K, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA, Garvey EP. Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase By S-methyl-L- thiocitrulline and S-ethyl-L-thiocitrulline. *J Biol Chem* 269:26677-26683, 1994.

Garbers DL. Guanylyl cyclase receptors and their endocrine, paracrine and autocrine ligands. *Cell* 74:1-4, 1992.

Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor released on activation of NMDA receptors suggest a role as intracellular messenger in the brain. *Nature* 336:385-388, 1988.

Geggel RI. Inhalation nitric oxide: a selective pulmonary vasodilatation for treatment of persistent pulmonary hypertension of the newborn. *J Pediatr* 123:76-79, 1993.

Gillespie JS, Lui X, Martin W. The effects of L-arginine and $N^G$-monomethyl-L-arginine on the response of the rat anococcygeus to NANC nerve stimulation. *Br J Pharmacol* 98:1080-1982, 1989.

Greenberg LH, Triyer E, Ferrendelli JA, Weiss B. Enzymatic regulation of the concentration of cyclic GMP in mouse brain. *Neuropharmacol* 17:737-745, 1978.

Grozdanovic Z, Nakos G, Mayer B, Grossrau R. A modified method allows for correlation between NADPH-diaphorase histochemistry and immunohistochemistry for the demonstration of neuronal nitric oxide synthase (nNOS). *Folia Histochem Cytobiol* 33:11-18, 1995a.

Grozdanovic Z, Nakos G, Dahrmann G, Mayer B, Gossrau R. Species-independent expression of nitric oxide synthase in the sarcolemma region of visceral and somatic striated muscle fibres. *Cell Tissue Res* 283:493-499, 1995b.

Griscavage JM, Rogers NE, Scheman MP, Ignarro LJ. Inducible nitric oxide synthase from a rat alveolar macrophage cell line inhibited by nitric oxide. *J Immunol* 151:6329-6337, 1993.

Handy RLC, Wallace P, Gaffen ZA, Whitehead KJ, Moore PK. The antinociceptive effect of 1-(2-trifluoromethylphenul)imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase in vitro, in the mouse. *Br J Pharmacol* 116:2349-2350, **1995.**

Handy RLC, Moore PK. Mechanism of the inhibition of neuronal nitric oxide synthase by 1-(2-trifluoromethylphenul)imidazole (TRIM). *Life Sci* 60:PL389-394, **1997.**

Hartell NA. cGMP acts within cerebellar Purkinje cells to produce long term depression via mechanisms involving PKC and PKG. *Neuro Rep* 5:833-836, **1994.**

Hattori Y, Campbell EB, Gross SS. Arginosuccinate synthase mRNA and activity are induced by immunostimulans in vascular smooth muscle. Role in the regeneration of arginine for nitric oxide synthase. *J Biol Chem* 269:9405-9408, **1994.**

Hecker M, Sessa WC, Harris HJ, Änggård EE, Vane JR. The metabolism of L-arginine and its significance for the biosynthesis of endothelium derived relaxing factors: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci USA* 87:8612-8616, **1990.**

Henderson AH, Lewis MJ, Sdhah AM, Smith JA. Endothelium, endocardium and cardiac contraction. *Cardiovasc Res* 26:305-308, **1992.**

Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biphys Res Commun* 157:87-94, **1988.**

Hölscher C, Doyle CA, McGlinchey L, Anwyl R, Rowan MR. A selective neuronal nitric oxide synthase inhibitor impairs spatial learning in the rat. Abstract from the *Society for Neuroscience Meeting* in San Diego, USA, November **1995.**

Hope BT, Michael GJ, Knigge KM, Vincent SR. Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc Natl Acad Sci USA* 88:2811-2814, **1991.**

Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC. Targeted distribution of the neuronal nitric oxide synthase gene. *Cell* 75:1273-1286, **1993.**

Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neuro Sci* 20:132-139, **1997.**

Ignarro LJ, Bush PA, Bugga GM, Wood KS, Fukuto JM, Rajfer J. Nitric oxide and cyclic GMP formation upon electrical gild stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biphys Res Commun* 170: 843-850, **1990.**

Ignarro LJ. Biosynthesis and metabolism if endothelium-derived nitric oxide. *Ann Rev Pharmacol Toxicol* 30:535-560, **1990.**

Janssens SP, Shimouchi A, Quertermous T, Bloch DB, Bloch KD. Cloning and expression of cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *J Biol Chem* 267:14519-14522, **1992.**

Joly GA, Narayanan K, Griffith OW, Killbourn RG. Characterization of the effects of two new arginine/citrulline analogues on constitutive and inducible nitric oxide synthases in rat aorta. *Br J Pharmacol* 115:491-497, **1995.**

Joo F, Temesvari P, Dux E. Regulation of the macromolecular transport in the brain microvessels: the role of cyclic GMP. *Brain Res* 278:165-174, **1983.**

Kawabata A, Umeda N, Takagi H. L-arginine exerts a dual role in nociceptive processing in the brain: involvement of the kytorphin-met-enkephalin pathway and NO-cyclic GMP pathway. *Br J Pharmacol* 109:73-79, **1993.**

Kiechle F, Malinski T. Nitric oxide: Biochemistry, pathophysiology and detection. *Am J Clin Pathol* 100:567-575, **1993.**

Klatt P, Heinzel B, John M, Kastner M, Böhme E, Mayer B. $Ca^{2+}$/calmodulin-dependent cytochrome c reductase activity of brain nitric oxide synthase. *J Biol Chem* 267:11374-11378, **1992.**

Klatt P, Schmidt K, Bruner F, Mayer B. Inhibitors of brain nitric oxide synthase. Binding kinetics, metabolism and enzyme inactivation. *J Biol Chem* 269:1674-1680, **1994a.**

Klatt P, Schmidt K, Leopold E, Schmidt K, Werner ER, Mayer B. The pteridine binding site of brain nitric oxide synthase. Tetrahidrobiopterin binding kinetics, specificity and allosteric interaction with substrate domain. *J Biol Chem* 269:13861-13866, 1994b.

Klatt P, Schmidt K, Werner ER, Mayer B. Determination of nitric oxide synthase cofactors: haem, FAD, FMN, and tetrahydrobiopterin. *Methods Enzymol* 268:358-365, 1996.

Knowles NW, Palacois M, Palmer RMJ, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylyl cyclase. *Proc Natl Acad Sci USA* 86:5159-5162, 1989.

Knowles RG, Moncada S. Nitric oxide as a signal in blood vessels. *Trends Biochem Sci* 17:399-402, 1992.

Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stampler JS. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir Cell Mol Biol* 9:371-377, 1993.

Kobzbik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372:546-548, 1994.

Koshland DE. The molecule of the year. *Science* 258:17861, 1992.

Kowal NW, Ferrante RJ, Beal MF, Richardson EO Jr, Sofroniniew MV, Cuello AC, Martin JB. Neuropeptide Y, somatostatin and reduced nicotinamide adenine dinucleotide phosphate diaphorase in the human striatum: a combined immunocytochemical and enzyme histochemical study. *Neurosci* 20:817-828, 1987.

Kowall NW, Mueller MP. Morphology and distribution of nicotinamide adenine dinucleotide phosphate (reduced form) diaphorase reactive neurons in human brain stem. *Neurosci* 26:645-654, 1988.

Kröncke KD, Fehsel K, Kolb-Bachofen V. Nitric oxide: cytotoxicity versus cytoprotection: why, when, and where? *Nitric oxide* 1:107-120, 1997.

Lambert LE, Whitten JP, Baron BM, Cheng HC, Doherty NS, McDonald IA. Nitric oxide in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* 48:69-75, 1991.

Lancaster JR Jr. A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* 1:18-30, 1997.

Lee TJ-F, Sarwinski S, Ische C, Lai FY. Inhibition of cerebral neurogenic vasodilatation by L-arginine and nitric oxide synthase inhibitors and its reversal by L-citrulline. *J Pharmacol Exp Ther* 276:353-358, 1996.

Leigh PN, Connick JH, Stone TW. Distribution of NADPH-diaphorase positive cells in the rat brain. *Comp Biochem Physiol* 97C:259-264, 1990.

Leone AM, Wiklund NP, Hokfelt T, Brundin L, Moncada S. release of nitric oxide by nerve stimulation in the human urogenital tract. *Neuro Rep* 5:733-736, 1994.

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen H-SV, Sucher NJ, Loscalzo J, Singel DJ, Stampler JS. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364:626-632, 1993.

Lowenstein CJ, Dinermann JL, Snyder SH. Nitric oxide, a physiologic messenger. *Ann Intern Med* 120:227-237, 1994.

Lundberg JM. Pharmacology of cotransmission in the autonomic nervous system: Integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol Rew* 48:113-178, 1996.

MacKenzie GM, Rose S, Bland-Ward PA, Jenner P, Marsden CD. Time course of inhibition of brain nitric oxide synthase by 7-nitro indazole. *NeuRo Rep* 5:1993-1996, 1994.

Marletta MA. Nitric oxide synthase structure and mechanism. *J Biol Chem* 268:12231-12234, 1993.

Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T. Molecular cloning and characterization of human endothelial cell nitric oxide synthase. *FEBS Lett* 307:287-293, **1992.**

Mayer B, Schmidt K, Humbert P, Böhme E. Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells $Ca^{2+}$-dependent converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem Biophys Res Commun* 164:678-685, **1989.**

Mayer B, Klatt P, Werner ER, Schmidt K. Molecular mechanisms of inhibition of porcine brain nitric oxide synthase by the antinociceptive drug 7-nitro indazole. *Neuropharmacol* 33:1253-1259, **1994.**

Mayer B. Biochemistry and molecular pharmacology of nitric oxide synthase. In: *Nitric oxide in the nervous system.* Ed. S.Vincent. Academ Press, pp. 22-42, **1995.**

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, Malagelada JR. Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* 23:724-728, **1993.**

Meldrum B, Gartwaite J. Excitatory amino acids neurotoxicity and neurodegenerative disease. *Trends Pharmacol Sci* 11:379-387, **1990.**

Meyer G, Wahle P, Castaneyra-Perdomo A, Ferres-Torres R. Morphology of neurons in the white matter of the adult human neocortex. *Exp Brain Res* 88:204-212, **1992.**

Misko TP, Moore WM, Kasten TP, Nickols GA, Cobett JA, Titon RG, McDaniel ML, Williamson JR, Currie MG. selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol* 233:119-125, **1993.**

Miki N, Kawabe Y, Kuriyama K. Activation of cerebral guanulate cyclase by nitric oxide. *Biochem Biophys Res Commun* 75:851-856, **1977.**

Mitchell HH, Shonle KA, Grindley HS. The origin of nitrates in the urine. *J Biol Chem* 24:461-490, **1916.**

Moncada S, Radomski MW, Palmer RMJ. Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* 37:2495-2501, **1988.**

Moncada S, Higgs EA. Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur J Clin Invest* 21:361-374, **1991.**

Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109-142, **1991.**

Moore PK, Wallance P, Gaffen ZA, Hart SL, Babbedge RC. 7-nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* 108:296-297, **1993a.**

Moore OK, Wallace P, Gaffen Z, Hart SL, Babbedge RC. Characterisation of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociception and cardiovascular effects. *Br J Pharmacol* 110:219-224, **1993b.**

Murphy S, Grzybicki DM, Simmons ML. Glial cells as nitric oxide sources and targets. In: *Nitric oxide in the nervous system.* Ed. S. Vincent, Acad Press, pp. 163-191, **1995.**

Nagafuji T, Sugiyama M, Muto A, Makino T, Miyauchi T, Nabata H. The neuroprotective effect of a potent and selective inhibitor of type I NOS (L-MIN) in a rat model of focal cerebral ischemia. *Neuro Rep* 31:1541-1545, **1995.**

Nakamura S, Kawamata T, Kimura T, Akiguchi I, Kameyama M, Nakamura N, Wakata Y, Kimura H. Reduced nicotinamide adenine dinucleotide phosphate-diaphorase histochemistry in the pontomesencephalic region of the human brain stem. *Brain Res* 455:144-147, **1988.**

Nakane M, Ischikawa M, Deguchi T. Light and electron microscopic demonstration of guanylate cyclase in rat brain. *Brain Res* 273:9-15, **1983.**

Nakane M, Schmidt HH, Pollock JS, Forsterman U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316:175-180, 1993.

Narayanan K, Griffith OW. Synthesis of L-thiocitrulline, L-homothiocitrulline and S-methyl-L-thiocitrulline: a new class of potent nitric oxide synthase inhibition. *J Med Chem* 37:885-887, 1994.

Narayanan K, Spack L, McMillan K, Kilourn RG, Hayward MA, Masters BS, Griffith OW. S-alkil-L-citrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity *in vivo*. *J Biol Chem* 270:11103-11110, 1995.

Nathan C, Hibbs JB. Role of nitric oxide synthase in macrophage antimicrobial activity. *Curr Opin Immunol* 3:65-70, 1991.

Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc Natl Acad Sci USA* 89:3030-3034, 1992.

Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526, 1987.

Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesise nitric oxide from L-arginine. *Nature* 333:664-666, 1988.

Palmer RMJ, Moncada S. A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem Biphys Res Commun* 158:348-352, 1989.

Peterson DA, Peterson DC, Archer S, Weir EK. The nonspecificity of specific nitric oxide synthase inhibitors. *Biochem Biophys Res Commun* 187:797-801, 1992.

Pollock JS, Försterman U, Mitchel JA, Warner TD, Schmidt HHHW, Nakane M, Murad F. Purification and characterisation of particulate endothelium-derived relaxing factor synthase from cultured and naive bovine aortic endothelial cell. *Proc Natl Acad Sci USA* 88:10480-10484, 1991.

Presta A, Liu J, Sessa WC, Stuehr DJ. Substrate binding and calmodulin binding to endothelial nitric oxide synthase coregulate its enzymatic activity. *Nitric Oxide* 1:74-87, 1997.

Provis JM, Mitrofanis J. NADPH-diaphorase neurons of human retina have a uniform topografical distribution. *Visual Neurosci* 4:619-623, 1990.

Radomski MW, palmer RMJ, Moncada S. An L-arginine:nitric oxide pathway present in human pletelets regulates aggregation. *Proc Natl Acad Sci USA* 87:5193-5197, 1990.

Rand MJ, Li GG. Nitric oxide in the autonomic nervous system. In: *Nitric oxide in nervous system.* Ed. S.Vincent. Acad Press pp 227-281, 1995.

Rengasamy A, Johnson RA. Regulation of nitric oxide synthase by nitric oxide. *Mol Pharmacol* 44:124-128, 1993.

Rogers NE, Ignarro LJ. Constitutive nitric oxide synthase from cerebellum is reversible inhibited by nitric oxide formed from L-arginine. *Biochem Biphys Res Commun* 189:242-249, 1992.

Rossanit R. Nitric oxide: a new area in intensive care. *Press Med* 23:855-858, 1994.

Rossaint R, Kelly K, Kaisers U. Present role of nitric oxide inhalation in severe lung failure. *Acta Anaesthesiol Scand* 109:88-92, 1996.

Sangruchi T, Kowall NW. NADPH diaphorase histochemistry of the human hypothalamus. *Neurosci* 40:713-724, 1991.

Schmidt HHHW, Gagne GD, Nakane M, Pollock JS, Miller MF, Murad F. Mapping of NO synthase in the rat suggests co-localization with NADPH diaphorase but not soluble guanylyl cyclase and novel paraneural functions for nitrinergic signal transduction. *J Histochem Cytochem* 49:1439-1456, 1992.

Schmidt HHHW, Lohmann SL, Walter U. The nitric oxide and cGMP signal transduction pathway. *Biochem Biphys Acta* 1178:153-175, 1993.

Schmidt HHHW, Walter U. NO at work. *Cell* 78:919-925, 1994.

Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increase coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circulation Res* 74:349-353, **1994.**

Silvagno F, Xia H, Bredt DS. Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J Biol Chem* 19:11204-11208, **1996.**

Snyder SH, Bredt DS. Nitric oxide as a neuronal messenger. *Trends Pharmacol Sci* 12:125-128, **1991.**

Snyder SH, Bredt DS. Biologiocal roles of nitric oxide. *Scientific American* 5:28-35, **1992.**

Snyder SH. More jobs for that molecule. *Nature* 372:504-505, **1994.**

Stadler J, Billiar TR, Curran RD, Stuehr DJ, Ochoa JB, Simmons RL. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. *Am J Physiol* 260:C910-C916, **1991.**

Southan GJ, Zingarelli B, O'Connor M, Salzman A, Szabo C. Spontaneous rearrangement of aminoalkilisothioureas into mercaptoalkylguanidines, a novel class of nitric oxide synthase inhibitors with selectivity towards the inducible isoform. *Br J Pharmacol* 117:619-632, **1996.**

Tang FR, Tan CK, Ling EA. The distribution of NADPH-d in the central grey region (lamina X) of rat upper thoracic spinal cord. *J Neurocytol* 24:735-743, **1995.**

Terenghi G, Riveros-Moreno V, Hudson LD, Ibrahim NBN, Polak JM. 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, **1993.**

Toda N. Non-adrenergic non-cholinergic innervation in monkey and human cerebral arteries. *Br J Pharmacol* 71:281-283, **1981.**

Tracey WR, Nakane M, Pollock JS, Fostermann U. Nitric oxide synthase in neuronal cells, macrophages and endothelium are NADPH diaphorases, but represent only a fraction of total cellular NADPH diaphorase activity. *Biochem Biphys Res Commun* 15:1035-1040, **1993.**

Tracey WR, Nakane M, Basha F, Carter G. In vivo pharmacological evaluation of two novel type II (inducible) nitric oxide synthase inhibitors. *Can J Physiol Pharmacol* 73:665-669, **1995.**

Vallance P, Collier J, Moncada S. Nitric oxide synthesised from L-arginine mediates endothelium-dependent dilatation in human veins in vivo. *Cardiovasc Res* 23:1053-1057, **1989.**

Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibition of nitric oxide synthesis in chronic renal failure. *Lancet* 339:572-575, **1992a.**

Vallance P, Leone A, Calver A, Moncada S. Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *Cardiovasc Pharmacol* 20 Suppl 12:S60-62, **1992b.**

Vallance P, Moncada S. Role of endogenous nitric oxide in septic shock. *New Horizons* 41:77-87, **1993.**

Vallance P, Moncada S. Nitric oxide-from mediator to medicines. *J Royal Coll Phys* London 28:209-219, **1994.**

Vincent SR, Hope BT. Neurons that say NO. *Trends Neuro Sci* 15:108-113, **1992.**

Vincent SR, Kimura H. Histochemical mapping of nitric oxide synthase in the rat brain. *Neurosci* 46:755-784, **1992.**

Vincent SR. Nitric oxide: a radical neurotransmitter in the central nervous system. *Prog Neurobiol* 42:126-160, **1994.**

Wang Y, Marsden PA. Nitric oxide synthase: biochemical and molecular regulation. *Curr Opin Nephrol Hypertens* 1:12-22, **1995.**

Wetts R, Vaughn JE. Choline acetyltransferase and NADPH diaphorase are co-expressed in rat spinal cord neurons. *Neurosci* 63:1117-1124, **1994.**

White KA, Marletta MA. Nitric oxide synthase is a cytochrome P-450 type haemoprotein. *Biochem* 31:6627-6630, **1992.**

Wolff DJ, Gribin BJ. The inhibition of the constitutive and inducible nitric oxide synthase isoforms by indazole agents. *Arch Biochem Biphys* 311:300-306, **1994**.

Woolfson R, Postin L. Effect of $N^G$-monomethyl-L-arginine on endothelium-dependent relaxation of human subcutaneous resistant arteries. *Clin Sci* 79:273-278, **1990**.

Yang Z, Von Sesseger L, Bauer T, Stilz P, Turina M, Lüscher F. Different activation of the endothelial L-arginine and cyclooxigenase pathway in the human internal mammary artery and saphenous vein. *Circ Res* 68:52-60, **1991**.

Zembowicz A, Vane JR. Induction of nitric oxide synthase by toxic shock syndrome toxin in a macrophage/monocyte cell line. *Proc Natl Acad Sci USA* 89:2051-2055, **1992**.

# CHAPTER 2

## PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF NITRIC OXIDE

NO is an ubiquitous cellular messenger that plays a role in a variety of biological processes. Several functions for this new regulatory molecule have been identified in the nervous system, in the endothelium-dependent vasodilatation and in the host-defence mechanisms (Snyder and Bredt, 1992). NO can be synthesised by a variety of tissues throughout the body, but the brain is the richest source of NOS under physiological conditions. Because both NOS and soluble guanylyl cyclase are widely distributed in the nervous system, it is likely that NO is associated with other mediator systems, not only in the CNS but also in the sensory and motor areas of the PNS, where NO is produced in wide variety of neurons (Moncada *et al.*, 1991). NO formation is linked to NMDA receptor activity in the brain and to neuronal nicotinic receptor activity in myenteric neurons (Bredt, 1996). NO signalling is not restricted to defined synapses and the actions of NO are not limited to anatomical structures or cerebral circulation, but provide transient functional localization based on transcellular diffusion (Bredt *et al.*, 1991). While NO has a short half-life (~7 sec), this diffusible intra- and intercellular messenger molecule possesses tremendous potential to modulate neurotransmission (Gally *et al.*, 1990).

NO is rapidly emerging as one of the main signal molecules in the CNS and PNS (Snyder and Bredt, 1992). NO is now recognized as a major, atypical, neuronal messenger (Bredt, 1996), acting both as a *neurotransmitter* and as a *second messenger* in diverse biological functions (Snyder and Bredt, 1991,1992; Moncada and Higgs, 1993; Bredt, 1996). In some parts of CNS and in PNS, NO might be formed presynaptically and thus act as a neurotransmitter. Within CNS, NO also acts as a retrograde messenger, being released postsynaptically to act on presynaptic nerve endings and astrocytes (Garthwaite, 1991).

### 2.1 Central Nervous System

Histochemical studies suggest a widespread role for the NO/cGMP system in the CNS (Bredt *et al.*, 1990; Springall *et al.*, 1992). Only 2% of brain neurons contain NOS, but these neurons ramify sufficiently to contact almost all brain cells (Bredt *et al.*, 1990; Dawson *et al.*, 1992). This latter point highlights the potential importance of NO in CNS. The overall NOS activity varies by about 8-fold over all of the brain regions (Forsterman *et al.*, 1990; East and Garthwaite, 1991). All regions of the brain have the potential to produce surprisingly high concentrations of NO, though these concentrations are far lower than what has been used *in vitro* to show toxicity (Varner and Beckman, 1995).

Within the brain NO can originate from four different sources: neurons, endothelium of cerebral vasculature, NANC nerves and immunostimulated microglia and astrocytes (Bruhwyler *et al.*, 1993). NOS is found in excitatory GLU or inhibitory gama-aminobutyric acid (GABA) neurons. Indeed, NO would appear to rank with these latter two as one of the most common of all neuronal messenger molecules. The differential staining of nerve cell bodies, dendrites and axon terminals in various brain regions may well indicate different modes of NO transmission. In some situations, NO would be expected to be released from the cell bodies and dendrites in response to $Ca^{2+}$ influx following synaptic activation. In other areas, where NOS is concentrated in nerve terminals, one might predict NO synthesis to be triggered by $Ca^{2+}$ influx following action potential invasion of the nerve ending. Given that

the NO receptor appears to be soluble guanylyl cyclase, the varied actions of NO in these different situations may well be mediated by multiple actions of cGMP and its targets in the neurons responding to NO (Vincent, 1995).

Physiological functions of NO in the CNS are implicated by the discrete and highly conserved distribution of NOS in subpopulation of central neurons of all mammalian brains so far examined (Hope *et al.*, 1991; Bredt *et al.*, 1991). Accordingly, some NO actions include:

- *Modulation of neurotransmitter release* (Schuman and Madison, 1994; Guevara-Guzman *et al.*, 1994; Montague *et al.*, 1994),
- *Memory and learning* (Bohme *et al.*, 1991, 1993; Snyder and Bredt, 1992; Schuman and Medison, 1991, 1994; Shibuki and Okada, 1991),
- *Vigilance* (Dzoljic and De Vries, 1994; Bagetta *et al.*, 1994; Nistico *et al.*, 1994; Dzoljic *et al.*, 1996) *and resetting of the circadian rhythm* (Amir *et al.*, 1995),
- *Nociception* (Meller *et al.*, 1990, 1993),
- *Control of sensory functions* (Breer and Shepherd, 1993; Koch *et al.*, 1994),
- *Intake of water and food* (Bruhwyler *et al.*, 1993; Amir, 1995),
- *Modulation of behaviour* (Chapman *et al.*, 1992; Nelson *et al.*, 1995),
- *Regulation of neuronal excitability, including seizures* (Kirkby *et al.*, 1996; Facciolo *et al.*, 1996; Proctor *et al.*, 1996) and *anxiety* (Facciolo *et al.*, 1996),
- *Involvement in trophic functions* (Williams *et al.*, 1994; Bredt and Snyder, 1994; Northington *et al.*, 1997),
- *Control of cerebrovascular system* (Bruhwyler *et al.*, 1993; Faraci and Brian, 1994; Van Gelderen and Saxena, 1994; Van Gelderen *et al.*, 1995),
- *Regulation of inflammation* (Salvemini *et al.*, 1995; Schneider-Schaulies *et al.*, 1993; Bohne *et al.*, 1994; Koprowski *et al.*, 1993),
- *Involvement in trauma* (Mesenge *et al.*, 1996),
- *Neurotoxicity* (Dawson *et al.*, 1991; Snyder and Bredt, 1992; Dawson and Dawson, 1996; Iadecola, 1997).

### *2.1.1 Neurotransmitter Release*

Experimental findings support a role for NO in the modulation of *neurotransmitter release,* affecting the presynaptic membrane as a retrograde messenger. The mechanism by which it acts might involve cGMP as a second messenger (O'Sullivan and Burgoyne, 1990). NO donors (i.e. sodium nitroprusside, nitrosothiols, etc) have been shown to stimulate the release of GLU (Prast and Phillipu, 1992) as well as aspartate (ASP). Furthermore, NO inhibits the uptake of GLU (Lonart and Johnson, 1994; 1995), while NOS inhibitors decreased the release of GLU (Montague *et al.*, 1994). Stimulation of excitatory neurotransmitters release might be of importance in neuropsychiatric diseases. The disturbance of the GLU activity underlies *schizophrenia* (Schmidt, 1990, 1991; Bruhwyle *et al.*, 1993). Sodium nitroprusside induces increase of the endogenous striatal dopamine (DA) release, attenuated by L-NMMA (Prast and Philippu, 1992; Zhu and Luo, 1992). These data implicate the possibility of manipulations of the CNS functions, through modulation of NO system and consequently DA, to correct DA deficiency in *Parkinson's disease* (Bruhwyler *et al.*, 1993; Connop *et al.*, 1996). Moreover, NO might be able to evoke the catecholamine release at sites located at some distance from the sites of NO formation (Youdim and Lavie, 1994). NO donors

stimulate release of acetylcholine (Ach) from basal forebrain (BF, Prast and Phillipu, 1992), while NOS inhibitors decreased the release of Ach (Prast and Philippu, 1992; Lonart *et al.*, 1992). The brain regions containing cholinergic systems are involved in cognitive function. It has been reported that NO is implicated in a number of acute and chronic neurodegenerative conditions associated with impaired memory, such as *Alzheimer's disease* (Meldrum and Garthwaite, 1990).

### *2.1.2 Memory and Learning*

As a retrograde messenger NO affects a long term potentiation (LTP, O'Dell *et al.*, 1991) and long term depression (LTD, Shibuki and Okada, 1991), the fundamental mechanisms of *learning and memory* formation by which a particular neuron 'remembers' the signals previously received (Snyder and Bredt, 1992; Schmidt and Walter, 1994; Vallance and Moncada, 1994). Memory involves a long-term increase or decrease in transmission across certain synapses after the repetitive stimulation of neurons. In memory models, particular neurons have been repetitively stimulated and then a persistent increase or decrease in synaptic transmission has been detected (Snyder and Bredt, 1992). NO released from the postsynaptic neuron acts as retrograde messenger on the presynaptic terminal to increase neurotransmitter release, enhance the postsynaptic response and increase firing of the target neuron. Thus, NO affects LTP and LTD (O'Dell *et al.*, 1991; Chapman *et al.*, 1992; Schuman and Medison, 1991, 1994). This activity-dependent synaptic efficiency seems to be the mechanism by which mammals learn and remember (Chapman *et al.*, 1992). Additionally, NO would have many actions which can act to alter both *short-term and long-term synaptic events* that change cellular function. Short-term plasticity can last for several hundred milliseconds, while synaptic strength can also be persistently enhanced or depressed for periods of hours to days during long-term plasticity (Bliss and Collingride, 1993; Linden, 1994). Because NO is freely diffusible it may affect the plasticity across many synapses in local domain (Schuman and Madison, 1994). *In vivo*, processes of *spatial learning and memory* were interrupted by NOS inhibitors, interfering with the acquisition and retention of learned behaviour tasks on spatial learning (Bohme *et al.*, 1991, 1993). LTD has been reported to be prevented by an NOS inhibitor and methylene blue, an inhibitor of guanylyl cyclase (Schuman and Medison, 1994).

### *2.1.3 Vigilance*

NO mediates a short term electrocortical activation, an alerting response important in control of the *arousal state* (Bagetta *et al.*, 1993). Cholinergic neurons arising from the mesopontine tegmental nuclei contain NOS (Vincent and Hope, 1992). Released NO is involved in mechanisms supporting neuroactivity that is conducible to *wakefulness* (Pape and Mager, 1994). Accordingly, a significant decrease of brain NO levels has been found during slow wave *sleep* (Burlet *et al.*, 1995) compared to wakefulness or rapid eye movement (Williams *et al.*, 1997), known as vigilance stages with increased neuronal activity. It was found that NOS activity in the rat brain is higher during the dark phase (active period), compared to the light phase (sleeping period, Kàpas *et al.*, 1995). Results are consistent with findings that NOS inhibitors decrease wakefulness (Dzoljic and de Vries, 1994; Dzoljic *et al.*, 1996). Furthermore, blockade of NMDA receptors, NO synthesis, or cGMP production in the

suprachiasmatic nucleus inhibited the stimulating effect of light on heart rate. This extends a role of NO in the brain *circadian rhythm* (Spessert *et al.*, 1995; Amir and Edelstein, 1997).

### *2.1.4 Pain Perception*

The NO system is involved is *nociception* (Schmidt and Walter, 1994). It has been shown that NO is involved in both spinal and supraspinal processing of pain (Moore *et al.*, 1991; Babbedge *et al.*, 1993a,b; Meller and Gebhart, 1993). In different *pain* models, NO induces spinal hyperalgesia, resulting in facilitation of peripheral inputs and central sensitization (Meller and Gebhart, 1993; Stanfa *et al.*, 1996). Along with the importance of activation of the NMDA receptor in the lumbar dorsal horn for the maintenance of thermal hyperalgesia and chronic pain (Woolf *et al.*, 1991; Meller et Gebhart, 1993), it has been demonstrated that the NO system in the spinal cord is required for the acute nociceptive effects of NMDA. Moreover, immunocytochemistry has demonstrated that the dorsal horn of the spinal cord is also a site of action of NO. This is consistent with data showing the ability of intrathecally administered NOS inhibitors to block hyperalgesia. Likewise, L-NAME produced a potent and stereoselective antinociceptive effect in the formalin-induced paw-licking test, the acetic acid-induced abdominal constriction test and the hot-plate test (Moore *et al.*, 1991). Furthermore, L-NAME retains a considerable antinociceptive effect following intracerebroventricular (i.c.v.) administration. This supports the hypothesis of central mechanisms of nociceptive NO action (Babbedge *et al.*, 1993a,b; Moore *et al.*, 1993a,b). Thus, NOS inhibition or inactivation of guanylyl cyclase might serve as useful analgesics (Meller et Gebhart, 1993; Moore *et al.*, 1993a,b; Gaffen *et al.*, 1994).

In addition, NO is involved in peripheral analgesia. The peripheral *analgesic effect* of Ach has also been proposed to be mediated via NO (Duarte *et al.*, 1990). Ach, L-Arg and sodium nitroprusside induced analgesia in an animal model, and L-NMMA prevented the analgesia induced by both Ach and L-Arg. Whether this is the result of NO generation in the nociceptors or in cells closely associated with them and whether the NO pathway controls the input of nociceptive information into the nervous system requires further investigation (Moncada *et al.*, 1991). After damage of the peripheral axons of sensory neurons, a marked up-regulation of the NOS expression in primary sensory neurons occurs in spinal ganglia (Hökfelt *et al.*, 1994).

### *2.1.5 Sensory Function*

NO may be involved in the *sensory functions*, including adaptive regulation of visual and olfactory signalling. It has been shown that by enhancing or attenuating the receptor responses of one neuron in an NO-dependent mechanism with strong stimulation (high odorant concentration, strong light), may either lead to the recruitment of adjacent neurons to augment signal intensity or may allow for adaptation (to odour, light intensity) or cross-adaptation (different odours; Breer and Sheperd, 1993; Koch *et al.*, 1994).

### *2.1.6 Food and Water Intake*

NOS inhibitors reduce food intake in food-deprived or benzodiazepine- chlordiazepoxide-treated mice. L-Arg reverses these effects of NOS inhibitors (Morley and Flood, 1991; Czech, 1996). Therefore, it has been suggested that, along with noradrenaline (NA) or adrenaline, NO could be an intercellular mediator for the physiologic modulation of *food*

*intake*, while NOS inhibitors might be suitable agents for the treatment of obesity (Bruhwyler *et al.*, 1993). This is in accordance with the finding that endogenous NO-stimulated release of NA (Guevara-Guzman *et al.*, 1994; Montague *et al.*, 1994) inhibits the uptake of NA (Lonart and Johnson, 1994; 1995), while NOS inhibitors decrease the release of NA and adrenaline (Montague *et al.*, 1994) and enhance NA uptake (Kiss *et al.*, 1996). While *water intake* may be influenced by different mechanisms (direct action of thirst neurons, release of substances that affect these neurons or altered water or electrolyte balance in the body), L-Arg is able to inhibit drinking in water-deprived rats, when injected into the preoptic area (Calapai *et al.*, 1992). It has been shown that NOS levels within the hypothalamo-neurohypophyseal system are increased (upregulated) during neurohormone demand, such as that following osmotic stimulation (Luckman *et al.*, 1997). In addition, concerning other *autonomic regulation*, it has been found recently that the human ventrolateral medulla, the region which is critically involved in cardiovascular, respiratory and autonomic functions, contains abundant NADPH-d reactive neurons, in close vicinity to catecholamine neurons (Benarroch and Smithson, 1997).

### *2.1.7 Behaviour*

NO has also been implicated in different forms of *behavioural control* (Bohme *et al.*, 1991, 1993; Chapman *et al.*, 1992; Hölscher and Rose, 1992). For example, studies with NOS knockout mice have shown an increase in *aggressive behaviour* and excessive inappropriate *sexual behaviour* in these animals (Nelson *et al.*, 1995). The importance of compensatory mechanisms in mice without NOS for contribution of NOS in cell physiology and behaviour is still unknown (Good, 1996). Moreover, NOS inhibitors decreased *locomotor activity* (Pudiak *et al.*, 1993; Sandi *et al.*, 1995; Star and Star, 1995) and the righting reflex (Dzoljic *et al.*, 1996).

### *2.1.8 Neuronal Excitability*

NOS inhibitors have shown contradictory results in affecting neuronal excitability, exerting anticonvulsant (Osonoe *et al.*, 1994; Van Leeuwen *et al.*, 1995) or proconvulsant (Starr and Starr, 1993; Rundfelt *et al.*, 1995) effects. However, it has been demonstrated that pro- or anti-convulsant activity of NOS inhibitors vary depending on numerous factors, including the model of seizure, dose, route and time of administration of NOS inhibitors (Kirkby *et al.*, 1996). These behavioural variations could very likely be a consequence of NO interacting in a regional-specific manner with the different neurotransmitter propagating system (Facciolo *et al.*, 1996). On the basis of the effects exerted by L-NAME, the influences of this NOS inhibitor on convulsive behaviours were linked to the variations of benzodiazepine binding activities in the limbic areas. These areas are, apart from their involvement in memory and spatial tasks, strongly linked with the control of sociosexual behaviours, especially of the aggressive-defensive type (Unmeto *et al.*, 1975). It has been shown that GABA-ergic terminals contain NOS (Valtschanoff *et al.*, 1993) and $GABA_A$ receptor activity is modulated by NO (Zarri *et al.*, 1994). On the basis of recent results it has been suggested that the interaction between the NOS inhibition and $GABA_A$ complex may exert an important neuroprotective role in the control of *convulsive and anxiolytic behaviours* (Facciolo *et al.*, 1996). In accordance with that hypothesis are results that NOS inhibitors, microinjected into dorsal central grey, exert anxiolytic effect (Guimaraes *et al.*, 1994). Additionally, NOS

inhibitors block seizures induced by $GABA_A$ antagonist, bicuculline (Proctor *et al.*, 1996). In that epileptic model the removal of local GABA-ergic inhibition allows seizures to occur as a result of unopposed glutaminergic excitation within limbic region. Whereas activation of NMDA GLU receptor triggers synthesis of NO (Snyder, 1991), NO may in turn act to potentiate the excitatory influence of GLU, acting as a retrograde messenger to stimulate depolarization-dependent release of GLU (O'Dell *et al.*, 1991; Schuman and Medison, 1994). cGMP has also been suggested to play a role in seizures, as perfusion of cGMP analogues into the hippocampal grafts triggers epileptiform activity (Freedman *et al.*, 1979) and cGMP levels increase in several brain region prior to the onset of seizure (Ferrendelli *et al.*, 1980). It has been reported that NO participates in both functional and dysfunctional consequences of excitatory neurotransmission (e.g. neuronal excitotoxicity and epilepsy) via cGMP (Dawson *et al.*, 1991). Based on this, NO may contribute to the epileptogenesis in response to excessive GLU-mediated excitation (Proctor *et al.*, 1996). In addition, EAA mediated convulsant activity may occur through the subsequent activation of NOS, as i.c.v. injections of exogenous L-Arg induce behavioural and electroencephalographic (EEG) activation (Mollace *et al.*, 1991). L-Arg also displays proconvulsant effects in rats treated with subconvulsive doses of NMDA and these effects are prevented by L-NAME. Solutions of NO instilled i.c.v. produce brief but violent convulsive episodes in rats (Smith *et al.*, 1991). Furthermore, results from animal models of epilepsy have shown that NO mediates the increase in cerebral blood flow (CBF) following application pentylenetetrazol (Faraci *et al.*, 1993) and bicuculline (Pereira de Vasconceros *et al.*, 1995). Recent data implicate a neuronal source of NO playing a role in the increase in CBF evoked by kainate-induced seizures (Montécot *et al.*, 1997) or cerebral vasodilatation in response to NMDA (Faraci and Brian, 1995). Accordingly, the role of NO in seizure phenomena is complex and still unclear.

### *2.1.9 Trophic Functions*

With regard to potential cellular sites of action, these include a number of enzymes involved in cellular metabolism and DNA synthesis. These actions of NO clearly play a role in the modulation of cellular activity and cellular proliferation. Thus, it has been reported that in the nervous system, NO may serve a trophic function, acting as a retrograde messenger to ingrown axons, thereby establishing other synaptic connections (Williams *et al.*, 1994; Bredt and Snyder, 1994; Northington *et al.*, 1997). Additionally, the transient expression of n-NOS in the nervous system may reflect a role in developmental processes (Bredt and Snyder, 1994; Mendez-Medina *et al.*, 1994). Likewise, it has been demonstrated that the activity of NOS showed a developmental pattern associated with the maturative processes of the brain (Lizasoain *et al.*, 1996) and retina (Ientile *et al.*, 1996). It is proposed that high levels of NO production occurs immediately before *synaptogenesis* (Ientile *et al.*, 1996).

### *2.1.10 Cerebral Circulation*

A unique feature of cerebral circulatory control is the weak functioning of adrenergic vasoconstrictor nerve and the dominant functioning of *NANC vasodilator nerves* (Kelly *et al.*, 1995). The results strongly support the hypothesis that NO acts as a neurotransmitter in the vasodilator nerves in the cerebral arteries (Toda *et al.*, 1990a,b; Toda and Okamura, 1991). NANC axons are abundant in the proximal anterior and middle cerebral arteries, but are less numerous in small pial arteries (Adachi *et al.*, 1992; Northington *et al.*, 1992; Toda, 1993;

Faraci and Brian, 1994; Van Gelderen and Saxena, 1994; Van Gelderen *et al.* 1995). The role of NO in the regulation of basal CBF and activity-dependent cerebrovascular dilatation in experimental models is supported by a significant amount of data (Faraci *et al.*, 1993; Dirnagl *et al.*, 1994; Faraci and Brian, 1994; Zhang *et al.*, 1994). NANC derived NO acts to maintain cerebrovascular tone and necessary CBF in the brain even in a circulatory emergency. It has been shown that characteristics in the production and action of NO are similar in cerebral NANC nerves and endothelial cells (Toda and Okamura, 1991). In some primate arteries, the vasodilator nerve function develops with age, although it has not been determined whether this is due to age-dependent maturation of the perivascular nerves or to the increased sensitivity with age of the smooth muscle to nitro-vasodilators. NOS inhibitors can impair the response to nerve stimulation but do not affect the relaxation caused by NO (Toda *et al.*, 1990a,b; Toda and Okamura, 1991). These data are sufficient to support the concept of neurohumoral transmission with NO from vasodilator nerves to cerebroarterial smooth muscle. NO released from endothelium also mediates the cerebroarterial dilatation in response to chemical (vasodilator substances) or physical (blood flow change) stimuli. Neurogenic responses via NO are similarly observed in primate and subprimate mammals, while the response mediated via endothelial-synthesised NO following activation of drug receptors frequently vary between animal species. Recent studies on cultured smooth muscle cells indicate that the NO production is also due to expression of i-NOS following cytokine or lipopolysaharide (LPS) stimulation (Buse *et al.*, 1992). Nevertheless, NO derived from both nerve and endothelium undoubtedly plays an important role in the regulation of cerebral vascular tone *in vitro* and *in vivo*. Changes in vascular functions caused by NOS inhibitors *in vivo* reflect the physiological role of NO released from nerve and/or endothelium under resting conditions (Toda, 1993).

In addition, anatomical and neurochemical evidence suggests a role of cholinergic BF neurons in the *regulation of cortical CBF* via the NO/cGMP pathway (Bruhwyler *et al.*, 1993). Excitotoxic destruction of BF neurons resulted in reduced cholinergic innervation of the cerebral cortex and a corresponding decrement in CBF when loss of cholinergic innervation exceeds 40% (Arneric, 1989). The most plausible mechanism suggested is that freely diffusible NO, as a second messenger transmitter, was released from BF neurons, activating guanylyl cyclase in adjacent cells (Dirnagl *et al.*, 1994; Ishizaki *et al.*, 1991; Toda and Okamura, 1991). Increasing substrate availability to NOS with intravenous L-Arg administration modestly enhanced the BF-elicited response, while a NOS inhibitor blocked the BF-elicited cortical CBF response (Raszkiewicz *et al.*, 1992).

Similarly, it has been shown that NO participates in the regulation of CBF and cerebro-vascular resistance (CVR) under normoxic condition (Kozniewska *et al.*, 1992; Wang *et al.*, 1995). Lately it has been suggested that NO plays an even more important role in regulating cerebral vascular tone and enhances CBF during hypoxemia than normoxemia (Crabb and Harding, 1996). Thus, in conditions of cerebral ischaemia n-NOS and its mRNA have been shown to increase (upregulated) in NANC neurons (Zhang *et al.*, 1994). However, one of the most important aspects of the cerebral circulation is the coupling of the cerebral metabolic activity and the CBF. NO appears as a key coupling molecule that links changes in CBF and metabolism. It has been shown that NOS inhibitors reduce neuronally induced regional CBF responses (Goadsby *et al.*, 1992; Northington *et al.*, 1992; Lindauer *et al.*, 1996).

Concerning *migraine*, pain is probably due to depolarization of perivascular NANC nerve terminals (Moskowitz and Macfarlane, 1993). Large cerebral blood vessels contain far more n-NOS than small ones. The connection between cerebral blood vessel diameter and n-NOS levels fits with physiologic studies showing a prominent NANC relaxation mechanism in large cerebral vessels, which can play a role in the pathophysiology of migraine (Toda, 1981; Snyder and Bredt, 1991).

Moreover, the findings using NOS inhibitors suggest that production of NO contributes to disruption of the *blood-brain barrier* during various inflammatory conditions (Hurst and Clark, 1997), multiple sclerosis (Johnson *et al.*, 1995; Thompson *et al.*, 1992), bacterial meningitis (Boje *et al.*, 1996), cerebral ischaemia (Chi *et al.*, 1994) and acute hypertension (Mayhan, 1995). In acute hypertension NOS inhibitors prevent NO-mediated disruption of the blood-brain barrier (Mayhan, 1995).

In addition to the endothelial-derived NO within the nervous system, it has been shown that NO is produced in other extraneuronal tissues. Glial cells (mainly microglia) also synthesize NO via i-NOS. Astrocytes express i-NOS and n-NOS, although at lower levels than neurons (Murphy *et al.*, 1995).

### *2.1.11 Inflammation*

During *systemic inflammation* induced by intraperitoneal injection of LPS, cerebral levels of i-NOS mRNA are affected, with increased expression in the anterior pituitary, chorioid plexus and meninges and, later on, in the paraventricular nucleus and arcuate nucleus. This is accompanied with production of NO in the brain parenchyma and in the CSF (Mitrovic *et al.*, 1994, 1995). Since cytokines and LPS are not present in the normal brain in the quantities that cause induction of the enzyme *in vitro*, results have shown that i-NOS expression will occur *in vivo* only under pathological condition, such as *inflammation/infection or injury*, while NOS inhibitors can have antiinflammatory effects (Salvemini *et al.*, 1995). Evidence for induction of i-NOS in CNS *in vivo* has only recently been found and are provided by models of acute CNS infections, autoimmune reactions, ischaemia, seizure, migraine, spinal hyperalgesia, opioid tolerance and traumatic injury. In a number of these studies, especially those using NOS inhibitors, it is not clear whether the NOS induced is i-NOS or c-NOSs (n-NOS or e-NOS). Therefore, selective NOS blocking drugs are essential for evaluating the role of NO in physiological and pathophysiological processes with particular clinical and therapeutic implications (Mesenge *et al.*, 1996).

During acute or chronic *viral infection* in the CNS a variety of cytokines are produced in increased amounts, including interferon gamma and interleukin-1 (Frei *et al.*, 1988; Beveniste, 1992; Schneider-Schaulies *et al.*, 1993). Studies demonstrate that i-NOS is expressed *in vivo* in the brain during acute viral infection (Adams *et al.*, 1990). Recently, such induction of i-NOS has been reported *in vivo* in other CNS infections, such as acute *cerebral toxoplasmosis* (Gazzinelli *et al.*, 1993) and reduced replication of *Toxoplasma gondii* with NO produced either by i-NOS or released from sodium nitroprusside (Bohne *et al.*, 1994). Additionally, a possible cytotoxic role for NO under such conditions has been suggested by the fact that macrophages expressing i-NOS and producing NO have been shown to be cytotoxic for cells infected with intracellular pathogens (Adams *et al.*, 1990) and tumour cells (Stuehr and Nathan, 1989). This toxicity could result in the promotion of the

clearance of virus from infected cells and/or contribute to tissue damage through cytotoxic effects on uninfected neighbour cells.

Furthermore, expression of i-NOS has been reported during *experimental allergic encephalitis* (EAE), an autoimmune demyelinating process considered as a model for *human multiple sclerosis* (MS; Koprowski *et al.*, 1993). When stimulated by interferon-α and interleukin-ß *in vitro* human astrocytes produce NO, whereas microglial cells generate oxygen radicals. i-NOS is present in MS lesions (Okuda *et al.*, 1995) and its inhibition attenuates the development of EAE in rodents (Zhao *et al.*, 1996). Brains of animals with EAE have been shown to express increased levels of cytokines (Benveniste, 1992). Additionally, cytokine-activated microglial cytotoxicity for oligodendrocytes *in vitro* has recently been shown to be mediated by NO (Merrill *et al.*,l., 1993). Therefore, a glial source and possible cytotoxic role for NO produced by i-NOS during CNS demyelination *in vivo* is suggested. In the experimentally produced autoimmune disease EAE, microglia destroy oligodendrocytes in a NO-dependent fashion (Merrill *et al.*, 1993). i-NOS-positive infiltrating macrophages have been found in necrotic areas, thus, the damage of myelin and oligodendrocytes in MS results in a cytokine-mediated increase in NO production by macrophages/microglia (Okuda *et al.*, 1995). Increased i-NOS expression has been found in rats developing EAE and this preceded detectable clinical signs and symptoms (Koprowski *et al.*, 1993). However, administration of L-Arg derived NOS inhibitors in experimental autoimmune neuritis and experimental autoimmune encephalomyelitis had only little or no effect (Zielasek *et al.*, 1995).

### *2.1.12 Trauma*

Similar to inflammatory processes in the CNS, *traumatic injury* of the brain results in an increased expression of cytokines such as interleukin-1 by resident cells and infiltrating macrophages, creating an environment conductive for induction of i-NOS *in vivo*. In two models of traumatic injury, cerebral stab wound and ventral spinal root avulsion, expression of i-NOS *in vivo* has been reported (Moncada *et al.*, 1991; Vallance and Moncada, 1994; Änggård, 1994; Dawson and Dawson, 1996). Additionally, NOS inhibitors reduced neurological deficit following traumatic brain injury (Mesenge *et al.*, 1996).

### *2.1.13 Neurotoxicity*

NO may be involved in pathologic changes occurring with *ischaemia and seizures* (Dawson *et al.*, 1991). It is suggested that NO plays a role in NMDA receptor-mediated excitotoxicity (Dawson *et al.*, 1991) while n-NOS inhibitors may be useful in the treatment of neurologic diseases in which excitotoxic mechanisms play a role (Buisson *et al.*, 1992; Dawson and Snyder, 1994; Schulz *et al.*, 1995). Thus, neuronal NO production appears to exacerbate acute ischaemic injury, whereas vascular NO protects after middle cerebral artery occlusion. The data emphasize the importance of developing selective inhibitors of the n-NOS (Huang *et al.*, 1994). Moreover, NOS has also been shown to release reactive oxygen intermediates in the presence of low concentrations of L-Arg or tetrahydrobiopterin (Heinzl *et al.*, 1992; Pou *et al.*, 1992). Furthermore, NO reacts with reactive oxygen intermediates such as superoxide and peroxide, forming even more toxic peroxynitrate anions, which have been implicated in the development of many diseases such as ischaemia reperfusion injury and inflammation (Beckman *et al.*, 1990; Radi *et al.*, 1991; Noronha-Dutra *et al.*, 1993; Kooy and

Royall, 1994). Therefore, it is possible that not only NO but also reactive oxygen radicals are involved in tissue injury, while NOS inhibitors could be protective (Ishii *et al.*, 1997).

Inhibition of NOS has yielded conflicting effects upon the outcome of *cerebral and spinal cord insults*. In contrast to results obtained with non-selective NOS inhibitors, studies using n-NOS and i-NOS inhibitors have uniformly showed reduction in infarct size. These findings suggest that both n-NOS and i-NOS activity are detrimental to the ischaemic brain, while e-NOS activity might be protective, at least in the early stages (Margaill *et al.*, 1997). Accordingly, immediately after ischaemia, intravascular administration of L-Arg or of NO donors as sodium nitroprusside and 3-morpholino sydnonimine reduces infarct size, improving blood flow in the penumbra, that surrounded ischaemic tissue. However, L-Arg is not effective when it is administered more than 30 min after induction of ischaemia, while NO generating drugs lose their efficacy more than 2 h post ischaemia. Evidently, beneficial effect of NO production at the vascular level is protective only during the very early stages of cerebral ischaemia (Iadecola, 1997). Consistent with increased glutaminergic transmission, NMDA excitability and consequent n-NOS activity in the brain ischaemia-reperfusion is resistance to GLU of the n-NOS knockout neuronal cultures and mice (Iadecola, 1997). Recent data support a neuroprotective role of NOS inhibitors in transient global ischaemia in the gerbil (Kohno *et al.*, 1997). A better understanding of the balance between the physiological functions of NO and the mechanisms of toxicity will determine the therapeutic potential of modulating NO production (Varner and Beckman, 1995).

The important role of GLU and $Ca^{2+}$ in mechanisms of *cell injury* induced by severe cerebral hypoxia or *ischaemia* has been emphasized (Chleide *et al.*, 1991; Dawson *et al.*, 1991). Convincing evidence related to the involvement of NO in neurotoxic processes has been obtained in experiments showing that production of NO following NMDA receptor activation induced extended damage in primary rat cortical cultures (Dawson *et al.*, 1991), and in rat hippocampal slices (Izumi *et al.*, 1992). Similarly, cortical cultures exposed to NO donors, sodium nitroprusside and S-nitroso-N-acetylpenicillamine exhibited a delayed neurotoxicity, which follows the same time course as NMDA neurotoxicity (Dawson and Dawson, 1996). Elimination of n-NOS through transgenic technology results in a culture that is resistant to NMDA neurotoxicity indicating that n-NOS neurons are the source of neurotoxic NO (Dawson and Dawson, 1996). Most neural destruction in stroke seems to result from massive release of GLU, which, activating NMDA receptors, with consecutive stimulation of NOS and increasing activity of the NO/cGMP system, causes excess excitation leading to neuronal death (Snyder and Bredt, 1992; Choi, 1993). This model is supported by the ability of NOS inhibitors to block the *neurotoxic effects* of GLU and NMDA in brain cultures (Dawson *et al.*, 1991). The results from culture have been translated into clinically relevant models, as in several species low doses of NOS inhibitors, administered after ligation of the middle cerebral artery, provide marked protection against stroke injury (Nowicki *et al.*, 1991). Thus, NO, whether endogenously synthesized or directly produced from an NO donor, seems to be a primary signal in the sequence of events leading to neuronal death (Loiacono *et al.*, 1992).

*Neurotoxic destruction* by NMDA of neurons in culture, a model for *stroke*, can kill 90% of neurons, whereas NADPH-d neurons are completely preserved (Snyder and Bredt, 1992). Furthermore, striatal neurons expressing NADPH-d are spared in *Huntington's chorea* (Ferrante *et al.*, 1985; Kowall *et al.*, 1987; Morton *et al.*, 1993), although a significant

decrease in the striatal neuropil NADPH-d staining occurs in this disease (Morton *et al.*, 1993). Moreover, NADPH-d positive neurons survive in greater numbers than neighbouring NADPH-d negative neurons in *Alzheimer's dementia* (Hyman *et al.*, 1992), *ischaemia* (Uemura *et al.*, 1990), some forms of *excitotoxicity* (Koh *et al.*, 1988) and in *amyotrophic lateral sclerosis* (ALS; Wetts and Vaughn, 1994). Something about NOS makes neurons resist neurotoxic damage. A paradox has arisen because the NOS product NO is also a result of GLU activity, while GLU itself is responsible for neurotoxicity. A possible explanation could be that NO is toxic for adjacent neurons (Snyder and Bredt, 1992, Rogers and Ignarro, 1992; Rengsamy and Johnson, 1993). However, expression of NOS does not necessarily make a neuron resistant to excitotoxicity (Endoh *et al.*, 1994). Highlighting that paradox could provide therapeutic opportunities for major neurologic pathological states, including Huntington's chorea, Alzheimer's dementia and stroke (Snyder and Bredt, 1992).

### 2.2 Non-Adrenergic Non-Cholinergic (NANC) Nerves

Nerves whose transmitter is neither Ach nor NA (NANC system; nitrergic neurons) are found in the cardiovascular, respiratory, gastrointestinal and genitourinary systems. In each of these systems, immunohistochemical studies have established that n-NOS is present in the NANC neurons (Bredt *et al.*, 1990), being concentrated in neuronal axons (Llewellyn-Smith *et al.*, 1992; Berezin *et al.*, 1994), and upregulated (increased in expression) after nerve injury (Wu *et al.*, 1994; Vizzard *et al.*, 1995). Smooth muscle cells contain the receptor for NO, soluble guanylyl cyclase. Thus, in specific pathways of the PNS, NO functions as a NANC neurotransmitter throughout the body, producing relaxation of smooth muscle in cerebral vasculature, respiratory, urogenital and gastrointestinal systems, where it is involved in vaso/bronchodilatation, gut peristalsis and penile erection (Burnstock, 1981; Bredt, 1996).

The peripheral NANC neurons usually use NO as one of many neuromediators to decrease smooth, cardiac and possibly skeletal muscle tone contractility and intracellular $Ca^{2+}$. *Cardiac NANC neurons* innervate the pacemakers and myocardium to mediate negative inotropic autonomic influences (Balligand *et al.*, 1993).

In human *bronchi in vitro*, bronchodilatation mediated by the NANC neurons is blocked by NOS inhibitors (Belvisi *et al.*, 1992) and results of NO inhalation by healthy humans (Gustafsson *et al.*, 1991) suggest the physiological role for NO in the mechanisms matching ventilation and perfusion (Wiklund *et al.*, 1990).

Within *the genitourinary system* NO relaxes smooth muscle of the upper and lower urinary tract (Persson *et al.*, 1993), uterus (Kurtzman *et al.*, 1993) and corpus cavernosum (Hibbs *et al.*, 1988). Recently NOS activity has been found in rat and human uterus (Kurtzman *et al.*, 1993), while administration of NOS inhibitors blocks labour in sheep (Heymann *et al.*, 1993). These results implicate that basal production of NO maintains the uterus in a quiescent state during pregnancy (Natuzzi *et al.*, 1993). NANC nerves have been immunohistochemically identified in human corpus cavernosum (Leone *et al.*, 1994), while NOS inhibitors block neurogenic relaxation of corpus cavernosum (Raifer *et al.*, 1992; Burnett at el., 1992). Thus, corpus cavernosum relaxes in response to stimulation of NANC nerves to cause penile erection. L-NMMA blocks erection in experimental animals (Burnett *et al.*, 1992), while NO donors promote erection in patients (Meyhoff *et al.*, 1992). It was previously thought that vasoactive substances, such as vasoactive intestinal peptide (VIP) and

substance P, were mediators of penile erection. Nevertheless, it has been demonstrated that NO is the major physiological mediator of penile erection.

In the *gastrointestinal system*, production of NO in response to nerve stimulation mediates adaptive relaxation of the stomach (the mechanism by which the gut accommodates food, Desai *et al.*, 1991), relaxation of sphincters (including sphincter Oddi; Kaufman *et al.*, 1993) and the relaxant part in the peristaltic cycle (Burleigh, 1992). In human gut, NANC neurons have been demonstrated in the myenteric plexus (Mearin *et al.*, 1993), sphincter Oddi (Kaufman *et al.*, 1993) and duodenal sphincter (Vanderwinden *et al.*, 1992).

In *striated muscle* of the oesophagus, NOS immunoreactivity is confined to NANC nerve fibres terminating on motor endplates (Worl *et al.*, 1997). In addition, besides the NANC system, in the *skeletal muscle*, NO is confined to the sarcolemma of fast-twitch myofibres, where it attenuates force development (Nakane *et al.*, 1993; Kobzbik *et al.*, 1994). Induction of NOS in adult *spinal motoneurons* has been associated with motoneuronal death and it has been suggested that NO is involved in cell death induced by deprivation of trophic factors (Kanda, 1996).

### 2.3 Cardiovascular System

Biological roles of NO were first recognized in the cardiovascular system. Furchgott and Zawadzki (1980) found that blood vessel relaxation in response to Ach requires the endothelium, which releases a labile substance that diffuses to the adjacent smooth muscle. This endogenous relaxing factor appeared to be NO, the active metabolite of nitroglycerin and other organic nitrates, as they all dilate blood vessels by stimulating cGMP formation through activation of guanylyl cyclase (Arnoldt *et al.*, 1977). Under physiological conditions, the major source of NO in the cardiovascular system is e-NOS and in some vessels n-NOS from NANC neurons. Only under pathological conditions, i-NOS is expressed in other cells including vascular smooth muscle, producing higher amounts of NO. Major roles of NO in cardiovascular system are control of vascular tone and platelet and leucocyte functions (Vallance and Moncada, 1994).

It is likely that NO-dependent vasodilator tone is entirely locally regulated and, as such, is probably one of the simplest and yet most fundamental adaptive mechanisms in the cardiovascular system. The available evidence, therefore, indicates that the cardiovascular system is in a state of constant active vasodilatation dependent on the generation of NO. Indeed, NO can now be considered the endogenous vasodilator (Moncada *et al.*, 1988).

#### *2.3.1 Endothelial cells*

Endothelial cells were found to generate sufficient NO from L-Arg to fully account for endothelial-derived relaxing activity (Palmer *et al.*, 1987). NO is crucial in the regulation of blood flow and blood pressure (Moncada *et al.*, 1991) and also inhibits platelet aggregation and adhesion (Radomski *et al.*, 1987); these actions are mediated via the stimulation of soluble guanylyl cyclase. Endothelium-dependent, L-NMMA-inhibitable relaxation has been demonstrated in isolated arteries, veins and microvasculature. Systemic infusion of NOS inhibitors increase blood pressure by inhibiting e-NOS (Calver *et al.*, 1993). Local administration of L-NMMA into the brachial artery of man reduces forearm blood flow by 40%. Thus, the resistance vessels are in a continuous state of NO-mediated vasodilatation. However, basal release of NO does not control the resting tone of peripheral veins, although

it may have an effect on central veins (Änggård, 1994). Endothelium-derived NO inhibits platelet adhesion and addition of L-NMMA to a perfused vascular bed *in vitro* increases the adhesion of platelets and leucocytes (Vallance *et al.*, 1989; Radomski *et al.*, 1987; Kubes *et al.*, 1991). However, short term administration of a NOS inhibitor to healthy animals (Remuzzi *et al.*, 1990) or humans (Vallance *et al.*, 1992) does not cause generalised platelet aggregation. These results implicate that endothelium-derived NO, together with prostacyclin, make a defence layer against the aggregation of activated platelets. Therefore, the physiological role of endothelium derived NO is to provide vasodilatation and prevent platelets and leucocytes adhesion (Änggård, 1994). Additionally, the NO system is present in human *platelets* and NO synthesised by that c-NOS may act as a negative feed-back system to limit the extent of activation of c-NOS (Radomski *et al.*, 1990).

Accordingly, endothelium maintains blood pressure and flow, while endothelium damage leads to cardiovascular disease (Calver *et al.*, 1993; Vene *et al.*, 1990). Genetic abnormality in familial hyperholesterolemia (Flavahan, 1992) and type I insulin-dependent diabetes mellitus (Calver *et al.*, 1992) cause an abnormal endothelium-dependent vascular response. Acquired endothelial dysfunction is associated with smoking and dietary hyperlipidaemia (Henderson, 1991). The net effect of impaired release of endothelial mediators such as NO and prostacyclin is loss of vasodilator tone and exaggerated and paradoxical vasoconstrictor response to mental and physical stress (Änggård, 1994).

In contrast, overproduction of NO by i-NOS induced in vascular smooth muscle, endothelial cells, leucocytes, endocardium and myocardium by endotoxins in sepsis and endotoxic shock causes generalised vasodilatation, hypotension and decreased myocardial contractility. This is blocked by NOS inhibitors, restoring blood pressure and vascular responsiveness to pressor agents in animal models and humans (Calver *et al.*, 1993; Laszlo *et al.*, 1995). Even in irreversible haemorrhagic shock, L-NAME overcomes vascular paralysis (Thiemermann *et al.*, 1993).

### *2.3.2. Myocardium*

Cardiac function is regulated by both sympathetic noradrenergic and vagal cholinergic nerves, but there is also an extensive NANC innervation (Pabla and Curtis, 1996). Recently, induced NOS was detected in fibres innervating conducting and contractile cardiocytes, cardiac ganglion cells and coronary arteries and both c-NOS and i-NOS activities are present in the human myocardium (De Belder *et al.*, 1993). c-NOS is present in myocardium and endocardium (Henderson *et al.*, 1992; Finkel *et al.*, 1992), while the cardiomyopathic ventricle expresses i-NOS (De Belder *et al.*, 1993, 1995). However, NOS inhibitors usually cause a fall rather than a rise in cardiac output, probably due to reflex changes in peripheral resistance and increased blood pressure (Klabunde *et al.*, 1991; Petros *et al.*, 1994). Further, NO deficiency induced myocardial infarction in hypercholesterolemic stroke-prone spontaneously hypertensive rats (Ikeda *et al.*, 1997), while acute inhibition of NO biosynthesis by L-NAME causes myocardial necrosis (Moreno *et al.*, 1997). However, in the genetically hypertensive rat strain cardiovascular structure has been more sensitive to NOS inhibition than either normal or spontaneously hypertensive rat strain (Ledinghham and Laverty, 1997). Recent results implicate that L-NOARG does not completely inhibit endothelial cell NO synthesis in human isolated small coronary arteries and that non-NO-dependent relaxation to bradykinin appears to be mediated by a $K^+$-sensitive

vasodilator mechanism, possibly endothelium-derived hyperpolaryzing factor (Kemp and Cocks, 1997).

Moreover, NO production is influenced by cyclical hormonal changes in women, with a significant increase at mid cycle, protecting against cardiovascular diseases in the premenopausal period (Kharitonov *et al.*, 1994).

## 2.4 Other Systems

### *2.4.1 Immune System*

It has been suggested that NO production originated as an ancient first-line defence against intracellular parasites. While most messenger activities of NO are based on cell-specific expression and regulation of $Ca^{2+}$-dependent c-NOSs (n-NOS and e-NOS), almost every cell is able to express $Ca^{2+}$-independent i-NOS during cell-mediated immune response (Nathan and Hibbs, 1991; Nathan and Xie, 1994). Thus, the cells produce NO as a biological weapon (Snyder and Bredt, 1992; Hölsher *et al.*, 1995).

Activation of the immune defence results in the induction of macrophage i-NOS, which generates large amounts of NO. When a macrophage encounters a pathogen, it engulfs the organism and kills it with NO, causing massive oxidative damage. NO is effective against various microbes (viruses, bacteria, parasites and helminths), as well as tumour cells and alloantigens (Knox *et al.*, 1994). A reaction between oxygen and NO leads to the formation of strong oxidants (nitrogen dioxide and peroxynitrates) that are even more toxic than NO itself. In some cases, the immunologic defence increases become toxic to the host, with severe oxidative damage, hypotension and shock. Thus, NO is a toxic mediator utilised for host defence, contributing to the local and systemic inflammatory response and potentially damaging the host cells (Änggård, 1994).

However, the induction of i-NOS and cell-mediated immune responses by cytokines or microbial products can involve specific reactions such as T-cell recognition of specific antigen, while the NO cell-mediated immune response is always nonspecific. NO produced by macrophages suppress lymphocyte function and may have a particular role in inhibiting certain subtypes of T lymphocytes (Liew *et al.*, 1991). Thus, NO may also have role as a immune modulator, regulating lymphocyte function. Other cells of the immune system, including neutrophils (Kirk *et al.*, 1990) and lymphocytes (Salvemini *et al.*, 1989), may also release NO acting as immune modulator.

### *2.4.2. Respiratory System*

NOS is present in lung epithelium and other pulmonary cells and NO may be a physiological mediator in the respiratory system (Jorens *et al.*, 1993), causing NANC nerve-dependent bronchodilatation (Barnes, 1993). Reduced NO production may be involved in pulmonary vasoconstriction conditions (Sprague *et al.*, 1992). Accordingly, NOS inhibitors increase pulmonary vascular resistance. Contrary, inhalation of NO abolishes pulmonary vasoconstriction in humans. In alveolar macrophages, NO constitutes an important first line of host defence against infection in the lung and NO is an essential component of the microbicidal activity of cytokine-activated macrophages (Persson *et al.*, 1994; Kharitonov *et al.*, 1994).

### *2.4.3. Gastrointestinal System*

In the gastrointestinal system NO transmission is present in the myenteric plexus of the stomach and intestine, responsible for gastric dilatation and peristalsis (Desai *et al.*, 1991). NO-transmission has been identified in the lower oesophageal, illeocolonic and internal and anal sphincters. In gut smooth muscle, the essential role of NO is particularly evidenced by development of a grossly enlarged stomach and spasm of sphincter and the circular muscle layer in mice lacking n-NOS (Huang *et al.*, 1993). Further data provides evidences that NO is released in the stomach and colonic wall during vagal nerve activity, at concentrations able to cause inhibition of smooth muscle contractions *in vivo* (Iversen *et al.*, 1997). Evidently, NO has important roles in the gastrointestinal tract maintaining adaptive dilatation, peristalsis, compartmentalisation and control of special internal sphincters (Deasi *et al.*, 1991; Niioka *et al.*, 1997).

### *2.4.4 Genitourinary System*

In the genitourinary system, NANC produced NO causes relaxation of corpus cavernosum and consecutive penile erection (Leone *et al.*, 1994), while during pregnancy it causes maintenance of uterine contractile quiescence (Yallampalli *et al.*, 1993). In the kidney, NO may have a local signalling role (Thorup *et al.*, 1996). It has been demonstrated that the macula densa synthesises NO in response to sodium reabsorption and this dilates the afferent arteriole to increase glomerular filtration (Wilcox *et al.*, 1992).

### *2.4.5 Endocrine System*

Results within the endocrine system show that NO stimulates release of insulin from pancreatic beta cells (Schmidt *et al.*, 1992), regulates release of renin from kidney (Reid and Chiu, 1995) and is involved in the regulation of thyroid hormone production (Millatt *et al.*, 1993). Additionally, there is morphological evidence of NANC innervation in the pancreas and adrenal gland (Bredt *et al.*, 1990). Along with the role of cGMP in hormone secretion, effects of NO in modulating levels of cGMP in the endocrine system are implicated. Accordingly, L-Arg has been used for many years for testing pituitary function and it stimulates the release of a variety of hormones such as growth hormone, prolactin, glucagon, somatostatin, catecholamines, insulin and pancreatic polypeptide (Barbul *et al.*, 1986).

However, recently it has been suggested that NO may play a role in the destruction of pancreatic beta cells during the development of autoimmune type I diabetes mellitus (Corbett *et al.*, 1993). Treatment with NOS inhibitors reduced macrophage infiltration into pancreas and prevents hyperglycaemia. Selective i-NOS inhibitors would be of potential value preventing immunogenic destruction and progression of autoimmune type I diabetes mellitus (Änggård, 1994).

Involvement of NO in peptide hormone release extends from the hypothalamo-pituitary site affecting the corticotrophin releasing hormone/corticotrophin system, to the adrenal cortex where adrenocorticoid synthesis is inhibited. This anti-adrenocorticoid effect of NO and the immunosuppressive effects of glucocorticoids upon cytokine-induced i-NOS expression can provide maintainance of the equilibrium of both processes that control the strength of systemic endocrine as well as immune responses.

### *2.4.6 Reproduction*

NO can promote reproduction on several levels, beginning with enhanced luteinizing hormone-releasing factor secretion, which induces mating behaviour. In males, additionally to relaxation of corpus cavernosum, NO may modulate motility of spermicytes (Shmidt and Walter, 1994). In pregnant females, NOS is expressed in the placental villi, regulating placental blood flow and is further induced with plasma progesterone levels. Afterwards NOS expression decreases to allow the delivery (Yallampalli *et al.*, 1993).

Evidently, NO systems have important roles in both, physiological and pathophysiological processes. This implicates the importance of NO systems in clinical medicine with promising therapeutic possibilities.

## 2.5 References


Adachi T, Inanami O, Sato A. Nitric oxide (NO) is involved in increased cerebral cortical blood flow following stimulation of the nucleus basalis of Meynert in anesthetised rats. *Neurosci Lett* 139:201-204, **1992.**

Adams LB, Hibbs JB, Taintor RR, Krahenbuhl JL. Microbiostatic effect of murine-activated macrophages from Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine. *J Immunol* 144:2725-2729, **1990.**

Adams ML, Kalicki JM, Meyer ER, Cicero TJ. Inhibition of the morphine withdrawal syndrome by a nitric oxide synthase inhibitor, $N^G$-nitro-L-arginine methyl ester. *Life Sci* 52:PL245-249, **1993.**

Amir S. Nitric oxide signalling in the hypothalamus. In: *Nitric oxide in the nervous system.* Ed. Vincent SR. Academic Press, New York, pp.151-162, **1995.**

Amir S, Edelstein K. A blocker of nitric oxide synthase, $N^G$-nitro-L-arginine methyl ester, attenuates light-induced Fos protein expression in rat suprachiasmatic nucleus. *Neurosci Lett* 224:29-32, **1997.**

Änggård E. Nitric oxide: mediator, murderer and medicine. *Lancet* 343:1199-1206, **1994.**

Arneric SP. Cortical cerebral blood flow is modulated by cholinergic basal forebrain neurons: effects of ibotenic acid lesions and electrical stimulation. In: *Experta Medica International Congress Series,* Eds: Seylaz J, MacKenzie ET, New York: Elsevier, vol:869, pp.381-384, **1989.**

Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide guanylate cyclase and increase guanosine 3'5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci USA* 74:3203-3207, **1977.**

Babbedge RC, Wallance P, Gaffen ZA, Hart ST, Moore PK. L-$N^G$-nitro arginine p-nitroanilide (L-NAPNA) is antinociceptive in the mouse. *Nero Rep* 4:307-310, **1993.**

Babedge RC, Hart SL, Moore PK. Anti-nociceptive activity of nitric oxide synthase inhibitors in the mouse: dissociation between the effect of L-NAME and L-NMMA. *J Pharm Pharmacol* 45:77-79, **1993.**

Balligand J-L, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signalling system. *Proc Natl Acad Sci USA* 90:347-351, **1993.**

Bagetta G, Iannone M, Del Duca C, Nistico G. Inhibition by N-nitro-L-arginine methyl ester of the electrocortical arousal response in rats. *Br J Pharmacol* 198:858-860, **1993.**

Barbul A. Arginine: biochemistry, physiology and therapeutic implications. *J Parent Ent Nut* 10:227-238, **1986.**

Barnes PJ. Nitric oxide and airways. *Eur Resp J* 6:163-165, **1993.**

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrate: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87:1620-1624, **1990.**

Belvisi MG, Stretton CD, Mivra M, Verleden MG, Tajdakarimi S, Yacoub MH, Barnes PJ. Inhibitory NANC nerves in human tracheal smooth muscle: a quest for the neurotransmitter. *J Appl Physiol* 73:2505-2510, **1992.**

Berezin I, Snyder SH, Bredt DS, Daniel EE. Ultrastructural localization of nitric oxide synthase in canine small intestine and colon. *Am J Physiol* 266:C981-C989, **1994.**

Benarroch EE, Smithson IL. Distribution and relationship of neuropeptide Y and NADPH-diaphorase in human ventrolateral medulla oblongata. *J Autonom Nerv System* 62:143-146, **1997.**

Benveniste EN. Inflammatory cytokines within the central nervous system: sources, functions and mechanism of action. *Am J Physiol* 263:C1-C16, **1992.**

Bhargava HN, Sanjay NT. Evidence for a role of nitric oxide of the central nervous system in morphine abstinence syndrome. *Pharmacol* 52:86-91, **1996.**

Bliss TVP, Collingridge GL. A synaptic model of memory: longterm potentiation in the hippocampus. *Nature* 361:31-39, **1993.**

Bohme GA, Bon C, Stutzmann JM, Doble A, Blanchard JC. Possible involvement of nitric oxide in long-term potentiation. *Eur J Pharmacol* 199:379-381, **1991.**

Bohme GA, Bon C, Lemaire M, Reibaud M, Piot O, Stutsmann JM, Doble A, Blanchard J-C. Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats. *Proc Natl Acad Sci USA* 90:9191-9194, **1993.**

Bohne W, Heesemann J, Gross U. Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antagonist: a possible role for nitric oxide in triggering stage conversion. *Infect Immun* 62:1761-1767, **1994.**

Boje KMK. Inhibition of nitric oxide synthase attenuates blood-brain barrier disruption during experimental meningitis. *Brain Res* 720:75-83, **1996.**

Breer, H, Schepherd GM. Implications of the NO/cGMP system for olfaction. *Trends Neurosci* 16:5-9, **1993.**

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

Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neuronal role for nitric oxide. *Nature* 347:768-770, **1990.**

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

Bredt DS, Snyder SH. Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. *Neuron* 13:301-313, **1994.**

Bredt DS. Molecular characterization of nitric oxide synthase. In: *Nitric oxide in the nervous system.* Ed: S.Vincent, Academic Press, pp 1-21, **1995.**

Bredt DS. Targeting nitric oxide to its targets. *Proc Soc Exp Biol Med* 211:41-48, **1996.**

Bruhwyler J, Chleide E, Liegeois JF, Carreer F. Nitric oxide: a new messenger in the brain. *Neurosci Biobehav Rev* 17:373-384, **1993.**

Buisson A, Plotkine M, Boulu RG. The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischemia. *Br J Pharmacol* 106:766-767, **1992.**

Burleigh DE. $N^G$-nitro-L-arginine reduces nonadrenergic noncholinergic relaxations of human gut. *Gastroenterology* 102:679-683, **1992.**

Burlet S, Jouvet M, Cespuglio R. Specific voltametric detection of brain nitric oxide through the rat sleep-waking cycle. In: *Sleep research,* Brain Information Service, Ed: Chase MH, Roth T, O'Conor C, Los Angeles, 24A, pp.67, **1995.**

Burnett AL, Tillman SL, Chang TS, Epstein JI, Lowenstein CJ, Bredt DS, Snyder SH, Walsh PC. Immunohistochemical localization of nitric oxide synthase in the autonomic innervation of the human penis. *J Urol* 150:73-76, **1993.**

Burnstock G. Neurotransmitters and trophic factors in the autonomic nervous system. *J Physiol* (Lond) 313:1-35, **1981.**

Busse R, Kaufman H, Zeiher A, Mulsch A. Inducible nitric oxide synthase in the human vasculature. In: *Biology of nitric oxide*, Part 1. Eds:Moncada S, Marletta MA, Hibbs JB Jr, Higggs EA. London: Portland Press pp.325-328, **1992.**

Buster BL, Weintrob AC, Townsend GC, Scheld WM. Potential role of nitric oxide in the pathophysiology of experimental bacterial meningitis in rats. *Infect Immun* 63:3835-3839, **1995.**

Calapai G, Squadrito F, Altavilla D, Zingarelli B, Campo GM, Cilia M, Caputi AP. Evidence that nitric oxide modulates drinking behaviour. *Neuropharmacology* 31:761-764, **1992.**

Calver A, Collier J, Moncada S, Vallance P. Effect of local intra-arterial N-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *J Hypertens* 10:1025-1031, **1992.**

Calver H, Collier J, Vallance P. Nitric oxide and cardiovascular control. *Exp Physiol* 78:303-326, **1993.**

Cappendijk SLT, De Vries R, Dzoljic MR. Inhibitory effect of nitric oxide (NO) synthase inhibitors on naloxone-precipitated withdrawal syndrome in morphine-dependent mice. *Neurosci Lett* 162:97-100, **1993.**

Cappendijk SLT, Duval SY, De Vries R, Dzoljic MR. Comparative study of normotenzive and hypertensive nitric oxide synthase inhibitors on morphine withdrawal syndrome in rats. *Neurosci Lett* 183:67-70, **1995.**

Chapman PF, Atkins CM, Allen MT, Haley JE, Steinmetz JE. Inhibition of nitric oxide synthesis impairs two different forms of learning. *Neuro Rep* 3:567-570, **1992.**

Chleide E, Bruhwyler J, Ishikawa K. Biochemistry of hypoxic damage in brain cells- roles of energy metabolism, glutamate and calcium ion. *Neurosci* 17:375-390, **1991.**

Chi OZ, Wei M, Sinha AK, Weiss HR. Effects of inhibition of nitric oxide synthase on blood-brain barrier transport in focal cerebral ischemia. *Pharmacol* 48:367-373, **1994.**

Choi DW. Nitric oxide: foe or friend to the injured brain? *Proc Natl Acad Sci USA* 90:9741-9743, **1993.**

Connop BP, Boegman RJ, Beninger RJ, Jhamandas K. Attenuation of malonate-induced degeneration of the nigrostriatal pathway by inhibitors of nitric oxide synthase. *Neuropharmacol* 35:459-465, **1996.**

Corbett JA, Mikhael A, Shmizy J, Frederick K, Misko TP, McDaniel ML, Kanagawa O, Unanue ER. Nitric oxide production in islets from nonobese diabetic mice: aminoguanidine-sensitive and -resistant stages in the immunological diabetic processes. *Proc Natl Acad Sci USA* 90:8992-8995, **1993.**

Coyle JT, Puttfarcken P. Oxidative stress, glutamate and neurodegenerative disorders. *Science* 262:689-695, **1993**

Crabb GJ, Harding R. Role of nitric oxide in the regulation of cerebral blood flow in the ovine foetus. *Clin Exp Pharmacol Physiol* 23:855-860, **1996.**

Czech DA. Possible involvement of nitric oxide in chlordiazepoxide-induced feeding in the mouse. *Pharmacol Biochem Behav* 55:327-331, **1996.**

Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* 88:6368-6371, **1991.**

Dawson T, Dawson V, Snyder SH. A novel neuronal messenger molecule in brain: the free radical, nitric oxide. *Ann Neurol* 323:297-311, **1992.**

Dawson TM, Snyder SH. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J Neurosci* 14:5147-5159, 1994.

Dawson VL, Dawson TM. Nitric oxide neurotoxicity. *J Chem Neuroanat* 10:179-190, 1996.

De Belder AJ, Radomski MW, Why HFJ, Richardson PJ, Bucknall CA, Salas E, Martin JF, Moncada S. Nitric oxide synthase activities in human myocardium. *Lancet* 341:84-85, 1993.

De Belder, Radomski MW, Why HJF, Richardson PJ, Martin JF. Myocardial calcium-independent nitric oxide synthase is present in dilated cardiomyopathy, myocarditis and postpartum cardiomyopathy but not in ischaemic or valvular heart disease. *Br Heart J* 74:426-430, 1995.

Desai KM, Sessa WC, Vane JR. Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid. *Nature* 351:477-479, 1991.

Dirnagl U, Niwa K, Lindauer U, Villringer A. Coupling of cerebral blood flow to neuronal activation: role of adenosine and nitric oxide. *Am J Physiol* 267:H296-H301, 1994.

Duarte IDG, Lorenzetti BB, Ferreira SH. Acetylcholine induces peripheral analgesia by the release of nitric oxide. In: *Nitric oxide from L-arginine - a bioregulatory system.* Ed: Moncada S and Higgs EA. Elsevier, Amsterdam, pp.165-170, 1990.

Dzoljic MR, De Vries R, Cappendijk SLT. Nitric oxide (NO) synthase inhibitors attenuated naloxone-precipitated withdrawal. *Regulatory Peptides* 1:S285-S286, 1994.

Dzoljic MR, De Vries. Nitric oxide synthase inhibition reduces wakefulness. *Neuropharmacology* 33:1505-1509, 1994.

Dzoljic MR, De Vries R, Van Leeuwen. Sleep and nitric oxide: effects of 7-nitro indazole, inhibitor of brain nitric oxide synthase. *Brain Res* 718:145-150, 1996.

East SJ, Garthwaite J. NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. *Neurosci Lett* 123:17-19, 1991.

Endoh M, Maiese K, Wagner JA. Expression of the neuronal form of nitric oxide synthase by CA1 hippocampal neurons and other nervous system neurons. *Neurosci* 63:679-689, 1994.

Erdemil G, Krnjevic K. Nitric oxide tonically depress a voltage- and Ca-dependent outward current in hippocampal slices. *Neurosci Lett* 201:57-60, 1995.

Facciolo MR, Tavolaro R, Chinellato A, Ragazzi E, Canonaco M, Fassina G. Effects of N-nitro-L-arginine methyl ester on benzodiazepine binding in some limbic areas of hyperlipidemic rats. *Pharmacol Biochem Behav* 54:431-437, 1996.

Faraci FM, Breese KR, Heistad DD. Nitric oxide contributes to dilatation of cerebral arterioles during seizures. *Am J Physiol* 265:H2209-H2212, 1993.

Faraci FM, Brian JE Jr. Nitric oxide and cerebral circulation. *Stroke* 5:692-706, 1994.

Faraci FM, Brian JE Jr. 7-nitroindazole inhibits brain nitric oxide synthase and cerebral vasodilatation in response to N-methyl-D-aspartate. *Stroke* 26:2172-2175, 1995.

Ferrendelli JA, Blank AC, Gross RA. Relationships between seizure activity and cyclic nucleotide levels in brain. *Brain Res* 200:93-103, 1980.

Ferrante RJ, Kowall NW, Beal MF, Martin JB, Bird ED, Richardson EP Jr. Morphologic and histochemical characteristics of a spared subset of striatal neurons in Huntington 's disease. *J Neuropath Exp Neurol* 46:12-27, 1985.

Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hathler BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 257:387-389, 1992.

Forstermann U, Gorsky LD, Pollock JS, Schmidt HH. Heller M, Murad F. Regional distribution of EDRF/NO-synthesizing enzyme(s) in rat brain. *Biochem Biphys Res Commun* 168:727-732, 1990.

Freedman R, Taylor D, Seiger A. Seizure and related epileptiform activity in hippocampus transplantated to the anterior chamber in the eye. II Modulation by cholinergic and adrenergic input. *Ann Neurol* 6:281-293, 1979.

Frei K, Leist TP, Meager A, Gallo P, Leppert D, Zinkernagel RM, Fontana A. Production B cell stimulatory factor-2 and interferon gamma in the central nervous system during viral meningitis and encephalitis. Evaluation in a murine model infection and in patients. *J Exp Med* 168:449-453, **1988.**

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376, **1980.**

Furuyama T, Inagaki S, Takagi H. Localizations of $\alpha_1$ and $\beta_1$ subunits of soluble guanylate cyclase in the rat brain. *Molec Brain Res* 20:335-344, **1993.**

Gaffen Z, Bland-Ward PA, Pitcher A, Wallace P, Moore PK. Augmented antinociception following 7-nitro indazole and flubiprofen in the conscious mouse. *Eur J Pharmacol* 271:445-452, **1994.**

Gally JA, Montague PR, Reeke GN, Edelman GM. The NO hypothesis: possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system. *Proc Natl Acad Sci USA* 87:3547-3551, **1990.**

Garthwite J. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neuro Sci* 14:60-67, **1991.**

Gazzinelli RT, Eltoum I, Wynn TA, Sher A. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alfa and correlates with the down-regulation expression of inducible nitric oxide synthase and other markers of macrophage activation. *J Immunol* 151:3672-3681, **1993.**

Goadsby PJ, Kaube H, Hoskin KL. Nitric oxide synthesis couples cerebral blood flow and metabolism. *Brain Res* 595:167-170, **1992.**

Good M. Targeting deletion of neuronal nitric oxide: a step closer to understanding its functional significance? *Trends Neuro Sci* 19:83-84, **1996.**

Guevara-Guzman R, Emson PC, Kendrick KM. Modulation of *in vivo* striatal transmitter release by nitric oxide and cyclic GMP. *J Neurochem* 62:807-810, **1994.**

Guimaraes FS, de Aguiar JC, Del Bel EA, Ballejo G. Anxiolytic effect of nitric oxide synthase inhibitors microinjected into the dorsal central grey. *Neuro Rep* 5:1929-1932, **1994.**

Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S. Endogenous nitric oxide is present in the exhaled air of rabbits, guinea-pigs and humans. *Biochem Biophys Res Commun* 181:852-857, **1991.**

Henderson AH. Endothelium in control. *Br Heart J* 65:116-125, **1991.**

Henderson AH, Lewis MJ, Sdhah AM, Smith JA. Endothelium, endocardium and cardiac contraction. *Cardiovasc Res* 26:305-308, **1992.**

Heinzl B, John M, Klatt P, Bohme E, Mayer B. $Ca^{2+}$/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. *Biochem J* 281:627-630, **1992.**

Heymann MA, Bootsataylor B, Roman C, Natuzzi ES. Nitroglycerin (NG) stops active labour in sheep. *Endothelium* 1:S75, **1993.**

Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biphys Res Commun* 157:87-94, **1988.**

Hökfelt T, Zhang X, Wiesefeld-Hallin Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci* 17:22-30, **1994.**

Hölscher C, Rose SPR. An inhibition of nitric oxide synthesis prevents memory formation in the chick. *Neurosci Lett* 145:165-167, **1992.**

Hölscher C, Doyle CA, McGlinchey L, Anwyl R, Rowan MR. A selective neuronal nitric oxide synthase inhibitor impairs spatial learning in the rat. Abstract, *Society for Neuroscience Meeting*, San Diego, USA, November **1995.**

Hope BT, Michael GJ, Knigge KM, Vincent SR. Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc Natl Acad Sci USA* 88:2811-2814, **1991.**

Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC. Targeted distribution of the neuronal nitric oxide synthase gene. *Cell* 75:1273-1286, **1993.**

Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265:1883-1885, **1994.**

Hurst RD, Clark JB. Nitric oxide-induced blood-brain-barrier dysfunction is not mediated by inhibition of mitochondrial respiratory chain activity and/or energy depletion. *Nitric Oxide* 1:121-129, **1997.**

Hyman BT, Marzloff K, Wenninger JJ, Dawson TM, Bredt DS, Snyder SH. Relative sparing of nitric oxide synthase-containing neurons in the hippocampal formation in Alzheimer's disease. *Ann Neurol* 32:818-820, **1992.**

Iadecola C, Faris PL, Hartman BK, Xu X. Localization of NADPH diaphorase in neurons of the rostral ventral medulla: possible role of nitric oxide in central autonomic regulation and oxygen chemoreception. *Brain Res* 603:173-179, **1993.**

Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neuro Sci* 20:132-139, **1997.**

Ientile R, Piccuirro V, Pedale S, Nucci C, Malecka B, Nistico G, Macaione S. Nitric oxide enhances amino acid release from immature chick embryo retina. *Neurosci Lett* 219:79-82, **1996.**

Ignarro LJ, Bush PA, Bugga GM, Wood KS, Fukuto JM, Rajfer J. Nitric oxide and cyclic GMP formation upon electrical gild stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biphys Res Commun* 170: 843-850, **1990.**

Ignarro LJ. Biosynthesis and metabolism if endothelium-derived nitric oxide. *Annu Rev Pharmacol Toxicol* 30:535-560, **1990.**

Ikeda K, Nara Y, Tagami M, Yamori Y. Nitric oxide deficiency induces myocardial infarction in hypercholesterolaemic stroke-prone spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol* 24:344-348, **1997.**

Ishii M, Yamamoto T, Shimizu S, Sano A, Momose K, Kuroiwa Y. Possible involvement of nitric oxide synthase in oxidative stress-induced endothelial injury. *Pharmacol Toxicol* 80:191-196, **1997.**

Ishizaki Y, Ma L, Morita I, Murota S. Astrocytes are responsive to endothelium-derived relaxing factor (EDRF). *Neurosci Lett* 125:29-30, **1991.**

Iversen HH, Celsing F, Leone AM, Gustafsson LE, Weiklund NP. Nerve-induced release of nitric oxide in the rabbit gastrointestinal tract as measured by *in vivo* microdialysis. *Br J Pharmacol* 120:702-706, **1997.**

Izumi Y, Clifford DB, Zorumski CF. Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science* 257:1273-1276, **1992.**

Janssens SP, Shimouchi A, Quertermous T, Bloch DB, Bloch KD. Cloning and expression of cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *J Biol Chem* 267:14519-14522, **1992.**

Johnston MV, McKinney M, Coyle JT. Neocortical cholinergic innervation: a description of extrinsic and intrinsic components in the rat. *Exp Brain Res* 43:159-172, **1981.**

Johanson AW, Land JM, Thompson EJ, Bolanos JP, Clark JB, Heales SRJ. Evidence for increased nitric oxide production in multiple sclerosis. *J Neurol Neurosurg Psychiatr* 58:107, **1995.**

Jorens PG, Vermeira PA, Herman AG. L-arginine dependent nitric oxide synthase: a new metabolic pathway in the lung and airways. *Eur Resp J* 6:258-266, **1993.**

Kaufman HS, Shermik MA, May CA, Pitt HA, Lillemoe KD. Nitric oxide inhibits resting sphincter of Oddi activity. *Am J Surg* 165:74-80, **1993.**

Kadowaki K, Kishimoto J, Leng G, Emsin PC. Up-regulation of nitric oxide synthase (NOS) gene expression together with NOS activity in the rat hypothalamo-hyopophysial system after chronic salt loading: evidence of a neuromodulatory role of nitric oxide synthase in arginine vasopressin and oxytocin secretion. *Endocrinology* 134:1011-1017, **1994.**

Kanda K. Expression of neuronal nitric oxide synthase in spinal motoneurons in aged rats. *Neurosci Lett* 219:41-44, 1996.

Kapás L, Ayers NA, Kruger JM. Circadian variation in brain nitric oxide synthase activity. In: *Sleep Research*, Ed:Chase MH, Roth T, O'Connor C, Brain Information Service, Los Angeles, 24A, pp.106, 1995.

Karatinos J, Rosse RB, Deutsch SI. The nitric oxide pathway: potential implications for treatment of neuropsychiatric disorders. *Clin Neuropharm* 18:482-499, 1995.

Kelly PA, Rittchie IM, Arbuthnott GW. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole: effects upon local cerebral blood flow and glucose use in the rat. *J Cereb Blood Flow Metab* 15:766-773, 1995.

Kemp BK, Coks TM. Evidence that mechanisms dependent and independent of nitric oxide mediate endothelium-dependent relaxation to bradykinin in human small resistance-like coronary arteries. *Br J Pharmacol* 120:757-762, 1997.

Kharitanov SA, Yates D, Robbins RA, Logan-Sinclair, Shinebroune EA, Barnes PJ. Endogenous nitric oxide id increased in the exhaled air of asthmatic patients. *Lancet* 343:133-135, 1994.

Kiechle FL, Malinski T. Nitric oxide: biochemistry, pathophysiology and detection. *Clin Chem* 100:567-575, 1993.

Kirk SJ, Regan MC, Barbul A. Cloned murine T lymphocytes synthesize a molecule with the biological characteristics of nitric oxide. *Biochem Biophys Res Commun* 173:660-665, 1990.

Kiss JP, Sershen H, Lajtha A, Vizi ES. Inhibition of neuronal nitric oxide synthase potentiates the dimethylphenylpiperazinium-evoked carrier-mediated release of noradrenaline from rat hippocampal slices. *Neurosci Lett* 215:115-118, 1996.

Klabunde RE, Ritger RC, Helgren MC. Cardiovascular actions of inhibitors of endothelium-derived relaxing factor (nitric oxide) formation/release in anaesthetized dogs. *Eur J Pharmacol* 199:51-59, 1991.

Knox LK, Stewart AG, Hayward PG, Morrison WA. Nitric oxide synthase inhibitors improve skin flap survival in the rat. *Microsurgery* 15:708-711, 1994.

Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372:546-548, 1994.

Koch KW, Lambrecht HG, Haberecht M, Redburn M, Redburn D, Schmidt HHHW. Functional coupling of a calcium/calmodulin dependent nitric oxide synthase and a soluble guanylyl cyclase in vertebrate photoreceptor cells. *EMBO J* 13:3312-3320, 1994.

Koh J-Y., Choi DW. Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. *J Neurosci* 8:2153-2163, 1988.

Kohno K, Higuchi T, Ohta S, Kohno K, Kumon Y, Sakaki S. Neuroprotective nitric oxide synthase inhibitor reduces intracellular calcium accumulation following transient global ischemia in the gerbil. *Neurosci Lett* 224:17-20, 1997.

Kooy NW, Royall JA. Agonist-induced peroxynitrite production from endothelial cells. *Arch Biochem Biophys* 310:352-359, 1994.

Koprowski H, Zheng YM, Heber-Katz E, Fraser N, Rorke L, Fu ZF, Hanlon C, Dietzchold B. *In vivo* expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc Natl Acad Sci USA* 90:3024-3027, 1993.

Kowal NW, Ferrante RJ, Beal MF, Richardson EO Jr, Sofroniniew MV, Cuello AC, Martin JB. Neuropeptide Y, somatostatin and reduced nicotinamide adenine dinucleotide phosphate diaphorase in the human striatum: a combined immunocytochemical and enzyme histochemical study. *Neurosci* 20:817-828, 1987.

Kozniewska E, Oseka M, Stys T. Effects of endothelium derived nitric oxide on cerebral circulation during normoxia and hypoxia in the rat. *J Cereb Blood Flow Metab* 12:311-317, 1992.

Kröncke KD, Fehsel K, Kolb-Bachofen V. Nitric oxide: cytotoxicity versus cytoprotection: why, when, and where? *Nitric oxide* 1:107-120, **1997.**

Kubes P, Suzuter M, Granger DN. Nitric oxide: an endogenous modulator of leucocyte adhesion. *Proc Natl Acad Sci USA* 88:4651-4655, **1991.**

Kurtzman J, Natuzzi ES, Buscher CA, Harrison M. Human preterm labour is associated with decreased nitric oxide synthase activity. *Endothelium* 1: S75, **1993.**

Laszlo F, Whittle BJ, Evans SM, Moncada S. Association of microvascular leakage with induction of nitric oxide synthase: effects of nitric oxide synthase inhibitors in various organs. *Eur J Pharmacol* 283:47-53, **1995.**

Lee TJ-F, Sarwinski S, Ische C, Lai FY. Inhibition of cerebral neurogenic vasodilatation by L-arginine and nitric oxide synthase inhibitors and its reversal by L-citrulline. *J Pharmacol Exp Ther* 276:353-358, **1996.**

Ledingham JM, Laverty R. Nitric oxide synthase inhibition with N-nitro-L-arginine methyl ester affects blood pressure and cardiovascular structure in the genetically hypertensive rat strain. *Clin Exp Pharmacol Physiol* 24:433-435, **1997.**

Leone AM, Wiklund NP, Hokfelt T, Brundin L, Moncada S. release of nitric oxide by nerve stimulation in the human urogenital tract. *Neuro Rep* 5:733-736, **1994.**

Liew FY, Li Y, Severn A, Millot S, Schmidt J, Salter M, Moncada S. A possible novel pathway of regulation by murine T helper type-2 cells (Th2) of Th1 cell activity via the modulation of the induction of nitric oxide synthase in macrophages. *Eur J Immunol* 21:2489-2494, **1991.**

Lindauer U, Megow D, Schultze J, Weber JR, Dirnagl U. Nitric oxide synthase inhibition does not affect somatosensory evoked potentials in the rat. *Neurosci Lett* 207-210, **1996.**

Lizasoain I, Weiner CP, Knowles RG, Moncada S. The ontogeny of cerebral and cerebellar nitric oxide synthase in the guinea pig and rat. *Pediatr Res* 39:779-788, **1996.**

Llewllyn-Smith IJ, Song ZM, Costa M,Bredt DS, Snyder SH. Ultrastructural localization of nitric oxide synthase activity in guinea-pig enteric neurons. *Brain Res* 577:337-342, **1992.**

Loicano RE, Beart PM. Hippocampal lesions induced by microinjection of the nitric oxide donor nitroprusside. *Eur J Pharmacol* 216:331-333, **1992.**

Lonart G, Wang J, Johnson KM. Nitric oxide induces neurotransmitter release from hippocampal slices. *Eur J Pharmacol* 220:271-272, **1992.**

Lonart G, Johnson KM. Inhibitory effects of nitric oxide on the uptake of [$^3$H]dopamine and [$^3$H]glutamate by striatal synaptosomes. *J Neurochem* 63:2108-2117, **1994.**

Lonart G, Johnson KM. Characterization of nitric oxide generator-induced hippocampal [$^3$H]-norepinephrine release. II The role of calcium, reverse norepinephrine transport and cyclic 3',5'-guanosine, monophosphate. *J Pharmacol Exp Ther* 275:14-22, **1995.**

Luckman SM, Huckett L, Bicknell RJ, Vpisin DL, Herbison AE. Up-regulation of nitric oxide synthase messenger RNA in an integrated forebrain circuit involved in oxytocin secretion. *Neurosci* 77:37-48, **1997.**

Margalli I, Allix M, Boulu RG, Plotkine M. Dose- and time-dependence of L-NAME neuroprotection in transient focal cerebral ischemia in rats. *Br J Pharmacol* 120:160-163, **1997.**

Mayhan WG. Role of nitric oxide in disruption of the blood-brain barrier during acute hypertension. *Brain Res* 686:99-103, **1995.**

McNamara JO, Role of neurotransmitters in seizure mechanisms in kindling model of epilepsy. *Fed Proc* 43:2516-2520, **1984.**

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, Malagelada JR. Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* 23:724-728, **1993.**

Meldrum B, Garthwaite J. Excitatory amino acids neurotoxicity and neurodegenerative disease. *Trends Pharmacol Sci* 11:379-387, **1990.**

Meller ST, Dykstra C, Gebhart GF. Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for N-methyl-D-aspartate produced facilitation of the nociceptive tail-flick reflex. *Eur J Pharmacol Sci* 11:379-387, **1990.**

Meller ST, Gebhart GH. Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain* 52:127-136, **1993.**

Mendez-Medina R, Ferres-Torres R, Meyer G. Transient NADPH-diaphorase activity in motor nuclei of the foetal human brain stem. *Neuro Rep* 5:758-760, **1994.**

Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol* 151:2132-2141, **1993.**

Mesenge C, Verrecchia C, Allix M, Bpulu PR, Plotkin M. Reduction of the neurological deficit in mice with traumatic brain injury by nitric oxide synthase inhibitors. *J Neurotrauma* 13:11-16, **1996.**

Meyhoff HH, Rosenkilde P, Bodker A. Non-invasive management of impotence with transcutaneous nitroglycerin. *Br J Urol* 69:88-90, **1992.**

Millatt LJ, Jackson R, Williams BC, Whitley GS Jr. Nitric oxide stimulates cyclic GMP in human thyrocytes. *J Mol Endocrinol* 10:163-169, **1993.**

Misko TP, Moore WM, Kasten TP, Nickols GA, Cobett JA, Titon RG, McDaniel ML, Williamson JR, Currie MG. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol* 233:119-125, **1993.**

Mitrovic B, Martin FC, Charles AC, Ignarro LJ, Anton PA, Shanahan F, Merrill JE. Neurotransmitters and cytokines in CNS pathology. *Prog Brain Res* 103:319-330, **1994.**

Mitrovic B, Ignarro LJ, Vinters HV, Akers MA, Schmidt I, Uittenbogaart C, Merrill JE. Nitric oxide induces necrotic but not apoptotic cell death in oligodendrocytes. *Neuroscience* 65:531-539, **1995.**

Mollace V, Bagetta G, Nistico G. Evidence that L-arginine possesses proconvulsant effects mediated through nitric oxide. *Nero Rep* 2:269-272, **1991.**

Moncada S, Radomski MW, Palmer RMJ. Endothelium-derived relaxing factor: Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem Pharmacol* 37:2495-2501, **1988.**

Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109-142, **1991.**

Moncada S, Higgs A. The L-arginine pathway. *N Engl J Med* 329:2002-2012, **1993.**

Montague PR, Gancayco CD, Winn MJ, Marchase RB, Friedlander MJ. Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. *Science* 263:973-977, **1994.**

Montécot C, Borredon J, Seylaz J, Pinard E. Nitric oxide of neuronal origin is involved in cerebral blood flow increase during seizures induced by kainate. *J Cereb Blood Flow Metab* 17:94-99, **1997.**

Moore PK, Oluyomi AO, Babbedge RC, Wallace P, art SL. L-N-nitroarginine methyl ester exhibits anti-nociceptive activity in the mouse. *Br J Pharmacol* 102:198-202, **1991.**

Moore PK, Wallance P, Gaffen ZA, Hart SL, Babbedge RC. 7-nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* 108:296-297, **1993a.**

Moore OK, Wallace P, Gaffen Z, Hart SL, Babbedge RC. Characterisation of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociception and cardiovascular effects. *Br J Pharmacol* 110:219-224, **1993b.**

Moreno H Jr, Nathan LP, Metze K, Costa SKP, Antunes E, Hyslop S, Zatz R, de Nucci G. Non-specific inhibitors of nitric oxide synthase cause myocardial necrosis in the rat. *Clin Exp Pharmacol Physiol* 24:349-352, **1997.**

Morley JE, Flood JF. Evidence that nitric oxide modulates food intake in mice. *Life Sci* 49:707-711, 1991.

Morton AJ, Nicholson LFB, Faull RLM. Compartmental loss of NADPH diaphorase in the neuropil of the human striatum in Huntington's disease. *Neurosci* 53:159-168, 1993.

Moskowitz MA, Macfarlane RC.Neurovascular and molecular mechanisms in migraine headaches. *Cerebrovasc Brain Res Metab Rev* 5:159-177, 1993.

Murphy S, Grzybicki DM, Simmons ML. Glial cells as nitric oxide sources and targets. In: *Nitric oxide in the nervous system.* Ed. S. Vincent, Acad Press, pp. 163-191, 1995.

Nagafuji T, Sugiyama M, Muto A, Makino T, Miyauchi T, Nabata H. The neuroprotective effect of a potent and selective inhibitor of type I NOS (L-MIN) in a rat model of focal cerebral ischemia. *Neuro Rep* 31:1541-1545, 1995.

Nakane M, Schmidt HH, Pollock JS, Forsterman U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316:175-180, 1993.

Nathan C, Hibbs JB. Role of nitric oxide synthase in macrophage antimicrobial activity. *Curr Opin Immunol* 3:65-70, 1991.

Nathan C, Xue Q. Nitric oxide synthase: roles, tolls and controls. *Cell* 78:915-918, 1994.

Natuzzi E, Ursell P, Buscher C, Heymann M, Harrison M, Riemer RK. Differential regulation of uterine NOS activity during gestation. *Endothelium* 1:S75, 1993.

Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc Natl Acad Sci USA* 89:3030-3034, 1992.

Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson TM, Snyder SH. Behavioral abnormalities in male mice lacking neuronal nitric oxide synthase. *Nature* 378:383-386, 1995.

Niioka S, Takeuchi T, Kishi M, Ishii T, Nishio H, Takewaki T, Hata F. Nonadrenergic, noncholinergic relaxation in longitudinal muscle of rat jejunum. *Jpn J Pharmacol* 73:155-161, 1997.

Nistiko G, Bagetta G, Iannone M, Del Duca C. Evidence that nitric oxide is involved in the control of electrocortical arousal. *Ann NY Acad Sci* 738:191-200, 1994.

Noronha-Dutra AA, Eperlein MM, Woolf N. Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing. *FEBS* 321:59-62, 1993.

Northington FJ, Matherne GP, Berne RM. Competitive inhibition of nitric oxide synthase prevents the cortical hyperaemia associated with peripheral nerve stimulation. *Proc Natl Acad Sci USA* 89:6649-6652, 1992.

Northington FJ, Tobin JR, Harris AP, Traystman RJ, Koehler RC. Developmental and regional differences in nitric oxide synthase activity and blood flow in the sheep brain. *J Cereb Blood Flow Metab* 17:109-115, 1997.

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

O'Dell TJ, Hawkins RD, Kandel ER, Arancio O. Tests of the role of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc Natl Acad Sci USA* 88:11285-11289, 1991.

O'Dell TJ, Huang PL, Dawson TM, Dinerman JL, Snyder SH, Kandel ER, Fishman MC. Blockade of LTP by inhibitors of nitric oxide synthase (NOS) in mice lacking neuronal NOS suggest a role for endothelial NOS. *Science* 265:542-546, 1994.

O'Donel P, Grace AA. Cortical afferents modulate striatal gap junction permeability via nitric oxide. *Neurosci* 76:1-5, 1997.

Okuda Y, Nakatsuji Y, Fujimura H, Esumi H, Ogura T, Yanagihara T, Sakada S. Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with

the severity of actively induced experimental allergic encephalitis. *J Neuroimmunol* 62:103-112, **1995.**

O'Sullivan AJ, Burgoune RD. Cyclic GMP regulates nicotine-induced secretion from cultured bovine adrenal chromaffin cells:effects of 8-bromo-cyclic GMP, atrial natriuretic peptide, and nitroprusside (nitric oxide). *J Neurochem* 54:1805-1808, **1990.**

Osone K, Mori N, Suzuki K, Osonoe M. Antiepileptic effects of inhibitors of nitric oxide synthase examined in pentylenetetrazol-induced seizures in rats. *Brain Res* 663:338-340, **1994.**

Pabla R, Curtis MJ. Endogenous protection against reperfusion-induced ventricular-fibrillation: role of neuronal versus non-neuronal sources of nitric oxide and species dependence in the rat versus rabbit isolated heart. *J Mol Cell Cardiol* 28:2097-2110, **1996.**

Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526, **1987.**

Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesise nitric oxide from L-arginine. *Nature* 333:664-666, **1988.**

Palmer RMJ, Moncada S. A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem Biphys Res Commun* 158:348-352, **1989.**

Pape HC, Mager R. Nitric oxide controls oscillatory activity in thalamocortical neurons. *Neuron* 9:441-448, **1992.**

Paul SM, Skolnick P. Rapid changes in brain benzodiazepine receptors after experimental seizures. *Science* 202:892-894, **1978.**

Persson K, Alm P, Johansson K, Larsson B, Andersson KE. Nitric oxide synthase in pig lower urinary tract: immunohistochemistry, NADPH diaphorase histochemistry and functional effects. *Br J Pharmacol* 110:521-530, **1993.**

Persson MG, Zetterstrom O, Agrenius V, Ihre E, Gustafsson LE. Single-breath measurements of nitric oxide: increased concentration in asthmatics and reduction in smokers. *Lancet* 343:146-147, **1994.**

Pereira de Vasconcels A, Baldwin RA, Wasterlain CG. Nitric oxide mediates the increase in local cerebral blood flow during focal seizures. *Proc Natl Acad Sci USA* 92:3175-3179, **1995.**

Petros A, Lamb G, Leone A, Moncada S, Bennett D, Vallance P. Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovasc Res* 28:34-39, **1994.**

Pogun S, Baumann MH, Kuhar MJ. Nitric oxide inhibits ($^3$H)dopamine uptake. *Brain Res* 641:83-91, **1994.**

Pou S, Pou WS, Bredt DS, Bredt DS, Snyder SH, Rosen GM. Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* 267:24173-24176, **1992.**

Prast H, Phillippu A. Nitric oxide release acetylcholine in the basal forebrain. *Eur J Pharmacol* 216:139-140, **1992.**

Prast H, Fischer H, Werner E, Werner-Felmayer G, Phillippu A. Nitric oxide modulate the release of acetylcholine in the rat ventral striatum of the freely moving rat. *Naunyn Schmiedeberg's Arch Pharmacol* 352:67-73, **1995.**

Proctor MR, Fornai F, Afshar JKB, Gale K. The role of nitric oxide in focally-evoked limbic seizures. *Neurosci* 76:1231-1236, **1996.**

Pudiak CM, Bozarth MA. L-NAME and MK-801 attenuate sensitization to the locomotor-stimulating effect of cocaine. *Life Sci* 53:1517-1524, **1993.**

Qian Y, Chao DS, Santillano DR, Cornwell TL, Nairn AC, Greengard P, Lincoln TM, Bredt DS. cGMP-dependent protein kinase in dorsal root ganglion: relationship with nitric oxide synthase and nociceptive neurons. *J Neurosci* 16:3130-3138, **1996.**

Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288:481-487, **1991.**

Radomski MW, Palmer RMJ, Moncada S. The anti-aggregating properties of vascular endothelium: interaction between prostacyclin and nitric oxide. *Br J Pharmacol* 92:639-646, **1987.**

Radomski MW, Palmer RMJ, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci USA* 87:10043-10047, **1990.**

Radomski MW, Martin JF, Moncada S. Synthesis of nitric oxide by the haemocytes of the American horseshoe crab (Limulus polypherus). *Philos Trans R Soc Lond* 334:129-133, **1991.**

Raifer J, Aronson WJ, Bush PA, Doren FS, Ignarro Lj. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. *N Engl J Med* 326:90-94, **1992.**

Raszkiewicz JL, Linville DG, Kerin JF, Wagenaar F, Arneric SP. Nitric oxide synthase is critical in mediating basal forebrain regulation of cortical cerebral circulation. *Neurosci Res* 33:129-135, **1992.**

Remuzzi G, Perico N, Zoja C, Corna D, Macconi D, Vigano G. Role of endothelium-derived nitric oxide in the bleeding tendency uremia. *J Clin Invest* 86:1768-1771, **1990.**

Rengasamy A, Johnson RA. Regulation of nitric oxide synthase by nitric oxide. *Mol Pharmacol* 44:124-128, **1993.**

Rogers NE, Ignarro LJ. Constitutive nitric oxide synthase from cerebellum is reversible inhibited by nitric oxide formed from L-arginine. *Biochem Biphys Res Commun* 189:242-249, **1992.**

Rundfeld C, Koch R, Richter A, Mevissen M, Gerecke U, Löschher W. Dose-dependent anticonvulsant and proconvulsant effects of nitric oxide synthase inhibitors on seizure threshold in a cortical stimulation model in rats. *Eur J Pharmacol* 274:73-81, **1995.**

Salvemini D, de Nucci G, Gryglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* 86:6328-6332, **1989.**

Salvemini D, Manning PT, Zweifel BS, Seibert K, Connor J, Currie MG, Needleman P, Masferrer JL. Dual inhibition of nitric oxide and prostaglanidin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. *Clin Invest* 96:301-308, **1995.**

Sandi C, Venero C, Guaza C. Decreased spontaneous motor activity and startle response in nitric oxide synthase inhibitor-treated rats. *Eur J Pharmacol* 277:89-97, **1995.**

Schmidt WJ, Bubser M, Hauber W. Excitatory amino acids and Parkinson's disease. *Trends Neurosci* 13:46-47, **1990.**

Schmidt WJ. The glutaminergic system as a target of antipsychotic drug action. In: Animal models in psychopharmacology: *Advances in pharmacological sciences*. Birkhauser Verlag Basel: Switzerland pp.289-293, **1991.**

Schmidt HHHW, Warner TD, Ishii K, Sheng H, Murad F. Insulin-secretion from pancreatic beta-cells caused by L-arginine-derived nitrogen oxides. *Science* 255:721-723, Reply: Science 258:1376-13798, **1992.**

Schmidt HHHW, Lohmann SL, Walter U. The nitric oxide and cGMP signal transduction pathway. *Biochem Biphys Acta* 1178:153-175, **1993.**

Schmidt HHHW, Walter U. NO at work. *Cell* 78:919-925, **1994.**

Schneider-Schaulies J, Schneider-Schaulies S, Ter Meulen V. Differential induction of cytokines by primary and persistent measles virus infection in human glial cells. *Virology* 195:219-228, **1993.**

Schulz JB, Matthews RT, Muqit MM, Browne SE, Beal MF. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *J Neurochem* 64:936-939, **1995.**

Schuman EM, Madison DV. A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* 254:1503-1506, **1991.**

Schuman EM, Madison DV. Nitric oxide and synaptic function. *A Rev Neurosci* 17:153-183, **1994**

Shibiki K, Okada D. Endogenous nitric oxide release required for long term synaptic depression in the cerebellum. *Nature* 349:326-328, **1991.**

Smith RP, Louis CA, Kruszyna H. Acute neurotoxicity of sodium azide and nitric oxide. *Fundam Appl Toxicol* 17:120-127, **1991.**

Snyder SH, Bredt DS. Nitric oxide as a neuronal messenger. *Trends Pharmacol Sci* 12:125-128, **1991.**

Snyder SH, Bredt DS. Biologiocal roles of nitric oxide. *Scientific American* 5:28-35, **1992.**

Snyder SH. More jobs for that molecule. *Nature* 372:504-505, **1994.**

Speesrt R, Layes E, Schollmayer A, Reuss S, Vollrath L. In the rat pineal gland, but not in the suprashiasmatic nucleus, the amount of constitutive neuronal nitric oxide synthase is regulated by environmental lighting conditions. *Biochem Biophys Res Commun* 212:70-76, **1995.**

Sprague R, Thiemermann C, Vane JR. Endogenous endothelium-derived relaxing factor opposes hypoxic pulmonary vasoconstriction and supports blood flow to hypoxic alveoli in anesthetised rabbits. *Proc Natl Acad Sci USA* 89:8711-8715, **1992.**

Springall DR, Riveros-Moreno V, Buttery L, Suburo A, Bishop AE, Merrett M, Moncada S, Polak JM. Immunological detection of nitric oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoform. *Histochemistry* 98:259-266, **1992.**

Stanfa LC, Misra C, Dickenson AH. Amplification of spinal nociceptive transmission depends on the generation of nitric oxide in normal and carrageen rats. *Brain Res* 737:92-98, **1996.**

Starr MS, Starr BS. Paradoxical facilitation of pilocarpine-induced seizures in the mouse by MK-801 and the nitric oxide synthesis inhibitor L-NAME. *Pharmacol Biochem Behav* 45:321-325, **1993.**

Starr MS, Starr BS. Do NMDA receptor-mediated changes in motor behaviour involve nitric oxide? *Eur J Pharmacol* 272:211-217, **1995.**

Stuehr DJ, Nathan CF. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumour target cells. *J Exp Med* 169:1543-1555, **1989.**

Thiemermann C, Szabo C, Mitchel JA, Vane JR. Vascular hyporeactivity to vasoconstrictor agents and haemodynamic decompensation in haemorrhagic shock is mediated by nitric oxide. *Proc Natl Acad Sci USA* 90:267-271, **1993.**

Thompson AJ, Miller D, Youl B, MacManus D, Moore S, Kingsley D, Kendall B, Feinstein A, McDonald WI. Serial gadolinium enhanced MRI in relapsing/remitting multiple sclerosis. *Neurology* 42:60-63, **1992.**

Thorup C, Eril A, Persson G. Macula densa derived nitric oxide in regulation of glomerular capillary pressure. *Kidney Int* 49:430-436, **1996.**

Toda N. Non-adrenergic non-cholinergic innervation in monkey and human cerebral arteries. *Br J Pharmacol* 71:281-283, **1981.**

Toda N, Okamura T. Mechanism underlying the response to vasodilator nerve stimulation in isolated dog and monkey cerebral arteries. *Am J Physiol* 259:H1511-H1517, **1990a.**

Toda N, Okamura T. Modification by L-N-monomethyl arginine (L-NMMA) of the response to nerve stimulation in isolated dog mesenteric and cerebral arteries. *Jpn J Pharmacol* 52:170-173, **1990b.**

Toda N, Okamura T. Role of nitric oxide in neurally induced cerebroarterial relaxation. *J Pharmacol Exp Ther* 258:1027-1032, **1991.**

Uemura Y, Kowall NW, Beal MF. Selective sparing of NADPH-diaphorase-somatostatin-neuropeptide Y neurons in ischemic gerbil striatum. *Ann Neurol* 27:620-625, **1990.**

Toda N. Mediation by nitric oxide of neurally induced human cerebral artery relaxation. *Experientia* 49:51-53, **1993.**

Unmento M, Olds ME. Effects of chlordiazepoxide, diazepam and chlorpromazine on conditional emotional behaviour and conditioned neuronal activity in limbic, hypothalamic and geniculate regions. *Neuropharmacol* 14:413-425, **1975.**

Vallance P, Collier J, Moncada S. Nitric oxide synthesised from L-arginine mediates endothelium-dependent dilatation in human veins *in vivo*. *Cardiovasc Res* 23:1053-1057, **1989.**

Vallance P, Benjamin N, Collier J. The effect of endothelium -derived nitric oxide on ex-vivo whole blood platelet aggregation in man. *Euro J Clin Pharmacol* 42:37-41, **1992.**

Vallance P, Moncada S. Nitric oxide-from mediator to medicines. *J Royal Coll Phys* London 28:209-219, **1994.**

Valtschanoff JG, Weinberg RJ, Kharaza VN, Nakane M, Schmidt HHHW. Neurons in rat hippocampus that synthase nitric oxide. *J Comp Neurol* 331:111-121, **1993.**

Vanderwinden JM, Maollux P, Schiffman SN, Vanderhaeghen JJ, De Laet. Nitric oxide synthase in infantile hypertrophic pyloric stenosis. *N Engl J Med* 327:511-515, **1992.**

Van Gelderen EM, De Bruijne ELE, Agteresch HJ, Saxena PR. The effect of nitric oxide donors on haemodynamics and blood flow distribution in the porcine carotid circulation. *Br J Pharmacol* 113:1303-1309, **1995.**

Van Leeuwen R, De Vries R, Dzoljic MR. 7-nitro indazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, **1995.**

Van Leeuwen R, De Vries R, Dzoljic MR. 7-nitro indazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, **1995.**

Varner PD, Beckman JS. Nitric oxide toxicity in neuronal injury and degeneration. In: *Nitric oxide in the nervous system.* Ed: Vincent SR. Academic Press, New York, pp.191-207, **1995.**

Vincent SR, Hope BT. Neurons that say NO. *Trends Neuro Sci* 15:108-113, **1992.**

Vincent SR, Kimura H. Histochemical mapping of nitric oxide synthase in the rat brain. *Neurosci* 46:755-784, **1992.**

Vincent SR. Nitric oxide: a radical neurotransmitter in the central nervous system. *Prog Neurobiol* 42:126-160, **1994.**

Vincent SR. Localization of nitric oxide neurons in the central nervous system. In: *Nitric oxide in the nervous system.* Ed: Vincent SR. Academic Press, New York, pp.83-103, **1995.**

Vizzard MA, Erdman SL, de Groat WC.Increased expression of neuronal nitric oxide synthase (NOS) in visceral neurons after nerve injury. *J Neurosci* 15:4033-4045, **1995.**

Wang O, Pelligrino DA, Baughman VL, Koenig HM, Albrecht RF. The role of neuronal nitric oxide synthase in the regulation of cerebral blood flow in normocapnia and hypercapnia in rats. *J Cereb Blood Flow Metab* 15:774-778, **1995.**

Wang Y, Marsden PA. Nitric oxide synthase: biochemical and molecular regulation. *Curr Opin Nephrol Hypertens* 1:12-22, **1995.**

Wetts R, Vaughn JE. Choline acetyltransferase and NADPH diaphorase are co-expressed in rat spinal cord neurons. *Neurosci* 63:1117-1124, **1994.**

Wiklund NP, Person MG, Gustafson LE, Moncada S, Hedqvist P. Modulatory role of endogenous nitric oxide in pulmonary circulation *in vivo*. *Eur J Pharmacol* 185:123-124, **1990.**

Wilcox CS, Welch WJ, Murad F, Gross SS, Taylor G, Levi R, Schmidt HHHW. Nitric oxide synthase in macula densa regulates glomerular capillary pressure. *Proc Natl Acad Sci USA* 89:11993-11997, **1992.**

Williams CV, Nordquist D, McLoon SC. correlation of nitric oxide synthase expression with changing patterns of axonal projections in the developing visual system. *J Neurosci* 14:1746-1755, **1994.**

Williams JA, Vincent S, Reiner P. Nitric oxide production in rat thalamus changes with behavioral state, local depolarization and brainstem stimulation. *J Neurosci* 17:420-427, **1997.**

Woolfson R, Postin L. Effect of $N^G$-monomethyl-L-arginine on endothelium-dependent relaxation of human subcutaneous resistant arteries. *Clin Sci* 79:273-278, 1990.

Worl J, Mayer B, Neuhuber WL. Spatial relationships of enteric nerve fibres to vagal motor terminals and the sarcolemma in motor endplates of the rat oesophagus: a confocal laser scanning and electron-microscopic study. *Cell Tissue Res* 287:113-118, 1997.

Wu W, Han K, Li L, Schinco FP. Implantation of PNS graft inhibits the induction of neuronal nitric oxide synthase and enhances the survival of spinal motoneurons following root avulsion. *Exp Neurol* 129:335-339, 1994.

Wu W, Li Y, Schinco FP. Expression of c-jun and neuronal nitric oxide synthase in rat spinal motoneurons following axonal injury. *Neurosci Lett* 179:157-161, 1994.

Wu W, Luizzi FJ, Schinco FP, Depto AS, Li Y, Mong JA, Dawson TM, Snyder SH. neuronal nitric oxide synthase is induced in spinal neurons by traumatic injury. *Neurosci* 61:719-726, 1994.

Yallampalli C, Garfield RE, Byam-Smith M. Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. *Endocrinology* 133:1899-1902, 1993.

Youdim MB, Lavie L. Selective MAO-A and B inhibitors, radical scavengers and nitric oxide synthase inhibitors in Parkinson's disease. *Life Sci* 55:2077-2082, 1994.

Zarri L, Bucossi G, Cupello A, Rappallino MV, Robello M. Modulation by nitric oxide of rat brain GABA receptors. *Neurosci Lett* 180:239-242, 1994.

Zhang X, Verge V, Wiesenfeld-Hallin Z, Ju G, Bredt D, Snyder SH, Hökfelt T. Nitric oxide synthase-like immunioreactivity in lumbar dorsal root ganglia and spinal cord of rat and monkey and effect of peripheral axotomy. *J Comp Neurol* 335:563-575, 1993.

Zhang ZG, Chopp M, Gautam S, Zaloga C, Zhang RL, Schmidt HH, Pollock JS, Forstermann U. Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurons after focal cerebral ischemia in rat. *Brain Res* 654:85-89, 1994.

Zhao WG, Tilton RG, Corbett JA, McDaniel ML, Misko TP, Williamson JR, Cross AH, Hickey WF. Experimental allergic encephalomyelitis in the rat is inhibited by aminoguanidine, an inhibitor of nitric oxide synthase. *J Neuroimmunol* 64:123-133, 1996.

Zhu ZX, Luo LG. Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. *J Neurochem* 59:932-935, 1992.

Zielasek J, Jung S, Gold R, Liew FY, Toyka KV, Hartung HP. Administration of nitric oxide synthase inhibitors in experimental autoimmune neuritis and experimental autoimmune encephalomyelitis. *J Neuroimmunol* 58:81-88, 1995.

# CHAPTER 3

## NITRIC OXIDE AND CLINICAL MEDICINE: THERAPEUTIC ASPECTS

Based on numerous biological roles of NO, its impact on clinical medicine is developing. However, the ubiquity of NO and its involvement in a wide variety of physiological and pathophysiological mechanisms implicate that drugs designed to modify NO biological activity may have distinct effects. The list of pathological conditions in which NO is an important mediator is increasing (Moncada and Higgs, 1993) and the changes of the L-Arg/NO/cGMP system could be implicated in a wide variety of diseases. Thus, further clinical applications of NO, its analogues or of newly developed NOS inhibitors are forthcoming. The therapeutic challenge would consist in manipulating the NO pathways selectively (Moncada *et al.*, 1991).

### 3.1 Diseases with Hypofunction of Nitric Oxide System

#### *3.1.1. Cardiovascular Diseases*

The most common conditions with hypofunction of the NO system are *hypertension and vasospastic diseases*. Decreased synthesis or action of NO has been implicated in virtually every pathophysiologic state associated with increased vascular tone, vasospasm or enhanced adhesion of platelets and leucocytes to the vessel wall (Ånggård, 1994). Likewise, the NO system is impaired in patients with essential hypertension (Calver *et al.*, 1993). Diminished basal NO-mediated vasodilatation has been demonstrated in patients not only with hypertension (Calver *et al.*, 1992a; Panza *et al.*, 1993), but also with hypercholesterolemia (Creager *et al.*, 1992), atheroma (Ludmer *et al.*, 1986) and angiopathy in diabetes mellitus (Calver *et al.*, 1992b; Johnstone *et al.*, 1993). In addition, endothelial functions, including NO release and actions, are clearly impaired in important vascular diseases, such as atherosclerosis, reperfusion injury and vasculopathy associated with angioplasty, bypass surgery and transplantation (Nathan, 1992; Bucala *et al.*, 1991; Hogan *et al.*, 1992; Busse and Fleming, 1993). However, treatment of hypertension may restore NO-mediated vasodilatation towards normal (Calver *et al.*, 1991a). Flow-dependent coronary dilatation by NO has been demonstrated in humans (Drexler *et al.*, 1989), while decreased endothelium-dependent relaxation has been reported in arteriosclerotic coronary arteries (Moncada, 1992).

Concerning therapy, *nitrovasodilators* have been in clinical practice for about 100 years and are still widely used in conditions such as coronary heart disease, congestive heart failure, hypertensive emergences, pulmonary hypertension, fibrinolysis, percutaneous coronary angioplasty and complications after cardiac catheterization (Moncada *et al.*, 1991). NO-containing vasodilatator drugs, including glyceryl trinitrate, other nitrate esters, sodium nitroprusside and a new one, molsidomine, have been used clinically to decrease systemic vascular resistance and blood pressure. However, only recently their mechanism of action via the liberation of NO, followed by increased cGMP, vascular smooth muscle relaxation and inhibition of platelet aggregation (Murad *et al.*, 1979; Ignarro, 1989) has come to light. In addition, it has been shown that NO donors, glyceryl trinitrate or sodium nitroprusside, also decrease myocardial contractility (Grocott-Mason *et al.*, 1993). However, nitrovasodilators preferentially dilate veins (MacAllister *et al.*, 1995a) and this is the basis for at least part of the efficacy of glyceryl trinitrate in the treatment of heart failure and coronary heart disease, explaining also unwanted postural hypotension as a side effect. The venoselectivity is most

easily explained by the observations that veins have low basal output of NO (Vallance *et al.*, 1989) and consequently the guanylyl cyclase in venous smooth muscle is upregulated. Likewise, results from animal studies implicate that nitrovasodilators have an exaggerated effect in vessels with damaged endothelium or impaired NO production (Moncada *et al.*, 1991). This effect also occurs in human coronary vessels *in vivo* and contributes to the anti-anginal effect of nitrovasodilators. The anti-platelet effect of conventional nitrovasodilators is smaller than dilatation, but the new drug S-nitroso-glutathione metabolises NO within platelets, inhibiting platelet aggregation at doses that cause only minimal vasodilatation (Radomski *et al.*, 1992). Recent results implicate a possible protective role for nitrovasodilators in stroke, but it is not yet clear whether this is a vascular, platelet or neuronal effect of the drugs (Vallance and Moncada, 1994).

New therapeutic approaches may be aimed specifically at preserving endothelial integrity and boosting a failing NO system by administration of L-Arg or preventing its destruction by using antioxidants. In advanced states of endothelial dysfunction it would not be possible to enhance endogenous NO synthesis. Therefore, substitution with exogenous NO generators would be beneficial. Traditional NO donors, such as nitroglycerine, will be replaced by new NO generators having both vasodilating and anti-adhesive effects without development of tolerance (Änggård, 1994).

Further enhancement of the NO system has been obtained by agonist-stimulated NO synthesis (Vallance and Moncada, 1994). c-NOS activators, such as Ach, bradykinin, substance P and 5-HT, activate e-NOS and stimulate endothelial cells to release NO. It has been suggested to develop such agonists to cause long-term stimulation of the NO system. For example, inhibition of angiotensin-converting enzyme (ACE) by captopril or enalapril increases bradykinin levels with subsequent stimulation of endothelium-dependent NO release. This is an additional mechanism of action of these drugs (Vanhoutte *et al.*, 1989). Moreover, it has been reported that new ACE inhibitors show potentiation of memory retention via endothelial-derived bradykinin, activated e-NOS and the subsequent formation of NO (Hock and Weimer, 1992).

Accordingly, new technology explores the possibility of enhancing endogenous NO production and activity, using it in treatment of common *vascular degenerative diseases* such as *atherosclerosis, vascular thrombosis and restenosis*. Moreover, the enhancement of the NO system could be achieved by increasing concentrations of L-Arg, incorporated into a physiologically acceptable vehicle for oral use or it may be administered intravascularly (Calver *et al.*, 1991). Infusion of L-Arg decreases blood pressure in healthy volunteers and patients with hypertension (Hishikawa *et al.*, 1992) and corrects endothelial dysfunction in patients with hyperholesterolemia (Creager *et al.*, 1992; Drexler *et al.*, 1991). Beneficial effects of dietary L-Arg supplementation in cardiovascular disease is under current investigation (Vallance and Moncada, 1994). A new form of NO donors, called S-nitrosothiols, because NO is attached to a thiol, release free NO into the blood. This shows that NO attached to thiol can provide a regulatory function in a new blood substitute (Stampler *et al.*, 1996). Since superoxide rapidly inactivates NO and it has been suggested that giving superoxide dismutase and blocking the endogenous production of superoxide might protect NO activity (Gryglewski *et al.*, 1986). Components of the NO metabolic pathway may be also manipulated by enhancing the amount of present NOS, either

specifically in the vascular lesion site or systemically in the vascular network (Vallance and Moncada, 1994).

Furthermore, development of an NO sensor on the tip of a catheter used for angioplasty would allow detection of local NO production after Ach or bradykinin treatment (Bassenge, 1992). This clinical test for determination of the functional state of the endothelial lining could be monitored in regions of arteriosclerosis before and after angioplasty. It could be used to determine the functionality of endothelial cells covering denuded vascular surfaces. The effect of nitrovasodilators could also be monitored with this ultramicrosensor for NO (Creager *et al.*, 1992; Drexler *et al.*, 1991). Once developed, the clinical assessment of NO production and effects in specific sites of the coronary vasculature would become a useful clinical tool (Kiechle and Malinski, 1993; Vallance *et al.*, 1995).

### *3.1.2. Respiratory Diseases*

NADPH-d and immunostaining have shown expression of c-NOS in human nerve elements and large vessel endothelium and i-NOS in alveolar macrophages in human lung (Kobzbik *et al.*, 1993). NOS is found in respiratory epithelial cells (Robbins *et al.*, 1993). In the respiratory system decrease of NO production by c-NOS provides a link between pulmonary hypertension and chronic lung disease, while abnormalities of NANC neurons supplying the airways could increase bronchial constriction (Dinh-Xuan *et al.*, 1993). Accordingly, NO was detected in exhaled air of humans (Gustafsson *et al.*, 1991). In addition, inhalation of NO gas by humans has resulted in the reversal of pulmonary vasoconstriction caused by hypoxia (Blomqvist *et al.*, 1993) or pulmonary hypertension (Kinsella *et al.*, 1992; Rossaint *et al.*,1993). Adult patients with severe respiratory distress syndrome treated with NO inhalation show reduced pulmonary artery pressure and increased arterial oxygenation (Rossaint *et al.*, 1993). Bilateral pulmonary infiltrates secondary to pneumococcal pneumonia were reversed by NO inhalation (Blomqvist *et al.*,1993). Recent results implicate that *inhaled NO* is beneficial in the treatment of *pulmonary hypertension*, common in neonatal and adult respiratory distress syndrome and after cardiopulmonary bypass surgery (Frostell *et al.*, 1991, 1993; Pepke-Zabe *et al.*, 1991; Rossiant *et al.*, 1993; Winberg *et al.*, 1994).

It has been found that *inhaled NO* in low concentrations 50-80 ppm (2-3.2 μmol/l) causes selective pulmonary vasodilatation, bronchodilatation, improving arterial oxygenation, without causing systemic vasodilatation. Inhalation of very low concentrations of NO gas (in order of 100 parts per billion) appears to cause selective vasodilatation of vessels supplying ventilated alveoli and this therapeutic strategy might be useful in certain patients with acute lung injury (Benzing *et al.*, 1995; Samama *et al.*, 1995) or other causes of pulmonary hypertension (Pepke-Zaba *et al.*, 1991; Gerlach *et al.*, 1993). Inhaled NO at low concentrations (<80 ppm) is rapidly taken up by the capillary blood of the lung, causing an increase in excretion of nitrates. Since the airways follow the pulmonary arteries into the lung as far as the precapillary level, it is easy to understand how some inhaled NO can diffuse over to resistance pulmonary arterioles. By acting on the abluminal side of the arteriolar vascular smooth muscle, NO causes specific vasodilatation in the well-ventilated segments of the lung. Following diffusion into the blood, it is rapidly inactivated by binding to haemoglobin and thus will not have any generalised vascular effect. This selective vasodilatation is observed by NO in pulmonary vasoconstriction and pulmonary

hypertension. There remain substantial issues to be addressed about long-term consequences of inhaled NO, but it is clear that breathing 20-40 ppm (about 0.6-1.6 μmol/l) for several days does not cause obvious toxicity in humans (Schmidt and Walter, 1994). Although inhaled NO has been successfully used in treatment of *acute pulmonary hypertension* (Higenbottam, 1993) it is still an experimental therapy. Some *chronic and severe pulmonary hypertension* appears to be associated with structural changes, rendering these conditions resistant to NO inhalation (Roger *et al.*, 1996). Inhalation of NO may be associated with risks. Although it is generally well tolerated, NO can cause tissue damage by free radical formation and inflammation due to increased capillary permeability. Precise control of NO concentration in the inspired gas of continuous flow respiratory devices provides the possibility of using inhaled NO with appropriate safety precautions (Hudome *et al.*, 1996). Inhalation of NO will develope in the future, because efficacy and safety evaluation will help to determine its position in treating acute pulmonary hypertension and *bronchial asthma* (Änggård, 1994; Dhillon *et al.*, 1996).

NO reacts with other free radicals and with some metals, but is remarkably unreactive with most biological molecules. While NO is a free radical, it is not necessarily highly reactive or destructive. NO has the highest diffusional coefficient of any biological molecule, being 1.4-fold higher than oxygen or CO at 37°C degree. Inhalation of NO from ambient air and nasopharynx epithelium, acts in concert with local synthesis in epithelial, endothelial and neuronal cells to optimize ventilation and regulate bronchial epithelial ciliary motility, mucus secretion and airway tone (Schmidt *et al.*, 1992 a). Contrary, concerning morbidity of the upper aerodigestive tracts, reduced salivary NO levels in smokers may potentiate roles in the pathogenesis of smoking related diseases (Bodis and Haregewoin, 1994).

### *3.1.3 Gastrointestinal Diseases*

No is also formed in the stomach from both exogenous and endogenous (epithelium, endothelium) sources, through the conversion of nutritional nitrate to nitrite by facultative anaerobic bacteria (Schmidt *et al.*, 1992a). Inhibition of NOS blocks mucosal hyperaemia and aggravates experimental *gastric ulceration*, suggesting a physiological cytoprotective effect of NO on mucosa that may provide resistance to *Heliobacter pylori* (Kim and Kim, 1996). However, gastric NO formation could have a mutagenic risk by its potential for nitrosamine formation from nutritional amine precursors.

The discovery of a deficient NOS in the pylorus in *infantile hypertrophic pylorospasm* may be the first example of absent NO production in gastrointestinal smooth muscle, while in *achalasia, Hirschsprung's disease,* NANC nerves are missing or lack n-NOS (Vanderwinden *et al.*, 1992; Mearin *et al.*, 1993; Schmidt and Walter, 1994). The absence of dilatatory activity of NO released locally in the pyloric muscle could explain the pylorospasm in children with this condition. This is consistent with diminished NO synthesis in opiate-induced constipation (Calignano *et al.*, 1992). Additional data suggest a *novel clinical approach* using local NO donors to control gastrointestinal motility and regulate sphincteric function (Slivka *et al.*, 1994). An ointment containing 0.5% nitroglycerine is recently introduced in *the* therapy of *haemorrhoids and fissures*, because nitroglycerine ointment releases NO, which opens the anal sphincter, allowing blood to flow from the area and relieve haemorrhoids and fissures (Gorfine, 1995).

### *3.1.4 Diseases of Genitourinary Tract*

Based on the findings that NOS is expressed in man's pelvic NANC neurons, innervating corpora cavernosa and in neuronal plexuses of the adventitial layer of the penile cavernosus arteries, NO is identified as a neuronal mediator of *penile erection* in men (Burnett *et al.*, 1993). NOS inhibitors abolish electrophysiologically produced penile erections in men (Raifer *et al.*, 1992). Accordingly, deficient NO-mediated relaxation of the corpus cavernosum may be associated with impotence (Saenz *et al.*, 1989). Application of NO donors causes relaxation of human corpus cavernosum and penile erection (Meyhoff *et al.*, 1992). Therefore, local application of NO donors have a beneficial effect in *impotence* among adult men, especially in those with diabetes mellitus. It has been suggested that erectile impotence in chronic diabetic males is a part of the peripheral neurological degeneration involving the NO pathway (Ignarro *et al.*, 1990; Änggård, 1994). Moreover, peripheral cGMP mechanisms have been found in testicular tissue and vas deferens derived NO may upregulate motility of spermiocytes via $Ca^{2+}$ influx (Schmidt and Walter, 1994). NO pathway may have a role in the control of human prostatic smooth muscle activity and/or in secretory neurotransmission (Hedlund *et al.*, 1997).

Concerning women, immunoreactive NOS nerve fibres have been found in the adventitia of the human uterine artery, where NO mediates vasodilatation (Toda *et al.*, 1994). In pregnant females NO regulates placental blood flow, nutrition and growth in uterus during preterm, contributing to the maintenance of uterine quiescence and maternal vasodilatation. NO is involved in the pathogenesis and clinical features of *hypertensive disorders during pregnancy* (Nobunaga *et al.*, 1996). Contrary, during parturition with uterine contraction, NO activity decreases to allow delivery (Yallampalli *et al.*, 1993; Morris *et al.*, 1995). Based on these results, nitroglyceryl patches have been applied successfully in blocking *painful uterine contractions* and *preterm labour* (Lee *et al.*, 1994).

Hypertension and immune dysfunction in patients with *chronic or end-stage renal failure* may be secondary to NOS blockade by an increased concentration of two endogenous L-Arg derived NOS inhibitors: L-NMMA and ADMA (Vallance *et al.*, 1992a). Patients with chronic renal failure have decreased plasma L-Arg concentrations and elevated plasma ADMA levels. NO deficiency leads to hypertension and renal damage (Baylis and Vallance, 1996). ADMA, as potent competitive inhibitor of NOS, might induce vasoconstriction, that can further cause some *complications of renal failure* (MacAllister *et al.*, 1996a,b). Moreover, inhibition of the activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes L-NMMA and ADMA, provides an alternative mechanism for modulating NO system (MacAllister *et al.*, 1996a,b).

Involvement of NO in regulation of bladder function increases new therapeutic possibilities for treatment of *incontinence*, because in the urinary system, NANC nerves control bladder outflow (Person *et al.*, 1993).

### *3.1.5 Oncology*

It has been shown that certain types of immunotherapy can induce NOS, such as injection of interleukin-2 in treatment of *renal tumours*. Elevated NO production could participate in the *anti-tumour effects* of this immunotherapy (Habeas *et al.*, 1992).

Regarding the major goal in the *anticancer therapy* to enhance the effectiveness of clinically used chemotherapeutic agents, NO delivery systems increase cisplatin cytotoxicity,

providing insight into strategies for participation of NO donors with cisplatin therapy (Wink *et al.*, 1997). Further therapy is planned by devising a successful method of attaching NO donors into synthetic biomaterial substrates, implants which could become "biologically alive". Such implants could be used to sensitize tumour cells to be more vulnerable to radiation, modulate inflammatory reactions, facilitate wound healing, boost the immune system (Chu, 1995). In addition, NOS inhibitors caused changes in tumour pathology, indicating promise for potential applications in therapy (Adams and Stratford, 1994).

### 3.2 Diseases with Hyperfunction of Nitric Oxide System

In contrast to previous pathological conditions, other diseases could involve hyperfunction of NO system. Examples of these are septic shock, chronic inflammation, autoimmune disease, and stroke, accompanied with abounding release of NO from i-NOS. However, usually NO produced by i-NOS is involved in host-defence mechanisms, killing of pathogens including leishmania, mycobacterium tuberculosis, malaria parasites and certain fungi, mediates non-specific immunity and is toxic to tumour cells (Moncada *et al.*, 1991; Habeas *et al.*, 1991). Nitrate and nitrite (stable breakdown products of NO) are produced by human macrophages, their amounts are variable and increased in *inflammatory conditions* (Habeas *et al.*, 1992). Food intake could not account for the increase of nitrate levels (Snyder and Bredt, 1992; Hölscher *et al.*, 1995).

#### *3.2.1 Sepsis*

Patients, adults and newborns, with *sepsis* have high levels of nitrogen oxides, nitrite and nitrate, in serum and urine (Marzinzig *et al.*, 1997), which may be useful in forecasting the severity of illness and occurrence of *septic shock* (Ochoa *et al.*, 1991; Shi *et al.*, 1993). It has been suggested that NO produced by i-NOS and pro-inflammatory cytokines contribute to reversible *myocardial depression* in patients with sepsis and congestive heart failure (Oddis *et al.*, 1997). In studies on the ability of NOS inhibitors to reverse the haemodynamic effects of septic shock, some of them have recently been studied in critically ill humans with some success (Schilling *et al.*, 1993). Preliminary studies in humans suggest that NOS inhibitors improve blood pressure and stabilize haemodynamics (Vallance and Moncada, 1993; Wolfe and Dasta, 1995). Likewise, L-NMMA restores hypotension in patients with septic shock (Petros *et al.*, 1994). Selective i-NOS blocking drugs prevent cytotoxic effects of endotoxin and some cytokines *in vitro* and would be useful drugs to prevent NO overproduction accompanied with toxicity and to *reverse septic hypotension* (Radomski *et al.*, 1993). Accordingly, selective i-NOS inhibitors, like aminoguanidine, that can block pathologic NO production without affecting physiological endothelial, neuronal or platelet function, are in current development (Misko *et al.*, 1993; Vallance and Moncada, 1994).

#### *3.2.2 Inflammation*

NO synthesis is increased in *general inflammatory conditions* (erythema, vascular leakiness). Similarly, an increased concentration of NO in the exhaled air of patient with *seasonal rhinitis* (Martin *et al.*, 1996) and *asthma* (Person *et al.*, 1994; Kharitonov *et al.*, 1994), may reflect *inflammation of the airways*, and exhaled NO may be a useful means for monitoring *asthma severity and treatment efficacy* (Kharitonov *et al.*, 1994). It is likely that excretion of NO from the lung represents an overflow from NO produced locally rather than NO extracted

from the blood. The measurement of NO in exhaled breath could, therefore become a useful indicator of pulmonary NO production in various lung diseases (Ånggård, 1994).

Induction of i-NOS by endotoxin or cytokines appears to be part of the *chronic inflammatory response* and could contribute to vasodilatation, vascular leakage and tissue damage in a number of inflammatory conditions. NOS inhibitors decreased such NO release. Increased concentrations of nitrite in synovial fluid and serum in patients with osteoarthritis and rheumatoid arthritis, have suggested increased NOS activity in *chronic rheumatic diseases* (Farrell *et al.*, 1992; Ueki *et al.*, 1996). NOS inhibitors suppressed adjuvant-induced arthritis (Connor *et al.*, 1995). There are data showing induction of i-NOS during chronic inflammatory diseases, in the gut of patients with *chronic ulcerative colitis and Crohn's disease* (Middleton *et al.*, 1993; Boughton-Smith *et al.*, 1993).

In the heart, increased production of NO following expression of the i-NOS could contribute to the pathogenesis of *myocarditis, dilated cardiomyopathy and postpartum cardiomyopathy* (De Belder *et al.*, 1993; 1995). The further cytotoxicity in the heart is caused by reaction of NO with superoxide forming peroxynitrate and contributing to tissue damage in inflammation and ischaemia-reperfusion (Downey *et al.*, 1990).

### *3.2.3 Endotoxemia*

Increased activity of the NO system may be involved in maintaining vasodilatation and systemic circulatory disturbances in patients with severe cirrhosis and *liver failure*, usually accompanied with endotoxemia (Sogni *et al.*, 1995; Matsumoto *et al.*, 1995). Likewise, over-production of NO in platelets has been suggested as a cause of the bleeding tendency of *uraemia* (Remuzzi *et al.*, 1990; Zoja *et al.*, 1991).

### *3.2.4 Autoimmunity*

It has been reported that the feed-back blockade on NOS catalytic activity by NO (but not oxidation products, such as nitrite, nitrate, peroxynitrate) serves not only to modulate NO production in the cell of origin but also in the neighbouring cells (Griscavage *et al.*, 1993; Rengasamy and Johns, 1993). This could be an important amplification mechanism in the response to invading pathogens. Alternatively, it may represent an uncontrolled process with disastrous consequences for host integrity. Unregulated NO production becomes self-destructive, like in *autoimmune diseases, immune rejection of allograft* and *graft versus host disease* (Schoedon *et al.*, 1993). Concerning autoimmunity, NOS inhibitors prevent anti-DNA immune complex *glomerulonephritis* and reduce the intensity of inflammatory *arthritis* (Weinberg *et al.*, 1994). Additionally, pancreatic beta cells have limited capacity for free radical scavenging and are thus highly sensitive to NO cytotoxicity. In pharmacologically induced models of *insulin-dependent diabetes mellitus*, progressive autoimmune insulitis, dysfunction and eventual killing of pancreatic beta cells correlate with the induction of i-NOS and has been decreased by NOS inhibitors. Therefore, NO has been added to the list of mediators involved in processes of *local and systemic inflammation* in humans (Green *et al.*, 1994).

*Anti-inflammatory glucocorticoids* inhibit induction of i-NOS but are ineffective once the enzyme is expressed (Radomski *et al.*, 1990). This can explain why glucocorticoids facilitate the spread of *infections, malignancy* and prevent the consequences of *delayed hypersensitivity* in conditions such as *transplant rejection and vasculitis* (Moncada *et al.*, 1991). Similarly,

the anti-fungal imidazole compound, econazole, inhibits i-NOS (Bogle and Vallance, 1996), while the anti-inflammatory and cytotoxic drug methotrexate blocks synthesis of NOS cofactor H4B (Gross *et al.*, 1992). These effects could participate in the therapeutic efficacy of drugs. Development of more specific *anti-inflammatory and immunosuppressive* agents that simultaneously block i-NOS accompanied with new therapeutic approaches would be beneficial for clinical practice (Salvemini *et al.*, 1995; Pfeilschifter *et al.*, 1996).

### 3.3 Disturbed Nitric Oxide System and Neuropsychiatric Diseases

Conditions which are not only associated with hypo- or hyper-function of NO system, but also with combined hypo/hyper-function of NO mechanisms, have been described.

#### *3.3.1 Brain Ischaemia*

In brain ischaemia-reperfusion damage the situation is more complex than in the other part of the body. NO formation initially is protective and increased, inducing collateral perfusion, but after time NO formation from c-NOSs ceases. Upon reoxygenation, NO production from i-NOS occurs, but is accompanied with the presence of peroxynitrate formation, leading to tissue damage. Precisely, the reaction of NO with superoxide forms peroxynitrate that causes cytotoxic oxidation (Beckman *et al.*, 1990), contributing to tissue injury in brain ischaemia-reperfusion (Lipton *et al.*, 1993). Evidence suggests that NO production is enhanced at all stages of cerebral ischaemia. At the onset of ischaemia, microvascular and parenchymal NO overproduction are driven by upregulation of e-NOS and n-NOS. At later times, i-NOS is responsible for the synthesis of NO. Thus, vascular-derived NO protects against brain injury, while neuronal-derived NO leads to neurotoxicity. In the presence of high levels of GLU following ischaemia-reperfusion, NOS containing cells would act like activated macrophages continuously releasing large amounts of NO killing neighbouring cells. While neurons are extremely sensitive toward NO, other cells are much less sensitive (Kröncke *et al.*, 1997). Consequently, n-NOS inhibitors, such as indazole derivatives, ameliorate the damage by impairing GLU-induced NO synthesis without interfering with the beneficial vascular effects of endothelial NO. At later times (>6 hours), i-NOS inhibitors, such as aminoguanidine, improve ischaemic-reperfusion tissue injury (Iadecola, 1997). Accordingly, comparing usefulness of different NOS inhibitors, it has been described that nitro-L-Arg and L-NAME worsen *focal ischaemic brain damage* due to particular blockade of e-NOS, while relatively selective n-NOS inhibitors, 7-NI (Dalkara *et al.*, 1994), ARL17477 or S-methyl-isothiureido-L-norvaline as well as more specific i-NOS inhibitor aminoguanidine reduce infarct size (Iadecola, 1997).

Evidently, manipulation of the NO system might be used to devise *new strategies* for *stroke treatment*. Results suggest that the effect of NO in ischaemic brain damage depends on the stage of evolution of the ischaemic process and on the cell type producing NO. In patients who reached medical attention within the first few hours of onset of ischaemia, NO donors might be useful, particularly when interventional approaches to restore blood flow are not feasible. Regarding the hypotension that they cause, the safest and most effective administration of these agents would be in an intensive-care unit. Later on, n-NOS inhibitors would be beneficial, perhaps in conjunction with GLU-receptors antagonists to increase neuroprotection. At later stages after onset of ischaemia (>12 hours), i-NOS inhibitors could be administered to block the deleterious effect of NO produced by i-NOS. Generally, most

patients with ischaemic stroke arrive at the emergency room many hours after the onset of symptoms and, therefore, i-NOS inhibitors would be particularly valuable. Although lubeluzole, a compound with some NOS inhibitory properties, is currently clinically tested in stroke patients, selective inhibitors of n-NOS and i-NOS for human use are not yet available (Iadecola, 1997). Additionally, a few basic conditions have to be fulfilled before NOS inhibitors can be used in clinical medicine. Because of the stage-dependent double-edged role of NO in ischaemic damage, evaluation of the time of onset of ischaemia and of the stage of evolution of ischaemic injury are necessary for successful treatment with NOS inhibitors. Magnetic resonance imaging could be useful in that estimation. Further research, such as establishment of clinical trials are needed to define the safety and therapeutic potentials of selective NOS inhibitors (Meldrum, 1995; Iadecola, 1997).

### *3.3.2 Migraine and Subarachnoid Haemorrhage*

Disorders of endothelium or neurally derived NO have been involved in the pathogenesis of migraine (Appenzeller, 1991) and vasospasm after subarachnoid haemorrhage (Edwards *et al.*, 1992). While in peripheral blood vessels, NO is produced by e-NOS, in cerebral vasculature additional NO is synthesised by n-NOS in NANC neurons, highly concentrated in adventitial layers. These nerve plexuses also include 5-HT neurons which constrict the arteries via 5-$HT_{1D}$ receptors that are responsible for clinically efficient anti-migraine drugs such as sumatriptan (Saxena, 1995). Therefore, selective vasodilatation of such cerebral vessels by NO may play a role in the pathophysiology of *migraine* (Snyder and Bredt, 1991). NO may play a key role in migraine and other vascular headaches since both, glyceryl trinitrate, as a NO donor, and histamine, increasing endothelium-derived NO, cause a pulsating dose-dependent headache like migraine. At relatively high doses of glyceryl trinitrate migraine sufferers develop a stronger and more migraine-like headache accompanied with more pronounced cerebral arterial dilatation than controls. After glyceryl-trinitrate infusion, non-migraineurs remain headache free while migraineurs develop a migraine-like attack. Accordingly, it has been suggested that migraine may be caused by increased amounts and/or affinity of an enzyme in the NO-triggered cascade of reactions. NO may also be involved in the pathogenesis of other vascular headaches, e.g. cluster and symptomatic vascular headaches (Olesen *et al.*, 1994). Recent results suggest that combined measurements of systemic levels of NO and endothelin-1 together with transcranial Doppler velocity data would provide useful information on the haemodynamic changes of cerebral blood flow regulation in migraineurs, thereby adding new insights into the mechanism of the migraine attack (Nattero *et al.*, 1996). In addition, while diminished NO or interference with its action is involved in vasospasm following *subarachnoid haemorrhage* (Watkins, 1995), it has been proposed that facilitating the release (Tanazawa *et al.*, 1996) and enhancing the effect of NO by using drugs that increase intracellular $Ca^{2+}$ (cyclopyazonic acid) and free radicals scavengers (superoxide dismutase) may therapeutically prevent the effect of cerebral vasospasm in subarachnoid haemorrhage (Hans, 1996).

### *3.3.3 Seizures*

Additionally to functions matching neuronal activity with nutritive blood flow and protection of neurons from degeneration, NO could be involved in *neurotoxicity* underlying *ischaemia and seizures* (Varner and Beckman, 1995). Firstly, NOS can be inhibited by its reaction

product NO (Rogers and Ignarro, 1992; Assreuy *et al.*, 1993; Buga *et al.*, 1993). Alternatively, it may represent an uncontrolled process with disastrous consequences for CNS integrity (Schoedon *et al.*, 1993). Secondly, results implicate that NO may protect neurons against overstimulation by GLU due to feed-back blockade of NMDA receptors, while neurotoxic effects may be mediated by peroxynitrate rather than NO itself (Lipton *et al.*, 1993). It has been reported that GLU, an excitatory neurotransmitter which initiates the synthesis of NO in CNS, accounts for synaptic transmission at more sites in the brain than any other neurotransmitter (Snyder and Bredt, 1992). However, cytotoxicity in experimental models of cerebral stroke/seizure postulates a release of GLU causing an overstimulation of NMDA receptors, leading to prolonged release of NO. It is likely that excessive NMDA receptor activation, with the consequent increase in intraneuronal $Ca^{2+}$, contributes to GLU neurotoxicity by enhanced production of NO (Dawson *et al.*, 1991; Moncada *et al.*, 1991). It has been suggested that NO contributes to the *epileptogenesis* in response to excessive GLU-mediated excitation (Proctor *et al.*, 1996). cGMP has been suggested to have a role in *seizures*, because the levels of this nucleotide increase in several brain regions prior to the onset of drug-induced convulsions (Ferrendelli *et al.*, 1980). Infusion of cGMP analogues onto grafts of hippocampus triggers prolonged epileptiform activity in the pyramidal neurons (Freedman *et al.*, 1979). Accordingly, NO participates in both functional and dysfunctional mechanisms of excitatory neurotransmission (neuronal excitotoxicity and epilepsy) via cGMP (Dawson *et al.*, 1991). It is therefore important to note that antagonists of EAA receptors or inhibitors of GLU release or NOS inhibitors have both antiepileptic actions and also protect against ischaemic damage which is thought to be mediated by excessive release of GLU (Snyder and Bredt, 1992; Meldrum, 1995). Recent results indicate that selective n-NOS inhibitors may have therapeutic benefit in stroke and neurologic damage associated with excessive GLU release (Snyder and Bredt, 1992).

An understanding of the role of NO in *seizures* is still in its infancy. Nevertheless, such improved understanding might contribute to innovations in the treatment of seizure disorders. Based on present results the precise mechanism of NO in the expression of seizures is still unclear and controversial (De Sarro *et al.*, 1991; Rundfelt *et al.*, 1995; Van Leeuwen *et al.*, 1995; Kirkby *et al.*, 1996). Furthermore, kindling phenomena and dysregulated mechanisms of cellular $Ca^{2+}$ ion homeostasis have been hypothesised to be involved in pathophysiology of *seizures and bipolar disorders* (Dubovski *et al.*, 1992). It is likely that excessive NMDA receptor activation, with the consequent increase in intraneuronal $Ca^{2+}$, contributes to GLU neurotoxicity by enhanced production of NO, playing a role in neuroexcitation during seizure or bipolar disorders (Moncada *et al.*, 1991; Karatinos *et al.*, 1995). In accordance with that are results that carabamazepine, an potent antiepileptic drug, inhibits influx of $Ca^{2+}$ ions and has beneficial therapeutic effects in patients with resistant epilepsy or manic-depressive disorders (Narasapur, 1983).

### *3.3.4 Schizophrenia*

Development of interventions for schizophrenia on the basis of the NO pathway has been suggested (Karson *et al.*, 1994). A study of the distribution of NADPH-d stained neurons in postmortem brains of five chronic schizophrenic patients, compared with brains of five age- and gender-matched controls, showed a decrease of these stained neurons in the superficial layers but an increase in the deep layers of the white matter of the dorsolateral prepiriform

cortex (Akbarian *et al.*, 1993a). Further data demonstrated lower numbers of NADPH-d stained neurons in the hippocampal formation but greater numbers in the parahippocampal and lateral lobe white matter in brains of seven chronic schizophrenic patients (Akbarian *et al.*, 1993 b). Related to previous studies (Prast and Philippu, 1992; Zhu and Luo, 1992) and these results (Lorrain and Hull, 1993), it has been suggested that NO plays a role in DA release in certain brain areas. Accordingly, as excess dopaminergic activity in mesolimbic structures has been implicated in the pathophysiology of psychosis, the NO pathway supports the possible link between abnormalities of central dopaminergic regulation and the pathogenesis of psychosis (Karatinos *et al.*, 1995).

### *3.3.5 Opioid Dependence and Opiate Withdrawal*

NO is also implicated in the function of opioids in the brain, in both their addictive and analgesic properties. Consequently, manipulations of the NO pathway can lead to new treatments of opioid dependence and opiate withdrawal (Karatinos *et al.*, 1995). Additional data supported the neuroexcitatory role of NO showing that NO donor isosorbide dinitrate aggravated the opioid withdrawal syndrome (Adams *et al.*, 1993), while NOS inhibitors attenuated naloxone induced withdrawal syndrome (Adams *et al.*, 1993; Cappendijk *et al.*, 1993, 1995; Dzoljic *et al.*, 1994). Thus, results implicate the idea that NOS inhibitors may be potentially useful in the treatment of opioid withdrawal syndrome (Bhargava and Sanjay, 1996).

### *3.3.6 Pain*

The central transmission of noxious input evoked by thermal, chemical and mechanical stimuli is blocked by the L-NAME (Radhakrishnan and Henry, 1993), implicating NO as a general mediator in central pain processing (Holthusen and Ding, 1997). Therefore, it has been suggested that NOS inhibitors could be potentially *novel analgesics* (Hao and Xu, 1996).

### *3.3.7 CNS Tumours*

A mutagenicity of NO towards human cells has been shown (Nguyen *et al.*, 1992), accompanied with findings of high levels of c-NOSs (n-NOS and e-NOS; Cobbs *et al.*, 1995). Thus, *selective NOS inhibitors* could be useful in blocking pathophysiological processes important to these tumours (Cobbs *et al.*, 1995).

### *3.3.8 Multiple Sclerosis*

The involvement of i-NOS and NO in MS has been demonstrated (Koprowski *et al.*, 1993; Johnson *et al.*, 1995). Induction of human i-NOS occurs in the demyelinated regions of MS brains (Bo *et al.*, 1994). NO may be directly toxic to the myelin-producing oligodendrocytes (Merrill *et al.*, 1993). Useful for clinical practice are also data obtained from analysis of peripheral blood from patients with MS, supporting a role of monocytic i-NOS in MS (López-Moratalla *et al.*, 1997).

Accordingly, production of NO by i-NOS is implicated in the pathogenesis of several types of brain diseases and injury, including focal cerebral ischaemia (Iadecola *et al.*, 1997), demyelinating diseases (Koprowski *et al.*, 1993; Iadecola *et al.*, 1995) and sepsis (Wong *et al.*, 1996). In some cases, use of *selective i-NOS inhibitors* has directly demonstrated that

i-NOS-derived NO contributes to the development of the pathological symptoms (Iadecola *et al.*, 1995). To prevent potentially deteriorated i-NOS activity, efforts are in progress to develop highly specific i-NOS-inhibitors. However, use of such agents has been limited due to the cross-inhibition of n-NOS and e-NOS. As an alternative to the use of enzyme inhibitors to prevent glial i-NOS expression, the means of regulation the transcription of the i-NOS gene have been examined (Murphy *et al.*, 1993; Nathan, 1992).

### *3.3.9 Other Neuropsychiatric Diseases*

Modulation of NO system may be useful for various neuropsychiatric conditions such as *depression, stress* (Illowsky and Kirch, 1988; Patel, 1994), *anorexia* and *bulimia neurosa* (Squadrito *et al.*, 1993, 1994), *sexual* (Kirkeby *et al.*, 1993; McCann *et al.*, 1994) and *sleep disorders* (Karatinos *et al.*, 1995; Williams *et al.*, 1997).

According to coupling local levels of neuronal activity in the brain to local blood flow and important influence on synaptic transmission (Snyder, 1992; Snyder and Bredt, 1992; Zorumski *et al.*, 1993), NO has been implicated in several other clinical conditions. Deficit in memory can be seen in a variety of neuropsychiatric diseases, including different types of *dementia and amnestic disorders.* The role that NO might have in the memory functions provides a possible physiological way for pharmacological intervention. Although evidence exists that NO can have memory-enhancing functions, the precise role in memory still is not fully clarified. However, recent data implicated the NO system in *Alzheimer's disease*, a cognitive disorder with prominent memory impairment. NADPH-d staining in the neuropil of the hippocampus is decreased in patients with Alzheimer's disease (Rebeck *et al.*, 1993). Since NO is the retrograde messenger responsible for increasing the synaptic efficiency of presynaptic glutaminergic neurons in the induction of LTP (Snyder and Bredt, 1992), the latter data could explain the impaired ability of patients with Alzheimer's disease to learn new information. Whether medications designed to modulate the NO system, such as NO donors, would improve memory performance in humans, would be an exciting topic for further study. The investigators have shown an involvement of NO in the induction of LTP rather than in its maintenance (Schuman and Medison, 1994). Therefore, the manipulations of the NO pathway should be primary designed to improve memory performance influencing memory acquisition (Karatinos *et al.*, 1995). Evidence for the involvement of the NO system in human neuropathology is steadily increasing. Striatal neurons stained by NADPH-d are spared in *Huntington's chorea* (Ferrante *et al.*, 1985; Kowall *et al.*, 1987; Morton *et al.*, 1993), although a dramatic decrease (~ 95%) in the striatal neurons that expressed NADPH-d occurs in this disease (Morton *et al.*, 1993). It has been proposed that over-production of NO could contribute to cell death in the nervous system, while inhibition of NOS by NO (Griscavage *et al.*, 1994) could be protective against that *neurotoxicity.* Regarding that the primary stimulus for NO synthesis in CNS is activation of glutaminergic NMDA receptor (Garthwaite, 1991), an involvement of NMDA receptors overactivity in numerous neurodegenerative conditions has been shown, including *Huntington's chorea, Alzheimer's dementia, stroke* and *ALS* (Bredt, 1996). However, NOS-containing neurons may possess a safety system against NO-mediated toxicity that allow them to survive in an environment rich in NO (Dawson *et al.*, 1991; Snyder and Bredt, 1992). NO-induced NMDA receptor blockade via negative feed back could be a protective mechanism against an excess of NMDA receptor stimulation (Manzoni *et al.*, 1992). Accordingly, NADPH-d positive

neurons survive in a greater numbers than neighbouring NADPH-d negative neurons not only in Huntington's chorea (Morton *et al.*, 1993) but also in Alzheimer's dementia (Hyman *et al.*, 1992), ischaemia (Uemura *et al.*, 1990), some forms of excitotoxicity (Koh *et al.*, 1988) and in amyotrophic lateral sclerosis (ALS), in which somatic motor neurons degenerate but autonomic motor neurons are relatively spared (Wetts and Vaughn, 1994). Both, NOS inhibitors and superoxide dismutase were neuroprotective, pointing out the importance of superoxide anion in subsequent neuronal damage. Therefore, increasing the amount of superoxide dismutase available for scavenging superoxide and reducing the amount of NO by administration of NOS inhibitors could reduce neurotoxicity caused by superoxide and NO, due to *NMDA overstimulation* (Dawson *et al.*, 1996).

Recent evidence suggests that *neurotoxic mechanisms* may play role in etiology of *Parkinson's disease*. Likewise, selective n-NOS inhibitors exert protection, implicating their use in novel therapeutic strategies for Parkinson's disease (Connop *et al.*, 1994, 1996; Schulz *et al.*, 1995). For example, in an animal model of Parkinson's disease, NOS inhibitors provide protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydrophyridine (MPTP)-induced striatal DA depletion (Santiago *et al.*, 1994; Prezedborski *et al.*, 1996). Confirming the role of NO in MPTP induced neurotoxicity, n-NOS null transgenic mice are resistant to MPTP induced striatal dopamine depletion (Prezedborski *et al.*, 1996). Additionally, in an animal model of Huntington's disease, NOS inhibitors are protective against 3-nitropropionic acid-induced or malonate-induced lesions (Schultz *et al.*, 1995).

The role of NO in cerebral damage in *AIDS dementia* has been reported (Snyder, 1993). AIDS dementia may be derived from neurotoxic effects of the coat protein of the HIV virus which kills neurons when acting in conjunction with GLU at NMDA receptors. NOS inhibitors block this form of *neurotoxicity* and thus may have a role in the therapy of AIDS dementia (Dawson *et al.*, 1993).

More recent studies have found that in normal human biopsies n-NOS and dystrophin are colocalized beneath the sarcolemma of muscle fibres (Kobzbik *et al.*, 1994). Results have shown that NO promotes relaxation through the cGMP pathway, while NOS inhibitors augmented contractile function of skeletal muscle (Kobzbik *et al.*, 1994). In biopsies from patients with *Duchenne muscular dystrophy,* the disruption of dystrophin is accompanied with dramatically reduced n-NOS levels, providing evidence for the importance of both NO and dystrophin for signalling in striated muscle. The selective enrichment of n-NOS in fast-twitch muscle fibres (Kobzbik *et al.*, 1994) could help to explain the preferential degeneration of this fibre type seen in Duchenne muscular dystrophy (Webster *et al.*, 1988). Translocation of n-NOS from sarcolemma to myocyte cytosol in dystrophic muscle may also have implications for the pathogenesis of NO in muscular dystrophy. Regarding the already reported role of endogenous NO in cytotoxicity (Nathan and Xue, 1994; Lipton and Rosenberg, 1994; Huang *et al.*, 1994), it is suggested that inappropriate cytosolic n-NOS activity in dystrophic muscle is toxic to myocytes. Free radical oxygen intermediates, which occur at high levels in skeletal muscle (Reid *et al.*, 1992a,b), are known to contribute to cytotoxic damage in various muscle diseases including Duchenne muscular dystrophy (Davison *et al.*, 1988). Abnormal n-NOS in dystrophic muscle may increase the toxic interaction of NO and superoxide and contribute to myofibre necrosis. During the states of great hyperactivity of muscle, hydroxyl free radicals formed from NO and superoxide could lead to muscle damage. In such circumstances, NOS inhibitors might display a therapeutic

benefit (Snyder, 1994). Selective loss of sarcolemmal n-NOS in patients with Duchene (Brenman *et al.*, 1995) and *Becker muscular dystrophy* implicates new possibilities for design of dystrophin-gene therapy (Chao *et al.*, 1996). Moreover, it has been shown that the relationship of dystrophin and sarcolemmal n-NOS in *Duchenne muscular dystrophy* is analogous to relationship of huntington-associated protein and brain n-NOS in *Huntington disease* (Li *et al.*, 1996).

Conceivably, **NO** can be a **double-edged sword** (Schmidt and Walter, 1994). On the one side, in the low, constitutive mode, it has beneficial effects, mediating and protecting neuronal activity. On the other side, in the high, unregulated mode, it is an indiscriminately damaging molecule (Änggård, 1994). The possibility that NO can exist in distinct oxidation-reduction states, with different biological actions, provides further elucidation of mechanisms for the neuroprotective and neurotoxic effects of NO (Lipton *et al.*, 1993). The designation 'nitric oxide' should be used for the reduced, negatively charged form of the molecule, while the oxidized, positively charged form, the nitrosonium ion. Accordingly, oxidized NO, in the form of the nitrosonium ion, reacts with NMDA receptor to block neurotransmission. Precisely, an oxidized, positively charged form can bind to NMDA-receptor complex (Lipton *et al.*, 1993, 1994), resulting in changes in sensitivity of the NMDA-receptor complex to the actions of GLU. Thus, NO exerts negative feed-back to the NMDA receptor, reducing intracellular $Ca^{2+}$ with a consecutive decrease of NOS activity. On the contrary, the neurotoxic actions of NO are ascribed to the reduced, negatively charged form of the molecule. This reduced form of NO reacts with the superoxide anion to form peroxynitrate, the final neurotoxic agent. Reduced form of NO-induced toxicity is a complex process involving multiple pathways generally leading to cell death (Burney *et al.*, 1997). In cerebral cortical cultures, conditions favouring the reduced form of the molecule give rise to neurotoxicity, while neuroprotective actions occur in the presence of positively charged nitrosonium ions. Nitrosonium ions also block NMDA receptor-mediated current (Lipton *et al.*, 19943). These evidences implicate specific therapeutic methods. Therefore, further elucidation of neuroprotective versus neurotoxic effects of NO could find new treatment opportunities (Änggård, 1994). Since NOS inhibitors showed up to 70% protection from neural *stroke* injury, the pharmaceutical industry should make an effort to develop NOS inhibitors as antistroke drugs. The ideal therapeutic agent should be one that prevents the formation of the reduced form of NO while enhancing the formation of the oxidized form of the molecule. Useful would be drugs which could be converted to nitric oxide, but only to the oxidized nitrosonium ion. Similar consideration would apply to drugs aimed at *AIDS dementia* and *neurodegenerative conditions,* including *Hungtinton's and Parkinson's diseases*, which may also involve overstimulation of NMDA receptors (Snyder, 1992; Snyder and Bredt, 1992). Therefore, it is suggested that modulations of the NO pathway may become useful and important for new therapeutic strategies for various diseases in clinical medicine, especially in neuropsychiatry.

### 3.4 References

Adams GE, Stratford IJ. Bioreductive drugs for cancer therapy: the search for tumour specificity. *Int J Radiat Oncol Biol Phys* 29:231-238, 1994.

Akbarian S, Bunney WE, Potkin SG. Altered distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase cell in frontal lobe of schizophrenics implies disturbances of cortical development. *Arch Gen Psychiatry* 50:169-177, **1993a.**

Akbarian S, Vinuela A, Kim JJ, Potkin SG, Bunney WE, Jones EG. Distorted distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase neurons in temporal lobe of schizophrenics implies anomalous cortical development. *Arch Gen Psychiatry* 50:178-186, **1993b.**

Änggård E. Nitric oxide: mediator, murderer and medicine. *Lancet* 343:1199-1206, **1994.**

Appenzeller O. Pathogenesis of migraine. *Med Clin N Am* 75:763-789, **1991.**

Assreuy J, Cunha FQ, Liewm FY, Moncada S. Feed back inhibition of nitric oxide synthase activity by nitric oxide. *Br J Pharmacol* 108:833-837, **1993.**

Babbedge RC, Bland-Ward PA, Hart SL, Moore PK. Inhibition of rat cerebellar nitric oxide synthase by 7-nitro indazole and related substituted indazoles. *Br J Pharmacol* 110:225-228, **1993.**

Bassenge E. Clinical relevance of endothelium-derived relaxing factor (EDRF). *Br J Clin Pharmacol* 34:37S-42S, **1992.**

Benzing A, Brautigam P, Geiger K, Loop T, Beyer U, Moser E. Inhaled nitric oxide reduces pulmonary transvascular albumin flux in patients with acute lung injury. *Anesthesiology* 83:1153-1161, **1995.**

Baylis C, Vallance P. Nitric oxide and blood pressure: effects of nitric oxide deficiency. *Curr Opin Nephrol Hypertens* 5:80-88, **1996.**

Blomqvist H, Wickerts CJ, Andreen M, Ulberg U, Örtqvist, Frostell C. Enhanced pneumonia resolution by inhalation of nitric oxide? *Acta Anaesthesiol Scand* 37:110-114, **1993.**

Bo L, Dawson TM, Wesselingh S, Mork S, Choi S, Kong PA, Pardo C, Hanley D, Trapp BD. Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. *Ann Neurol* 36:778-786, **1994.**

Bodis S, Haregewoin A. Significantly reduced salivary nitric oxide levels in smokers. *Ann Oncol* 5:371-372, **1994.**

Bogle RG, Vallance P. Functional effects of econazole on inducible nitric oxide synthase: production of a calmodulin-dependent enzyme. *Br J Pharmacol* 117:1053-1058, **1996.**

Boughton-Smith N, Evans SM, Hawkey CJ, Cole AT, Balsitis M, Whittle BJ, Moncada S. Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet* 342:338-340, **1993.**

Bredt DS. Targeting nitric oxide to its targets. *Proc Soc Exp Biol Med* 211:41-418, **1996.**

Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchene muscular dystrophy. *Cell* 82:743-752, **1995.**

Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest* 87:432-438, **1991.**

Buga GM, Griscavage JM, Rogers NE, Ignarro Lj. Negative feedback regulation of endothelial cell function by nitric oxide. *Circ Res* 73:808-812, **1993.**

Burnett AL, Lowenstein CJ, Bredt DS, Chang DS, Snyder SH. Nitric oxide: a physiologic mediator of penile erection. *Science* 257:401-403, **1992.**

Burnett AL, Tillman SL, Chang TS, Epstein JI, Lowenstein CJ, Bredt DS, Snyder SH, Walsh PC. Immunohistochemical localization of nitric oxide synthase in the autonomic innervation of the human penis. *J Urol* 150:73-76, **1993.**

Burney S, Tamir S, Gal A, Tannenbaum RS. A mechanistic analysis of nitric oxide-induced cellular toxicity. *Nitric Oxide* 1:130-144, **1997.**

Calignano A, Moncada S, di Rosa M. Endogenous nitric oxide modulates morphine-induced constipation. *Biochem Biophys Res Commun* 181:889-893, **1992.**

Calver A, Collier J, Vallance P. Dilator actions of arginine in human peripheral vasculature. *Clin Sci* 81:695-700, 1991.

Calver A, Collier J, Moncada S, Vallance P. Effect of local intraarterial N-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *J Hypertens* 10:1025-1031, 1992a.

Calver A, Collier J, Vallance P. Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J Clin Invest* 90:2548-2554, 1992b.

Calver H, Collier J, Vallance P. Nitric oxide and cardiovascular control. *Exp Physiol* 78:303-326, 1993.

Calver A, Collier J, Vallance P. Forearm blood flow response to a nitric oxide synthase inhibitor in patients with treated essential hypertension. *Cardiovasc Res* 28:1720-1725, 1994a.

Calver A, Harris A, Maxwell JD, Vallance P. Effect of local inhibition of nitric oxide synthesis on forearm blood flow and dorsal hand vein size in patients with alchocolic cirrhosis. *Clin Sci* 86:203-208, 1994b.

Charles IG, Palmer RMJ, Hickery MS, Bayliss MT, Chubb AP, Hall VS, Moss DW, Moncada S. 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, 1993.

Chao DS, Gorospe JR, Brenman JE, Rafael JA, Peters MF, Froehner SC, Hoffman EP, Chamberrlain JS, Bredt DS. Selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy. *J Exp Med* 184:609618, 1996.

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1:623-634, 1988.

Chu CC. Cornell fiber scientist's inventions aid medicine, health care industries. *Science Daily Magazine Ithaca*, NY, 12/24/1995.

Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA. Expression of nitric oxide synthase in human central nervous system tumours. *Cancer Res* 55:727-730, 1995.

Connop BP, Rolfe NG, Boegman RJ, Jhamandas K, Beninger RJ. Potentiation of NMDA-mediated toxicity on nigrostriatal neurons by a low dose of 7-nitro indazole. *Neuropharmacol* 33:1439-1445, 1994.

Connop BP, Boegman RJ, Beninger RJ, Jhamandas K. Attenuation of malonate-induced degeneration of the nigrostriatal pathway by inhibitors of nitric oxide synthase. *Neuropharmacol* 35:459-465, 1996.

Connor JR, Manning PT, Settle SL, Moore WM, Jerome GM, Webber RK, Tjoeng FS, Currie MG. Supression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur J Pharmacol* 273:15-24, 1995.

Creager MA, Gallagher SJ, Girerd XJ. L-arginine improves endothelium-dependent vasodilatation in hypercholesterolemic humans. *J Clin Invest* 90:1248-1253, 1992.

Davison A, Tibbits G, Shi ZG, Moon J. Active oxygen in neuromuscular disorders. *Mol Cell Biochem* 84:199-216, 1988.

Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc Natl Acad Sci USA* 88:6368-6371, 1991.

Dawson TM, Dawson VL, Snyder SH. Nitric oxide as a mediator of neurotoxicity. *NIDA Res Monogr* 136:258-271, 1993.

Dawson VL, Kizushi VM, Huang PL, Snyder SH, Dawson TM. Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J Neurosci* 16:2479-2468, 1996.

De Belder, Radomski MW, Why HJF, Richardson PJ, Bucknall CA, Salas E, Martin JF, Moncada S. Nitric oxide synthase activity in human myocardium. *Lancet* 341:84-85, 1993.

De Belder AJ, Radomski MW, Why HFJ, Richardson PJ, Martin JF. Myocardial calcium-indipendent nitric oxide synthase activity is present in dilated cardiomyopathy, myocarditis and postpartum cardiomyopathy but not in ischemic or valvular heart disease. *Br Heart J* 74:426-430, 1995.

De Sarro GB, Di Paola ED, De Sarro A, Vidal MJ. Role of nitric oxide in the genesis of excitatory amino acid-induced seizures from the deep prepiriform cortex. *Fundam Clin Pharmacol* 5:503-511, 1991.

Dhillon JS, Kronck JB, Singh NC, Johnson CC. A portable nitric oxide scavenging system designed for use on neonatal transport. *Crit Care Med* 24:1068-1071, 1996.

Din-Xuan AT, Pepke-Zaba J, Butt AY, Cremona G, Higenbotam TW. Impairment of pulmonary-artery endothelium-dependent relaxation in chronic obstructive lung disease is not due to dysfunction of endothelial cell membrane receptors nor to L-arginine deficiency. *Br J Pharmacol* 109:587-591, 1993.

Downey JM. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Ann Rev Phys* 52:487-504, 1990.

Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hyperholesterolemic patients by L-arginine. *Lancet* 338:1546-1550, 1991.

Dubovski S, Murphy J, Christiano J, Lee C. The calcium second messenger system in bipolar disorders: data supporting new research directions. *J Neuropsychiatry Clin Neurosci* 4:3-14, 1992.

Edwards DH, Byrne JV, Griffith TM. The effect of chronic subarachnoid haemorrhage on basal endothelium derived relaxing factor activity in intrathecal cerebral arteries. *J Neurosurg* 76:830-837, 1992.

Farrell AJ, Blake DR, Palmer RMJ, Moncada S. Increased concentration of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann Rheum Dis* 51:1219-1222, 1992.

Feinstein DL, Galea E, Reis DJ. Suppression of glial nitric oxide synthase induction by heat shock: effects on proteolytic degradation of IkB-$\alpha^1$. *Nitric Oxide*, 1:167-176, 1997.

Ferrante RJ, Kowall NW, Beal MF, Martin JB, Bird ED, Richardson EP Jr. Morphologic and histochemical characteristics of a spared subset of striatal neurons in Huntington 's disease. *J Neuropath Exp Neurol* 46:12-27, 1985.

Ferrendelli JA, Blank AC, Gross RA. Relationship between seizure activity and cyclic nucleotide levels in brain. *Brain Res* 200:93-103, 1980.

Frostell CG, Fratacci MD, Wain JC, Zapol WM. Inhaled nitric oxide: selective pulmonary vasodilatator reversing hypoxic pulmonary vasoconstriction. *Circulation* 83:2038-2047, 1991.

Garthwaite J, Gartwaite G, Palmer RMJ, Moncada S. NMDA receptor activation induces nitric oxide synthase from arginine in rat brain slices. *Eur J Pharmacol* 172:413-416, 1989.

Garthwaite J. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neuro Sci* 14:60-67, 1991.

Gerlach H, Rossaint R, Papper D. Time course and dose-response of nitric oxide inhalation for systemic oxygenation and pulmonary hypertension in patients with adult respiratory distress syndrome. *Eur J Clin Invest* 23:499-502, 1993.

Gorfine SR. Treatment of benign anal disease with topical nitroglycerin. *Diseases of the Colon and Rectum* 38:453-457, 1995.

Green IC, Cunninghem JM, Delaney CA, Elphik MR, Mabley JG, Green MHL. Effects of cytokines and nitric oxide donors on insulin secretion, cyclic GMP and DNA damage: relation to nitric oxide production. *Biochem Soc Transact* 22:30-37, 1994.

Griscavage JM, Rogers NE, Scheman MP, Ignarro LJ. Inducible nitric oxide synthase from a rat alveolar macrophage cell line inhibited by nitric oxide. *J Immunol* 151:6329-6337, 1993.

Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ. Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. *J Biol Chem* 269:21644-21649, 1994.

Grocott-Mason R, Lewis MJ, Shah AM. Sodium nitroprusside (SNP) directly influences relaxation rate in the intact heart. *Br Heart J* 69:S10, 1993.

Gross SS, Levi R. Tetrahydrobiopterin synthesis: an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J Biol Chem* 267:25722-25729, 1992.

Gryglewski RJ, Palmer RMJ, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 320:454-456, 1986.

Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S. Endogenous nitric oxide is present in the exhaled air of rabbits, guinea-pigs and humans. *Biochem Biophys Res Commun* 181:852-857, 1991.

Hans P. Pharmacological therapeutic prospects of cerebral vasospasm. *Ann Fr Anesth Reanim* 15:374-381, 1996.

Hao JX, Xu XJ. Treatment of a chronic allodynia-like response in spinally injured rats: effects of systemically administered nitric oxide synthase inhibitors. *Pain* 66:313-319, 1996.

Hedlund P, Ekström, Larsson B, Alm P, Anderson K-E. Heme oxygenase and NO-synthase in the human prostate- relation to adrenergic, cholinergic and peptide-containing nerves. *J Autom Nerv System* 63:115-126, 1997.

Hibbs JB Jr, Westenfelder C, Taintor R, Vavrin Z, Kablitz C, Baranawski RL, Mc Murry MP, Kuha JP. Evidence for cytokine-induced nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. *J Clin Invest* 89:867-877, 1992.

Higenbottam T. Inhaled nitric oxide: a magic bullet? *Quart J Med* 86:555-558, 1993.

Hishikawa K, Nakaki T, Tsuda M, Esumi H, Ohshima H, Suzuki H, Saruta T, Kato R. Effect of systemic L-arginine administration on hemodynamics and nitric oxide release in man. *JPN Heart J* 33:41-48, 1992.

Hock FJ, Wiemer G. Involvement of nitric oxide formation in the action of ramipril and ramipril-octil in an inhibitory avoidance task in mice. *Drug Dev Res* 27:229-237, 1992.

Hogan M, Cerami A, Bucala R. Advanced glycosylation and products block the antiproliferative effect of nitric oxide: Role in the vascular and renal complications of diabetes mellitus. *J Clin Invest* 90:1110-1115, 1992.

Hölscher C, Doyle CA, McGlinchey L, Anwyl R, Rowan MR. A selective neuronal nitric oxide synthase inhibitor impairs spatial learning in the rat. Abstract, *Society for Neuroscience Meeting*, San Diego, USA, November 1995.

Holthusen H, Ding Z. Nitric oxide is not involved in vascular nociception of noxious physical stimuli in humans. *Neurosci Lett* 227:111-114, 1997.

Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265:1883-1885, 1994.

Hudome SM, Ergenekon EN, Darrow KA, Richard RB, Snider MT, Marks KH. Precise control of nitric oxide concentration in the inspired gas of continuous flow respiratory devices. *Pediatr Pulmonol* 22:182-187, 1996.

Hyman BT, Marzloff K, Wenninger JJ, Dawson TM, Bredt DS, Snyder SH. Relative sparing of nitric oxide synthase-containing neurons in the hippocampal formation in Alzheimer's disease. *Ann Neurol* 32:818-820, 1992.

Iadecola C, Faris PL, Hartman BK, Xu X. Localization of NADPH diaphorase in neurons of the rostral ventral medulla: possible role of nitric oxide in central autonomic regulation and oxygen chemoreception. *Brain Res* 603:173-179, 1993.

Iadecola C, Zhang F, Xu X. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am J Physiol* 268:R286-R292, 1995.

Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neuro Sci* 20:132-139, **1997.**

Ignarro LJ. Endothelium-derived nitric oxide: actions and properties. *J FASEB* 3:31-36, **1989.**

Ignarro LJ. Biosynthesis and metabolism if endothelium-derived nitric oxide. *Annu Rev Pharmacol Toxicol* 30:535-560, **1990** a.

Ignarro LJ, Bush PA, Bugga GM, Wood KS, Fukuto JM, Rajfer J. Nitric oxide and cyclic GMP formation upon electrical gild stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biphys Res Commun* 170: 843-850, **1990** b.

Illowski BP, Kirch DG. Polydipsia and hyponatremia in psychiatric patients. *Am J Psychiatry* 145:675-683, **1988.**

Johnson AW, Land JM, Thompson EJ, Bolanos JP, Clark JB, Heals SJR. Evidence for increased nitric oxide production in multiple sclerosis. *J Neurol Neurosurg Psychiatr* 58: 107-111, **1995.**

Johnston MT, Creager SJ, Scales KM, Cusco JA, Byron K, Lee BA, Creager MA. Impaired endothelium-dependent vasodilatation in patients with insulin dependent diabetes mellitus. *Circulation* 88:2510-1516, **1993.**

Kadota O, Ohta S, Kumon Y, Sakaki S, Matsuda S, Sakaki M. Basic fibroblast growth factor-like immunoreactivity in the rat basilar artery with reference to co-localization with NADPH-diaphorase in the trigeminal ganglion. *Neurosci Lett* 178:201-205, **1994.**

Karatinos J, Rosse RB, Deutsch SI. The nitric oxide pathway: potential implications for treatment of neuropsychiatric disorders. *Clin Neuropharm* 18: 482-499, **1995.**

Karson CN, Mrak R, Garcia-Rill E, Griffin WS. Nitric oxide synthase in schizophrenia. In: Neuropharmacology II. *Nitric oxide in the nervous system* (Abstracts). Ed: Garthwaite J, Snyder S, Lipton S, Oxford, England:Elsevier/Pergamon, Abstract:2.15, **1994.**

Kharitanov SA, Yates D, Robbins RA, Logan-Sinclair, Shinebroune EA, Barnes PJ. Endogenous nitric oxide id increased in the exhaled air of asthmatic patients. *Lancet* 343:133-135, **1994.**

Kiechle FL, Malinski T. Nitric oxide: biochemistry, pathophysiology and detection. *Clin Chem* 100:567-575, **1993.**

Kinsella JP, Neish SR, Shaffer E, Abman SH. Low-dose inhalational nitric oxide in persistent pulmonary hypertension of the newborn. *Lancet* 340:819-820, **1992.**

Kim H, Kim KH. Effects of a nitric oxide donor and nitric oxide synthase inhibitors on acid secretion of isolated rabbit gastric glands. *Pharmacol* 53:331-339, **1996.**

Kirkby RD, Carroll DM, Grossman AB, Subramaniam S. Factors determining proconvulsant and anticonvulsant effects of inhibitors of nitric oxide synthase in rodents. *Epilepsy Res* 24:91-100, **1996.**

Kirkeby H, Svane D, Poulsen J, Tottrup A, Forman A, Anderson K. Role of the L-arginine/nitric oxide pathway in relaxation of isolated human penile cavernous tissue and circumflex veins. *Acta Physiol Scand* 149:385-392, **1993.**

Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stampler JS. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir cell Mol Biol* 9:371-377, **1993.**

Kobzbik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372:546-548, **1994.**

Koh J-Y., Choi DW. Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. *J Neurosci* 8:2153-2163, **1988.**

Koprowski H, Zheng YM, Heber-Katz E, Fraser N, Rorke L, Fu ZF, Hanlon C, Dietzchold B. *in vivo* expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc Natl Acad Sci USA* 90:3024-3027, **1993.**

Kowal NW, Ferrante RJ, Beal MF, Richardson EO Jr, Sofroniniew MV, Cuello AC, Martin JB. Neuropeptide Y, somatostatin and reduced nicotinamide adenine dinucleotide phosphate diaphorase in the human striatum: a combined immunocytochemical and enzyme histochemical study. *Neurosci* 20:817-828, 1987.

Kröncke KD, Fehsel K, Kolb-Bachofen V. Nitric oxide: cytotoxicity versus cytoprotection: why, when, and where? *Nitric oxide* 1:107-120, 1997.

Kumar KV, Das UN. Are free radicals involved in the pathobiology of human essential hypertension? *Free Radic Res Commun* 19:59-66, 1993.

Lee C, Campbell S, Jauniax E, Brown R, Ramsay B, Gibb D, Moncada S, Martin JF. arrest of preterm labour and prolongation of gestation with glyceryl trinitrate, a nitric oxide donor. *Lancet* 343:1325-1326, 1994.

Li XJ, Sharp AH, Li SH, Dawson TM, Snyder SH, Ross CA. Huntingtin-associated protein (HAP1): discrete neuronal localization in the brain resemble those of neuronal nitric oxide synthase. *Proc Natl Acad Sci USA* 93:4839-4844, 1996.

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen H-SV, Sucher NJ, Loscalzo J, Singel DJ, Stampler JS. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364:626-632, 1993.

Lizasoain I, Weiner CP, Knowles RG, Moncada S. The ontogeny of cerebral and cerebellar nitric oxide synthase in the guinea pig and rat. *Pediatr Res* 39:779-788, 1996.

Lorrain D, Hull E. Nitric oxide increases dopamine and serotonin release in the medial preoptic area. *Neuropharm Neurotoxicol* 5:87-89, 1993.

López-Mortalla N, Gonzalez A, Soledad Aymerich M, López-Zabalza MJ, Pio R, de Castro P, Santiago E. Monocyte inducible nitric oxide synthase in multiple sclerosis: regulatory role of nitric oxide. *Nitric Oxide* 1:95-104, 1997.

Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* 315:1046-1051, 1986.

Mac Allister R, Vallance P. Nitric oxide in essential and renal hypertension. *J Am Soc Nephrol* 5:1057-1065, 1995 a.

MacAllister RJ, Calver AL, Riezebos J, Collier J, Vallance P. Relative potency and arteriovenous selectivity of nitrovasodilators on human blood vessels: an insight into the targeting of nitric oxide delivery. *J Pharmacol Exp Ther* 273:154-160, 1995 b.

MacAllister RJ, Parry H, Kimpto M, Ogawa T, Russel RJ, Hodson H, Whitley GS, Vallance P. Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. *Br J Pharmacol* 119:1533-1540, 1996 a.

MacAllister RJ, Rambausek MH, Vallance P, Williams D, Hoffmann KH, Ritz E. Concentration of dimethyl-L-arginine in the plasma of patients with end-stage renal failure. *Nephrol Dial Transplant* 11:2449-2452, 1996 b.

McCann S, Karanth S, Agulia MC. The role of NO in the control of hypothalamic pituitary function. In: Neuropharmacology II. *Nitric oxide in the nervous system.* (Abstracts). Eds: Garthwaite J, Snyder S, Lipton S. Oxford, England: Elsevier/Pergamon, abstract 2 of free oral communications, 23 July, 1994.

Martin U, Bryden K, Devoy M, Howarth P. Increased levels of exhaled nitric oxide during nasal and oral breathing in subjects with seasonal rhinitis. *J Allergy Clin Immunol* 97:768-772, 1996.

Marzinzig M, Nussler AK, Stadler J, Marzinzing E, Barthlen W, Nussler NC, Beger HG, Morris SM Jr, Bruckner UB. Improved methods to measure end products of nitric oxide in biological fluids: nitrite, nitrate and S-nitrosothiols. *Nitric Oxide* 1:177-189, 1997.

Matsumoto A, Ogura K, Hirata Y, Kakoki M, Watanabe F, Takenaka K, Shiratory Y, Momomura S, Omata M. Increased nitric oxide in the exhaled air of patients with decompensated liver cirrhosis. *Ann Intern Med* 123:110-113, **1995.**

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, Malagelada JR. Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* 23:724-728, **1993.**

Meldrum B, Garthwaite J. Excitatory amino acids neurotoxicity and neurodegenerative disease. *Trends Pharmacol Sci* 11:379-387, **1990**

Meldrum BS. Cytoprotective therapies in stroke. *Curr Opin Neurol* 8:15-23, **1995.**

Merrill JE, Ignaro LJ, Sherman MP, Melinek J, Lane TE. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol* 151:2132-2141, **1993.**

Meyhoff HH, Rosenkilde P, Bodker A. Non-invasive management of impotence with transcutaneous nitroglycerin. *Br J Urol* 69:88-90, **1992.**

Middleton ST, Shorthouse M, Hunter JO. Increased nitric oxide synthesis in ulcerative colitis. *Lancet* 341:465-466, **1993.**

Minami Y, Kimura H, Aimi Y, Vincent SR. Projections of nitric oxide synthase-containing fibres from the sphenopalatine ganglion to cerebral arteries in the rat. *Neurosci* 60:7456-759, **1994.**

Misko TP, Moore WM, Kasten TP, Nickols GA, Cobett JA, Titon RG, McDaniel ML, Williamson JR, Currie MG. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol* 233:119-125, **1993.**

Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109-142, **1991.**

Moncada S. The L-arginine-nitric oxide pathway. *Acta Physiol Scand* 145:201-227, **1992.**

Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 329:2002-2012, **1993.**

Moore PK, Wallace P, Gaffen Z, Hart SL, Babbedge RC. Characterization of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociceptive and cardiovascular effects. *Br J Pharmacol* 110:219-224, **1993.**

Moore PK, Babbedge RC, Wallace P, Gaffen ZA, Hart SL. 7-nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in mouse without increasing blood pressure. *Br J Pharmacol* 108:296-297, **1993.**

Morris NH, Carroll S, Nicolades KH, Steer PJ, Warren JB. Exhaled nitric oxide concentration and amniotic fluid nitrite concentration during pregnancy. *Eur J Clin Invest* 25:138-141, **1995.**

Morton AJ, Nicholson LFB, Faull RLM. Compartmental loss of NADPH diaphorase in the neuropil of the human striatum in Huntington's disease. *Neurosci* 53:159-168, **1993.**

Murad F, Arnildt WP, Mittal CK, Braughler JM. Properties and regulations of guanylate cyclase and some proposed functions for cyclic GMP. *Adv Cyclic Nucletide Res* 11:175-204, **1979.**

Narasapur S, Naylor G. Methylene blue: a possible treatment for manic depressive psychosis. *J Affect Disord* 5:155-161, **1983.**

Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 6:3051-3064, **1992.**

Nathan C, Xie Q. Nitric oxide synthase: roles, tolls and controls. *Cell* 78:915-918, **1994.**

Nattero G, Menozzi G, Inconis T, Paradisi L. Nitric oxide, endothelin-1, and transcranial Doppler in migraine. Findings in interictal conditions and during migraine attack. *Headache* 36:307-311, **1996.**

Ngyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc Natl Acad Sci USA* 89:3030-3034, **1992.**

Nobunaga T, Tokugawa Y, Hashimoto K, Kimura T, Matsuzaki N, Nitta Y, Fujita T, Kidoguchi KI, Azuma C, Saji F. Plasma nitric oxide levels in pregnant patients with preeclampsia and essential hypertension. *Gynecol Obstet Invest* 41:189-193, **1996.**

Ochoa JB, Udekwu AO, Billiar TR. Nitrogen oxide levels in patients following trauma and during sepsis. *Ann Surg* 214:621-626, **1991.**

Oddis CV, Finkel MS. Cytokines and nitric oxide synthase inhibitors as mediators of adrenergic refractoriness in cardiac myocytes. *Eur J Pharmacol* 320:167-174, **1997.**

Olesen J, Thomsen LL, Iversen H. Nitric oxide is a key molecule in migraine and other vascular headaches. *Trends Pharmacol Sci* 15:149-153, **1994.**

Panza JA, Casino PR, Kilcoyne C, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation* 87:1468-1474, **1993.**

Patel J. Polydipsia, hyponatremia and water intoxication among psychiatric patients. *Hosp Community Psychiatry* 45:1073-1074, **1994.**

Pepke-Zabe J, Higenbottam TW, Dinh-Zuan AT, Stone D, Wallwork J. Inhaled nitric oxide causes selective pulmonary vasodilatation in patients with pulmonary hypertension. *Lancet* 338:1173-1174, **1991.**

Persson K, Alm P, Johansson K, Larsson B, Andersson KE. Nitric oxide synthase in pig lower urinary tract: immunohistochemistry, NADPH diaphorase histochemistry and functional effects. *Br J Pharmacol* 110:521-530, **1993.**

Persson MG, Zetterstrom O, Agrenius V, Ihre E, Gustafsson LE. Single-breath measurements of nitric oxide: increased concentration in asthmatics and reduction in smokers. *Lancet* 343:146-147, **1994.**

Petros A, Lamb G, Leone A, Moncada S, Bennett D, Vallance P. Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovasc Res* 28:34-39, **1994.**

Pfeilschifter J, Eberhardt W, Hummel R, Kunz D, Muhl H, Nitsch D, Pluss C, Walker G. Therapeutic strategies for the inhibition of inducible nitric oxide synthase-- potential for a novel class of anti-inflammatory agents. *Cell Biol Int* 20:51-58, **1996.**

Prast H, Phillippu A. Nitric oxide release acetylcholine in the basal forebrain. *Eur J Pharmacol* 216:139-140, **1992.**

Prezedborski S, Jackson-Lewis V, Yokoyama R, Shibata T, Dawson VL, Dawson TM. Role of neuronal nitric oxide synthase in 1-methyl-4-phenyl-1,2,3,6-tetrahydrophyridine (MPTP)-induced dopaminergic neurotoxicity. *Proc Natl Acad Sci USA* 93:4565-4571, **1996.**

Radhakrishnan V, Henry JL. L-NAME blocks response to NMDA, substance P and noxious cutaneous stimuli in cat dorsal horn. *Neuro Rep* 4:323-326, **1993.**

Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrate-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266:4244-4250, **1991.**

Radomski MW, Palmer RMJ, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci USA* 87:10043-10047, **1990.**

Radomski MW, Rees DD, Dutra A, Moncada S. S-nitrosoglutathione inhibits platelet activation *in vitro* and *in vivo*. *Br J Pharmacol* 107:745-749, **1992.**

Radomski MW, Vallance P, Whitley G, Foxwell N, Moncada S. Platelet adhesion to human vascular endothelium is modulated by constitutive and cytokine induced nitric oxide. *Cardiovasc Res* 27:1380-1382, **1993.**

Rebeck GW, Marzloff K, Hyman BT. The pattern of NADPH-diaphorase staining, a marker of nitric oxide synthase activity, is altered in the perforant pathway terminal zone in Alzheimer's disease. *Neurosci Lett* 152:165-168, **1993.**

Reid IA, Chiu YJ. Nitric oxide and the control of renin secretion. *Fundam Clin Pharmacol* 9:309-323, **1995.**

Remuzzi G, Perico N, Zoja C, Corna D, Macconi D, Vigano G. Role of endothelium-derived nitric oxide in the bleeding tendency uremia. *J Clin Invest* 86:1768-1771, **1990.**

Rengasamy A, Johnson RA. Regulation of nitric oxide synthase by nitric oxide. *Mol Pharmacol* 44:124-128, **1993.**

Robbins RA, Hamel FG, Floreani AA, Gossman GL, Nelson KJ, Balenky S, Rubinstein J. Bovine bronchial epithelial cells metabolize L-arginine to L-citrulline: Possible role of nitric oxide synthase. *Life Sci* 52:709-716, **1993.**

Roger N, Barbera JA, Farre R, Cobos A, Roca J, Rodriguez-Roisin R. Effect of nitric oxide inhalation on respiratory system resistance in chronic obstructive pulmonary disease. *Eur J Resp* 9:190-195, **1996.**

Rogers NE, Ignarro LJ. Constitutive nitric oxide synthase from cerebellum is reversible inhibited by nitric oxide formed from L-arginine. *Biochem Biphys Res Commun* 189:242-249, **1992.**

Rossaint R, Falke KS, Lopez F, Slama K, Pison U, Zapol WA. Inhaled nitric oxide for the adult respiratory distress syndrome. *N Engl J Med* 328:399-405, **1993.**

Rothman SM, Olney JW. Excitotoxicity and the NMDA receptor. *Trends Neuro Sci* 10:299-302, **1987.**

Rundfeld C, Koch R, Richter A, Mevissen M, Gerecke U, Löschher W. Dose-dependent anticonvulsant and proconvulsant effects of nitric oxide synthase inhibitors on seizure threshold in a cortical stimulation model in rats. *Eur J Pharmacol* 274:73-81, **1995.**

Saenz de Tajeda I, Goldstein I, Azadzoi K, Krane RJ, Cohen R. Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *N Engl Med* 320:1025-1030, **1989.**

Salvemini D, Manning PT, Zweifel BS, Seibert K, Connor J, Currie MG, Needleman P, Masferrer JL. Dual inhibition of nitric oxide and prostaglanidin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. *Clin Invest* 96:301-308, **1995.**

Samama CM, Diaby M, Fellahi JL, Mdhafar A, Eyraud D, Arock M, Guillosson JJ, Coriat P, Rouby JJ. Inhibition of platelet aggregation by inhaled nitric oxide in patients with acute respiratory distress syndrome. *Anesthesiology* 83:56-65, **1995.**

Santiago M, Nachado A, Cano J. Effect of L-arginine/nitric oxide pathway on MPP+ induced cell injury in the striatum of rats. *Br J Pharmacol* 111:837-842, **1994.**

Saxena PR. Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacol Ther* 66:339-368, **1995.**

Shilling J, Cakmakcki N, Battig U, Geroulanos S. A new approach in the treatment of hypotension in human septic shock by N-monomethyl-L-arginine, an inhibitor of the nitric oxide synthase. *Intensive Care Med* 19:227-231, **1993.**

Schmidt HHHW, Gagne GD, Nakane M, Pollock JS, Miller MF, Murad F. Mapping of NO synthase in the rat suggests co-localization with NADPH diaphorase but not soluble guanylyl cyclase and novel paraneural functions for nitrinergic signal transduction. *J Histochem Cytochem* 49:1439-1456, **1992a.**

Schmidt HHHW, Warner TD, Ishii K, Sheng H, Murad F. Insulin-secretion from pancreatic B-cells caused by L-arginine-derived nitrogen oxides. Science 255:721-723, Reply: *Science* 258:1376-13798, **1992b.**

Schmidt HHHW, Walter U. NO at work. *Cell* 78:919-925, **1994.**

Schoedon G, Schneemann M, Blau N, Edgell C-JS, Schaffner A. Modulation of human endothelial cell tatrahydrobiopterin synthesis by activating and deactivating cytokines: new perspectives on endothelium-derived relaxing factor. *Biochem Biophys Res Commun* 196:1343-1348, **1993.**

Schultz JB, Matthews RT, Muqit MMK, Browne SE, Beal MF. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP induced neurotoxicity in mice. *J Neurochem* 64:936-939, **1995.**

Schuman EM, Madison DV. Nitric oxide and synaptic function. *A Rev Neurosci* 17:153-183, **1994.**

Schulz JB, Matthews RT, Jenkins BG, Ferrante RJ, Siwek D, Henshaw DR, Cipolloni PB, Mecocci P, Kowall NW, Rosen BR, Flint Beal M. Blockade of neuronal nitric oxide synthase protects against excitotoxicity *in vivo*. *J Neurosci* 15:8419-85429, **1995a.**

Schulz JB, Matthews RT, Muqit MM, Browne SE, Beal MF. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *J Neurochem* 64:936-939, **1995b.**

Shi Y, Li HQ, Shen CK, Wang JH, Qin SW, Liu R, Pan J. Plasma nitric oxide levels in newborn infants with sepsis. *J Pediatr* 123:435-438, **1993.**

Slivka A, Chuttani R, Carr-Locke DL, Kobzbik L, Bredt DS, Locsalzo J, Stampler JS. Inhibition of sphincter of Oddi function by the nitric oxide carrier S-nitrosos-N-acetylcysteine in rabbits and humans. *J Clin Invest* 94:1792-1798, **1994.**

Snyder SH. Nitric oxide and neurons. *Curr Opin Neurobiol* 2:323-327, **1992.**

Snyder SH. Second messengers and affective illness. Focus on the phosphoinositide cycle. *Pharmacopsychiatry* 25:25-28, **1992.**

Snyder SH, Bredt DS. Biologiocal roles of nitric oxide. *Scientific American* 5:28-35, **1992.**

Snyder SH. Janus faces of nitric oxide. *Nature* 364:577, **1993.**

Snyder SH. More jobs for that molecule. *Nature* 372:504-505, **1994.**

Sogni P, Garnier P, Gadno A, Moreau R, Dall'Ava-Santucci J, Dinh-Xuan AT, Lebrec D. Endogenous pulmonary nitric oxide production measured from exhaled air is increased in patients with severe cirrhosis. *J Hepatol* 23:471-473, **1995.**

Squiadrito F, Calapai G, Cucinotta D. Anoretic activity of NG-nitro-L-arginine, an inhibitor of brain nitric oxide synthase, in obese Zucker rats. *Eur J Pharmacol* 230:125-128, **1993.**

Squadrito F, Calapai G, Altavilla D. Food deprivation increases brain nitric oxide synthase and depresses brain serotonin levels in rats. *Neuropharmacol* 33:83-86, **1994.**

Stampler S. Haemoglobin plus nitric oxide regulates blood pressure. *Nature* 380:205-206, **1996.**

Tanazawa T, Suzuki Y, Anzai M, Tsugane S, Takayasu M, Shibuya M. Vasodilatation by intrathecal lipopolysacharide of the cerebral arteries after subarachnoid haemorrhage in dogs. *Acta Neurochir* 138:330-337, **1996.**

Toda N, Kimura T, Yoshida K, Bredt DS, Snyder SH, Yoshida Y, Okamura T. Human uterine arterial relaxation induced by nitroxidergic nerve stimulation. *Am J Physiol* 266:H1446-1450, **1994.**

Ueki Y, Miyake S, Tominaga Y, Eguchi K. Increased nitric oxide levels in patients with rheumatoid arthritis. *J Rheumatol* 23:230-236, **1996.**

Uemura Y, Kowall NW, Beal MF. Selective sparing of NADPH-diaphorase-somatostatin-neuropeptide Y neurons in ischemic gerbil striatum. *Ann Neurol* 27:620-625, **1990.**

Vallance P, Collier J, Moncada S. Nitric oxide synthesised from L-arginine mediates endothelium-dependent dilatation in human veins *in vivo*. *Cardiovasc Res* 23:1053-1057, **1989.**

Vallance P, Moncada S. Hyperdynamic circulation in cirrhosis: a role for nitric oxide? *Lancet* 337:776-778, **1991.**

Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibition of nitric oxide synthesis in chronic renal failure. *Lancet* 339:572-575, **1992a.**

Vallance P, Leone A, Calver A, Moncada S. Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *Cardiovasc Pharmacol* 20 Suppl 12:S60-62, **1992b.**

Vallance P, Moncada S. Role of endogenous nitric oxide in septic shock. *New Horizons* 1:77-87, **1993.**

Vallance P, Moncada S. Nitric oxide-from mediator to medicines. *J Royal Coll Phys* London 28:209-219, **1994.**

Vallance OP, Patton S, Bhagat K, Mac Allister R, Radomski M, Moncada S, Malinski T. Direct measurement of nitric oxide in human beings. *Lancet*, 346:15:153-154, **1995.**

Vanhoutte PM, Auch-Scwelk W, Biondi ML, Lorenz RR, Schini VB, Vidal MJ. Why are converting enzyme inhibitors vasodilatators? *Br Clin Pharmacol* 28:S95-S104, **1989.**

Vanderwinden JM, Maoltux P, Schiffman SN, Vanderhaeghen JJ, De Laet. Nitric oxide synthase in infantile hypertrophic pyloric stenosis. *N Engl J Med* 327:511-515, **1992.**

Van Leeuwen R, De Vries R, Dzoljic MR. 7-nitro indazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, **1995.**

Varner PD, Beckman JS. Nitric oxide toxicity in neuronal injury and degeneration. In: *Nitric oxide in the nervous system.* Ed: Vincent SR. Academic Press, New York, pp191-207, **1995.**

Watkins LD. Nitric oxide and cerebral blood flow: an update. *Cerebrovasc Brain Metab Rev* 7:324-337, **1995.**

Webster C, Silberstein L, Hays AP, Blau HM. Fast muscle fibres are preferentially affected in Duchene muscular dystrophy. *Cell* 52:503-513, **1988.**

Weinberg JB, Granger DL, Pisetsky DS, Seldin MF, Misukonis MA, Mason SN, Pippen AM, Ruiz P, Wood ER, Gilkeson GS. The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-/pr/pl mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N-monomethyl-L-arginine. *J Exp Med* 179:651-660, **1994.**

Wetts R, Vaughn JE. Choline acetyltransferase and NADPH diaphorase are co-expressed in rat spinal cord neurons. *Neurosci* 63:1117-1124, **1994.**

Williams JA, Vincent S, Reiner P. Nitric oxide production in rat thalamus changes with behavioral state, local depolarization, and brainstem stimulation. *J Neurosci* 17:420-427, **1997.**

Winberg P, Lundell BP, Gustafsson LE. Effect of inhaled nitric oxide on raised pulmonary vascular resistance in children with congenital heart disease. *Br Heart J* 71:282-286, **1994.**

Wink DA, Cook JA, Cristodoulou D, Krishna M, Pacelli R, Kim S, DeGraff W, Gamson J, Vodovotz Y, Russo A, Mitchell JB. Nitric oxide and some nitric oxide donor compounds enhance the cytotoxicity of cysplatin. *Nitric Oxide* 1:88-94, **1997.**

Wolfe TA, Dasta JF. Use of nitric oxide synthase inhibitors as a novel treatment for septic shock. *Ann Pharmacother* 29:36-46, **1995.**

Wong ML, Rettori V, al-Shekhlee A, Bongiorno PB, Canteros G, McCann SM, Gold PW, Licinnio J. *Nature* 2:581-584, **1996.**

Yallampalli C, Garfield RE, Byam-Smith M. Nitric oxide inhibits uterine contractility during pregnancy but not during delivery. *Endocrinology* 133:1899-1902, **1993.**

Zhu ZX, Luo LG. Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. *J Neurochem* 59:932-935, **1992.**

Zoja C, Nori SU, Corna D, Vigano G, Perico N, de Gaetano G, Remuzzi G. L-arginine, the precyrsor of nitric oxide, abolishes the effect of oestrogens on bleeding time in experimental uremia. *Lab Invest* 65:479-483, **1991.**

Zorumski CF, Izumi Y. Nitric oxide and hippocampal synaptic plasticity. *Biochem Pharmacol* 46:777-785, **1993.**

# PART II

# NITRIC OXIDE AND BRAIN
## Neuropharmacological experiments

# CHAPTER 4

## NITRIC OXIDE AND SEIZURES
### *Anticonvulsant Activity of New and Potent Inhibitors of Nitric Oxide Synthase*


### 4.1 Abstract
The effects of new and potent NOS inhibitors, S-methyl-L-thiocitrulline (S-Me-TC), 3-bromo 7-nitro indazole (3-Br-7-NI) and 1-(2-trifluoromethylphenyl) imidazole (TRIM), were examined on the pilocarpine-induced seizures in mice. 3-Br-7-NI and TRIM decreased the frequency of status epilepticus and mortality, while TRIM, in addition, significantly reduced the incidence of seizures. The latencies to onsets of seizures, status epilepticus and mortality were significantly prolonged by all three NOS inhibitors, while duration of seizures was reduced by 3-Br-7-NI and TRIM. These data suggest an excitatory effect of NO in the neuronal structures involved in the pilocarpine-induced seizures.


### 4.2 Introduction
In the CNS NO is considered as a retrograde messenger being involved in the modulation of neuronal excitability. Accordingly, it has been shown that L-Arg-derived NOS inhibitors, $N^G$-nitro-L-arginine methyl ester (L-NAME), $N^G$-nitro-L-arginine (L-NOARG) and $N^G$-monomethyl-L-arginine (L-NMMA) affect seizure threshold. However, both proconvulsant and anticonvulsant effects have been reported.

L-NAME worsened seizures induced by kainate (Kirkby *et al.*, 1996a; Rigaud-Monnet *et al.*, 1994), N-methyl-D-aspartate (NMDA; Buisson *et al.*, 1993) and pilocarpine (Starr and Starr, 1993) in rats and mice. Similarly, L-NMMA reduced the convulsive threshold in the kainate-induced convulsions in mice (Przegalinski *et al.*, 1994). Potent NOS inhibitor L-NOARG increased the severity of kainate-induced seizures in mice and rats (Penix *et al.*, 1994) and potentiated epileptic activity in rats induced by various convulsant compounds, such as quinolinate (Haberny *et al.*, 1992), bicuculline (Wang *et al.*, 1994) and pilocarpine (Maggio *et al.*, 1995).

In contrast, L-NAME antagonized pentylenetetrazol-induced kindling (Becker *et al.*, 1995) and seizures induced by various excitatory drugs: kainic acid (De Sarro *et al.*, 1991), quinolinic acid (Nakamura *et al.*, 1995), NMDA (De Sarro *et al.*, 1991), pentylenetetrazol (Osonoe *et al.*, 1994), cocaine (Przewlocka *et al.*, 1994) and picrotoxin (Kirkby *et al.*, 1996b). Similarly, L-NOARG protected rats against oxygen- (Zhang *et al.*, 1993) and pentylenetetrazol-induced convulsions (Osonoe *et al.*, 1994).

Recently, NOS inhibitors which are not L-Arg derivatives became available. Some of them are relatively selective inhibitors of NOS, affecting predominantly n-NOS, without increasing blood pressure. 7-NI is a relatively selective inhibitor of n-NOS, which does not affect blood pressure (Moore *et al.*,1993) but influences local cerebral blood flow (Kovach *et al.*, 1994). This drug attenuates kainate-elicited convulsions in rats (Mülsch *et al.*, 1994) or pilocarpine- (Van Leeuwen *et al.*, 1995) and picrotoxin- (Kirkby *et al.*, 1996b) induced seizures in mice. However, this subject remains still controversial, since 7-NI does not affect bicuculline- and pentylenetetrazol-induced seizures (Penix *et al.*, 1994) or even worsened kainate-induced convulsions in mice (Kirkby *et al.*, 1996b).

In order to elucidate further the role of NO in epilepsy we have examined the effect of three new, potent and relatively selective n-NOS inhibitors, which are not derivatives of

L-Arg, 3-Br-7-NI, TRIM and S-Me-TC on pilocarpine-induced seizures in mice (Turski *et al.*, 1984). 3-Br-7-NI exhibits greater potency than 7-NI as an inhibitor of n-NOS *in vitro* (Bland-Ward and Moore, 1995). TRIM has shown selectivity for inhibition of n-NOS compared with e-NOS *in vitro* and lack of vasopressor activity in the anaesthetized mouse (Handy *et al.*, 1995). S-Me-TC is more selective for rat n-NOS compared to rat e-NOS (Furfine *et al.*, 1994). In addition, S-Me-TC is the most potent NOS inhibitor described to date with strong pressor activity (Narayanan *et al.*, 1994).

It seems that animal model of epilepsy is important factor determining proconvulsant or anticonvulsant effects of NOS inhibitors (Kirkby *et al.*, 1996 b). We selected the pilocarpine model of seizures, because biochemical analysis of brains from animals subjected to pilocarpine-induced seizures provided evidence for an involvement of EAA in the initiation of epileptogenesis. This lead to the suggestion that blocking excitatory amino acid receptors, or their associated second messenger systems could be the feasible way of arresting seizure activity in human status epilepticus (Walton *et al.*, 1990). Furthermore, it has been shown that cholinomimetics-induced seizures are accompanied by a widespread damage of brain structures (forebrain, neocortex, olfactory cortex, thalamus, amygdaloid complex, substantia nigra and particularly hippocampal formation), resembling that frequently observed in autopsied brains of human epileptics (Turski *et al.*, 1984). Therefore, we found that pilocarpine-induced epilepsy is an appropriate animal model to examine the role of NO in seizures.

## 4.3 Methods

### *4.3.1 Animals*

Experiments were performed on adult, male Swiss mice (28-35 g). A week prior to the experiments, animals were housed in groups of 5-10 in a room with controlled temperature (21$^0$C), humidity (55%), on a standard light-dark cycle (light: 07.00-19.00 h) and with free access to food and water.

### *4.3.2 Drug Administration Schedule*

Mice (n=100) were divided in 5 equal groups. All drugs, except scopolamine, were administered intraperitoneally. Two groups of animals received vehicle (saline 0.1 ml or dimethyl sulfoxide, DMSO, 0.025 ml). Each of the other three groups was treated with one of the NOS inhibitors. In all 100 animals seizures were induced by pilocarpine (300 mg/kg) following the method of Turski *et al.* (1984). Pilocarpine was administered 30 min after injection of vehicle or NOS inhibitor. The dose of all three NOS inhibitors, used in this study, was 120 mg/kg. The selected dose for NOS inhibitors, which had a similar molecular weight (S-Me-TC, 266; 3-Br-7-NI, 242; TRIM, 212), was based on our preliminary experiments and biologically active concentrations of these drugs used by other authors (Bland-Ward and Moore, 1995; Furfine *et al.*, 1994). In order to minimize peripheral cholinergic effects, scopolamine (1 mg/kg) was injected subcutaneously (s.c.) 30 min prior to administration of pilocarpine (Turski *et al.*, 1984).

### *4.3.3 Behavioural Assessments*

Behavioural assessments took place between 09.00-18.00 h and were carried out in transparent, plexiglas compartments (40x25x15 cm). Prior to administration of the drug each

animal was habituated to the environment for 30 min. After habituation the mice were removed, injected with the corresponding drug and rapidly returned to the experimental cage.

Convulsions were evaluated by observation of the frequencies and latencies to occurrence of seizures, status epilepticus and mortality. Latency was defined as the time measured from injection of pilocarpine until the onset of the corresponding seizure parameter. Continuous seizures of 3 min and more in duration were defined as a status epilepticus. The animals were observed for a period of 3 h following pilocarpine injection. This relative long period of observation was selected, because a few published experiments *in vivo* were not sufficiently informative in respect to the duration of activity of these new NOS inhibitors.

### *4.3.4 Ethical Approval*

The experiments and protocol of this study were approved by the Faculty Commission for experiments, handling and care of animals.

### *4.3.5 Chemical Agents*

1-(2-trifluoromethylphenyl)imidazole (TRIM, *RBI*) and 3-bromo 7-nitro indazole (3-Br-7-NI, *Affiniti*, UK) were dissolved in dimethyl sulfoxide (DMSO, *Merck*). S-methyl-L-thiocitrulline (S-Me-TC; *Alexis*, Switzerland), pilocarpine hydrochloride (*Sigma*) and scopolamine methylnitrate (*Sigma*) were dissolved in saline (0.9% NaCl). Drug solutions were prepared before each experiments.

### *4.3.6 Statistics*

Frequencies of seizure, status epilepticus and mortality were compared by Fisher's exact probability test or *Chi*-squared test. The latencies of these parameters were compared by two-tailed Student's *t-test* and for 3-Br-7-NI and TRIM data were analysed by *ANOVA* followed by multiple comparisons, *Dunnett* test. Statistical significance was accepted at a probability (P) value of 0.05.

## 4.4 Results

### 4.4.1 *Control Animals*

Two groups of animals (n=20 each) injected with vehicle in identical volume used to dissolve the drugs (saline 0.1 ml or DMSO 0.025 ml), did not show any behavioural changes. Administration of pilocarpine (300 mg/kg), 30 min following vehicle (saline or DMSO), produced in both control groups a sequence of behavioural alterations consisting of an initial akinesia, tremor of the whole body, ataxic lurching, with progression into motor seizures with clonus of the upper extremities, rearing and falling. This was usually accompanied by stereotyped behaviours like repeated head twitches, grooming and occasionally jumping fits. Seizures occurred in almost all control animals (100% in saline and 95% in DMSO group, Fig. 4.1). Mean latency of the seizures was 9-10 min (10.2±0.4 min in saline and 9.1±0.8 min in DMSO group; Table 4.1). The paroxysmal activity, in the form of motor seizures, lasted about 11 min (10.8±1.2 min in saline and 11.3±1.3 min in DMSO group; Table 4.2). In the majority of animals, convulsions developed into a status epilepticus (80% for saline and 75% for DMSO group; Fig. 4.1) with a mean latency of about 17 min (16.9±1.2 min in saline and 16.8±1.3 min in DMSO group; Table 4.1) and of about 4 min in duration (4.1±0.8 min in saline and 3.9±1.2 min in DMSO group, Table 4.2). Status epilepticus was

generally lethal to mice (80% for saline and 75% for DMSO group; Fig. 4.1). Mean latency to mortality in both groups was about 19 min (19.3±0.9 min in saline and 18.8±1.2 min in DMSO group; Table 4.1).

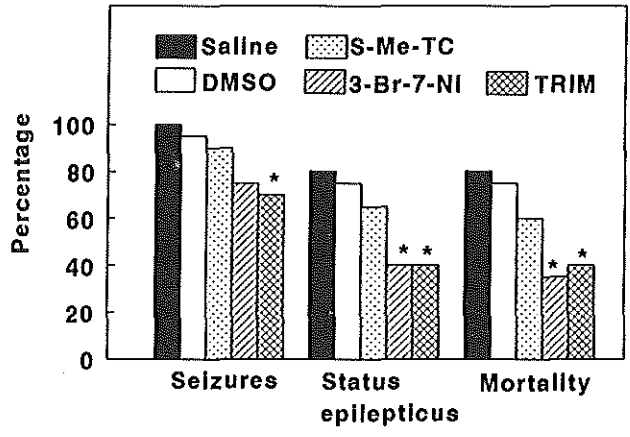


*Figure 4.1 Frequency of pilocarpine (300 mg/kg)-induced seizures, status epilepticus and mortality in mice (n=100, divided in 5 equal groups) pretreated (30 min prior to pilocarpine) with vehicle (saline, 0.1 ml or DMSO, 0.025 ml) or NOS inhibitors (120 mg/kg), S-methyl-L-thiocitrulline (S-Me-TC), 3-bromo 7-nitro indazole (3-Br-7-NI) and 1-(2-trifluoromethyl-phenyl)imidazole (TRIM). All drugs were administered intraperitoneally. The data are expressed as percentages of the effect of pilocarpine in vehicle-treated animals. Significance at p<0.05 level (binomial test: Fisher's and Chi-squared test) to vehicle-treated group.*

**Table 4.1** *Latencies (min) to pilocarpine (300 mg/kg)-induced seizures, status epilepticus and mortality in mice, pretreated (30 min prior to pilocarpine) with vehicle (saline, 0.1 ml or DMSO, 0.025 ml) or NOS inhibitors (120 mg/kg), S-methyl-L-thiocitrulline (S-Me-TC), 3-bromo 7-nitro indazole (3-Br-7-NI) and 1-(2-trifluoro-methyl-phenyl)imidazole (TRIM). All drugs were administered intraperitoneally.*

| | Vehicle | | NOS Inhibitor | | |
|---|---|---|---|---|---|
| | Saline | *DMSO* | *S-Me-TC* | *3-Br-7-NI* | *TRIM* |
| Seizure | 10.2±0.4 | 9.1±0.8 | 31.8±1.2* | 21.1±2.3* | 22.2±1.8* |
| Status epilepticus | 16.9±1.2 | 16.8±1.3 | 33.7±1.5* | 32.5±2.6* | 33.5±2.4* |
| Mortality | 19.3±0.9 | 18.8±1.2 | 50.2±5.6* | 37.2±2.5* | 37.4±2.1* |

Values are means ± S.E.M, 5 groups, $n$=20 in each group.
* p<0.05 significant differences from control ($t$-test, *ANOVA*, *Dunnett* test).

**Table 4.2** *Duration (min) of pilocarpine (300 mg/kg)-induced seizures and status epilepticus in mice, pretreated (30 min prior to pilocarpine) with vehicle (saline, 0.1 ml or DMSO, 0.025 ml) or NOS inhibitors (120 mg/kg), S-methyl-L-thiocitrulline (S-Me-TC), 3-bromo 7-nitro indazole (3-Br-7-NI) and 1-(2-trifluoromethylphenyl)imidazole (TRIM). All drugs were administered intraperitoneally.*

| | Vehicle | | NOS Inhibitor | | |
|---|---|---|---|---|---|
| | Saline | *DMSO* | *S-Me-TC* | *3-Br-7-NI* | *TRIM* |
| Seizure | 10.8±1.2 | 11.3±1.3 | 9.1±1.1 | 5.1±0.8* | 4.7±1.6* |
| Status epilepticus | 4.1±0.8 | 3.9±1.2 | 4.9±2.4 | 3.7±1.9 | 3.7±1.9 |

Values are means ± S.E.M, 5 groups, $n$=20 in each group.
* $p<0.05$ significant differences from control ($t$-test, *ANOVA*, *Dunnett* test).

### *4.4.2 Animals Treated with NOS Inhibitors*

Pretreatment of animals (3 groups, n=20 each group) with NOS inhibitors, S-Me-TC, 3-Br-7-NI and TRIM (120 mg/kg, each), 30 min prior to pilocarpine (300 mg/kg) decreased the mortality in mice (to 35-60%), reduced the occurrence of status epilepticus (to 40-65%) and seizures (to 70-90%; Fig. 4.1). Latencies of seizures, status epilepticus and mortality were significantly prolonged (2-3 times) following administration of NOS inhibitors (Table 4.1). Duration of paroxysmal motor seizures, but not the duration of status epilepticus, was significantly reduced by 3-Br-7-NI (5.1±0.8 min) and TRIM (4.7±1.6 min.), compared to the control (DMSO 11.3±1.3, Table 4.2).

## 4.5 Discussion

The present data show that new and potent non-L-Arg derived NOS inhibitors, 3-Br-7-NI, TRIM and S-Me-TC, attenuate pilocarpine-induced seizures in mice. It has been shown that pilocarpine-induced seizures involve predominantly limbic system (Turski *et al.*, 1984). This provides evidence for the involvement of NO in motor limbic epilepsy. The results of this study are consistent with the finding that L-Arg-derived NOS inhibitor L-NAME reduces the severity of seizures induced by acetylcholinesterase inhibitor, tacrine (Bagetta *et al.*, 1992). The idea of involvement of NO in the cholinergic model of seizure is supported by the evidence of the excessive release of EAA in pilocarpine-induced epilepsy (Walton *et al.*, 1990) and by the fact that L-Arg, the precursor of NO, potentiates epileptogenic properties of NMDA (De Sarro *et al.*, 1993; Mollace *et al.*, 1991). In addition, recent studies indicate that NO affects the transporters of various neurotransmitters, including those which exert an excitatory function in the CNS. Accordingly, NO can inhibit GLU (Lonart et Johnson, 1994; 1995) and NA (Lonart and Johnson, 1995; Miller and Hoffman, 1994) uptake, while 7-NI enhances the function of NA uptake carrier (Kiss *et al.*, 1996). Our data are in line with these studies, since decrease of NO synthesis by NOS inhibitors may facilitate the uptake of

excitatory neurotransmitters, leading to their inactivation and decreased concentration in the synaptic space. This may explain (at least partially) the decrease of neuronal excitability following administration of NOS inhibitors.

Although S-Me-TC is the most potent NOS inhibitor described to date (Narayanan *et al.*,1995), 3-Br-7-NI and TRIM are more efficient in reducing the duration of seizures and the frequency of seizures, status epilepticus and mortality. The reason is not known but several possibilities could be considered. One of the explanations might be that S-Me-TC, as the most potent NOS inhibitor, blocks more completely the negative feedback mechanism exerted by NO on the NMDA receptor (Hoyt *et al.*, 1992; Izumi *et al.*, 1992; Kirkby *et al.*, 1996a; Rundfelt *et al.*, 1995). Activation of NMDA receptors and corresponding glutaminergic transmission may lead to an increase of neuronal excitation. In addition, S-Me-TC acting as an inhibitor of e-NOS (Joly *et al.*, 1995; Narayanan *et al.*, 1994), induces vasoconstriction and reduces tissue perfusion and oxygenation. The resulting impairment of cerebrovascular autoregulation may cause a disbalance between increased metabolic demand and reduced blood flow during seizure with further spread of epilepsy (Kirkby *et al.*, 1996b; Narayanan *et al.*, 1994). Furthermore, an increase of systemic blood pressure may affect the neuronal excitability in the CNS. It has been reported that rise in blood pressure produced mechanically (by occlusion of thoracic aorta or by balloon inflation into descending aorta) or by hypertensive agents can stimulate wakefulness and cortical arousal (Baust *et al.*, 1967; Ebenezer, 1994; Furfine *et al.*, 1994). Pressure-induced arousal is mediated by afferent impulses from peripheral pressoreceptors (Bowes *et al.*, 1981). Therefore, it could be expected that potent pressor effect of S-Me-TC may attenuate its own anticonvulsant properties. This may contribute to the less prominent anticonvulsant effect of S-Me-TC, compared to other two NOS inhibitors examined in this study. This implicates that insufficient antiepileptic action of S-Me-TC could be due to both, increased activity of NMDA receptors (due to weaker negative feedback by NO) and significant cerebral and peripheral vasoconstriction (due to inhibition of e-NOS). Nevertheless, S-Me-TC is more potent than the other two NOS inhibitors in delaying the occurrence of convulsions, status epilepticus and mortality. This suggests that the main action of S-Me-TC, and probably of the other two NOS inhibitors, is on the mechanism of onset and genesis of epilepsy, rather than on the mechanism of maintenance of seizures. Based on experiments in which L-Arg potentiated EAA-induced seizures, De Sarro *et al.* (1993) suggested also that NO might contribute mainly to the genesis of seizure activity. Concerning the role of cyclic nucleotide levels in the brain, it has been suggested that cGMP levels increased by pentylenetetrazol may have a role in seizure onset and/or propagation, whereas cAMP may be involved in processes that attenuate or terminate seizures (Ferrendelli *et al.*, 1980). The fact that NO increases cGMP levels (Bagetta *et al.*, 1993; Garthwaite, 1991) could explain why the most potent NOS inhibitor used in this study affects mainly genesis and propagation of seizure.

Although the effects of NOS inhibitor on seizures is predominantly of central origin (neuronal and/or vascular), the peripheral component should also be considered. Recent results have shown that n-NOS is expressed at higher levels in the skeletal muscle (Nakane *et al.*, 1993) and it is localized in fast-twitch muscle fibres at the sarcolemma (Kobzik *et al.*, 1994). An inverse correlation has been shown between n-NOS activity and skeletal muscle force. Contractile function is augmented by blockers of NOS and it has been suggested that NO produced near sarcolemma opposes contractile force of skeletal muscle (Kobzik *et al.*,

1994). Evidently, there remains a possibility that motor expression of seizures following administration of NOS inhibitors could be modified by both, central and peripheral (sarcolemmal) n-NOS. However, the participation of the inhibition of sarcolemmal n-NOS in motor seizure phenomena has not been studied yet, but evidently this aspect deserves serious consideration.

In *conclusion*, pilocarpine-induced seizures in mice can be attenuated by NOS-inhibitors, 3-Br-7-NI, TRIM and in a lesser extent with S-Me-TC. We suggest that NO acts as a central endogenously active substance with proconvulsant properties in the motor limbic structures in mice. However, at this time the involvement of peripheral NO in the modulation of motor seizure phenomena cannot be excluded. The new, potent and relative selective NOS inhibitors used in this study, could be useful tools to examine further the role of central and peripheral NO in epilepsy.

### 4.6 References

Bagetta G, Iannone M, Scorsa A M, Nisticò G. Tacrine-induced seizures and brain damage in LiCl-treated rats can be prevented by N-nitro-L-arginine methyl ester. *Eur J Pharmacol* 213:301-304, **1992.**

Bagetta G, Massoud R, Rodino P, Federici G, Nisticò, G. Systemic administration of lithium chloride and tacrine increases nitric oxide synthase activity in the hippocampus of rats. *Eur J Pharmacol* 237:61-64, **1993.**

Baust W, Heinemann H. The role of baroreceptors and blood pressure in the regulation of sleep and wakefulness. *Exp Brain Res* 3:12-24, **1967.**

Becker A, Grecksch G, Schröder H. N-omega-nitro-L-arginine methyl ester interferes with pentylenetetrazol-induced kindling and has no effect on changes in glutamate binding. *Brain Res* 688:230-232, **1995.**

Bland-Ward P. A, Moore PK. 7-Nitro indazole derivatives are potent inhibitors of brain, endothelium and inducible isoforms of nitric oxide synthase. *Life Sci* 57: PL 131-135, **1995.**

Bowes G, Townsend ER, Bromwley SM, Kozar LF, Philipson EA. Role of the carotid body and of afferent vagal stimuli in the arousal response to airway occlusion in sleeping dogs. *Am Rev Resp Dis* 123:644-647, **1981.**

Buisson A, Lakhmeche N, Verrechia C, Plotkine M, Boulu RG. Nitric oxide: an endogenous anticonvulsant substance. *Neuroreport* 4:444-446, **1993.**

De Sarro GB, Di Paola ED, De Sarro A, Vidal MJ. Role of nitric oxide in the genesis of excitatory amino acid-induced seizures from the deep prepiriform cortex. *Fundam Clin Pharmacol* 5:503-511, **1991.**

De Sarro, G B, Di Paola E D, De Saro A, Vidal MJ. L-arginine potentiates excitatory amino acid-induced seizures elicited in the deep prepiriform cortex. *Eur J Pharmacol* 12:151-158, **1993.**

Ebenezer IS. The effects of subcutaneous administration of arginine-8-vasopressin on the electroencephalogram of conscious rats are mediated by peripheral vasopressin $V_1$ receptors. *Methods Find Exp Clin Pharmacol* 16:315-321, **1994.**

Ferrendelli JA, Blank AC, Gross RA. Relationships between seizure activity and cyclic nucleotide levels in brain. *Brain Res* 200:93-103, **1980.**

Fevell JE, Johnson P. Acute increase in blood pressure cause arousal from sleep in lambs. *Brain Res* 311:259-265, **1984.**

Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA, Garvey EP. Potent and selective inhibition of human nitric oxide synthase. Selective inhibition of neuronal nitric oxide synthase by S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline. *J Biol Chem* 269:26677-26683, 1994.

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

Handy RLC, Wallace P, Gaffen ZA, Whitehead KJ, Moore PK. The antinociceptive effect of 1-(2-trifluoromethylphenyl)imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase *in vitro*, in the mouse. *Br J Pharmacol* 116:2349-2350, 1995.

Haberny KA, Pou S, Eccles CU. Potentiation of quinolinate-induced hippocampal lesions by inhibition of NO synthesis. *Neurosci Lett* 146:187-190, 1992.

Hoyt KR, Tang LH, Aizenman E, Reynolds IJ. Nitric oxide modulates NMDA-induced increases in intracellular $Ca^{2+}$ in cultured rat forebrain neurons. *Brain Res* 592:310-316, 1992.

Izumi Y, Clifford DB, Zorumski CF. Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science* 257:1273-1276, 1992.

Joly GA, Narayanan K, Griffith O W, Kilbourn RG. Characterisation of the effects of two new arginine/citrulline analogues on constitutive and inducible nitric oxide synthases in rat aorta. *Br J Pharmacol* 115:491-497, 1995.

Kiss JP, Sershen H, Lajtha A, Vizi ES. Inhibition of neuronal nitric oxide synthase potentiates the dimethylphenylpiperazinium-evoked carrier-mediated release of noradrenaline from rat hippocampal slices. *Neurosci Lett* 215:115-118, 1996.

Kirkby RD, Forbes RA, Subramaniam S. Modification of kainate-induced behavioral and electrographic seizures following inhibition of nitric oxide synthase in mice. *Epilepsy Res* 24:79-90, 1996.

Kirkby RD, Carroll DM, Grossman AB, Subramaniam S. Factors determining proconvulsant and anticonvulsant effects of inhibitors of nitric oxide synthase in rodents. *Epilepsy Res* 24:91-100, 1996.

Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372:546-548, 1994.

Kovach A-GB, Lohinai Z, Marczis J, Balla I, Dawson TM, Snyder SH. The effect of haemorrhagic hypotension and retransfusion and 7-nitro indazole on rCBF, NOS catalytic activity, and cortical NO content in the cat. *Ann NY Acad Sci* 738:348-368, 1994.

Lonart G, Johnson KM. Inhibitory effects of nitric oxide on the uptake of [$^3$H]dopamine and [$^3$H]glutamate by striatal synaptosomes. *J Neurochem* 63:2108-2117, 1994.

Lonart G, Johnson KM. Characterization of nitric oxide generator-induced hippocampal [$^3$H]-norepinephrine release. II. The role of calcium, reverse norepinephrine transport and cyclic 3',5'-guanosine monophosphate. *J Pharmacol Exp Ther* 275:12-22, 1995.

Maggio R, Fumagalli F, Donati E, Barbier P, Racagni G, Corsini GU, Riva M. Inhibition of nitric oxide synthase dramatically potentiates seizures induced by kainic acid and pilocarpine in rats. *Brain Res* 679:184-187, 1995.

Miller KJ, Hoffman BJ. Adenosine A3 receptors regulate serotonin transport via nitric oxide and cGMP. *J Biol Chem* 269:27351-27356, 1994.

Mollace V, Bagetta G, Nisticò G. Evidence that L-arginine possesses proconvulsant effects mediated through nitric oxide. *Neuro Rep* 2:269-272, 1991.

Moore PK, Babbedge RC, Wallace P, Gaffen Z, Hart SL. 7-Nitro indazole an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* 108:296-297, 1993.

Mülsch A, Busse R, Mordvintcev PI, Vanin AF, Nielsen EO, Scheel-Krüger J, Olesen SP. Nitric oxide promotes seizure activity in kainate-treated rats. *Neuro Rep* 5:2325-2328, 1994.

Nakamura T-A, Yamada K, Hasegawa T, Nabeshima T. Possible involvement of nitric oxide in quinolinic acid-induced convulsions in mice. *Pharmacol Biochem Behav* 51:309-312, 1995.

Nakane M, Schmidt HH, Pollock JS, Forstermann U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316:175-180, 1993.

Narayanan K, Griffith OW. Synthesis of L-thiocitrulline, L-homothiocitrulline and S-methyl-L-thiocitrulline: a new class of potent nitric oxide synthase inhibitors. *J Med Chem* 37:885-887, 1994.

Narayanan K, Spack L, McMillan K, Kilbourn RG, Hayward MA, Masters BS, GriffithOW. S-alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity *in vivo*. *J Biol Chem* 270:11103-11110, 1995.

Osonoe K, Mori N, Suzuki K, Osonoe M. Antiepileptic effects of inhibitors of nitric oxide synthase examined in pentylenetetrazol-induced seizures in rats. *Brain Res* 663:338-340, 1994.

Penix LP, Davis W, Subramaniam S. Inhibition of NO synthase increases the severity of kainic acid-induced seizures in rodents. *Epilepsy Res* 18:177-184, 1994.

Przegalinski E, Baran L, Siwanowicz J. The role of nitric oxide in the kainate-induced seizure in mice. *Neurosci Lett* 170:74-76, 1994.

Przewlocka B, Lason W, Machelska H, Przewlocki R. The effects of cocaine-induced seizures on the proenkephalin mRNA in the mouse hippocampus: a possible involvement of the nitric oxide pathway. *Neurosci Lett* 168:81-84, 1994.

Rigaud-Monnet A-S, Pinard E, Borredon J, Seylaz J. Blockade of nitric oxide synthesis inhibits hippocampal hyperaemia in kainic acid-induced seizures. *J Cereb Blood Flow Metab* 14:581-590, 1994.

Rundfeld C, Koch R, Richter A, Mevissen M, Gerecke U, Löschher W. Dose-dependent anticonvulsant and proconvulsant effects of nitric oxide synthase inhibitors on seizure threshold in a cortical stimulation model in rats. *Eur J Pharmacol* 274:73-81, 1995.

Starr MS, Starr BS. Paradoxical facilitation of pilocarpine-induced seizures in the mouse by MK-801 and the nitric oxide synthesis inhibitor L-NAME. *Pharmacol Biochem Behav* 45:321-325, 1993.

Turski WA, Cavalheiro EA, Bortolotto ZA, Mello LM, Schwarz M, Turski L. Seizures produced by pilocarpine in mice: a behavioral, electroencephalographic and morphological analysis. *Brain Res* 321:237-253; 1984.

Van Leeuwen R, De Vries R, Dzoljic MR. 7-Nitro indazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, 1995.

Walton NY, Gunawan S, Treiman DM. Brain amino acid concentration changes during status epilepticus induced by lithium and pilocarpine. *Exp Neurol* 108:61-70, 1990.

Wang Q, Theard MA, Pelligrino DA, Baughman VL, Hoffman WE, Albrecht RF, Cwik R, Paulson OB, Lassen NA. Nitric oxide (NO) is an endogenous anticonvulsant but not a mediator of the increase in cerebral blood flow accompanying bicuculline-induced seizures in rats. *Brain Res* 658:192-198, 1994.

Zhang J, Su Y, Oury TD, Piantadosi CA. Cerebral amino acid, norepinephrine and nitric oxide metabolism in CNS oxygen toxicity. *Brain Res* 606:56-62, 1993.

# CHAPTER 5

## NITRIC OXIDE AND LOCOMOTION
### *New and Potent Inhibitors of Nitric Oxide Synthase Reduce Motor Activity in Mice*


**5.1 Abstract**

Potent inhibitors of NOS, 3-bromo-7-nitro indazole (3-Br-7-NI), 1-(2-trifluoromethylphenyl) imidazole (TRIM), S-methyl-L-thiocitrulline (S-Me-TC) and 7-nitro indazole (7-NI), reduced locomotion in mice. These results indicate that activity of NOS and corresponding NO release are of importance for spontaneous locomotion.


**5.2 Introduction**

Recent evidence suggests that NO plays an important role in motor activity. NOS inhibitor, $N^G$-nitro-L-arginine-methyl-ester (L-NAME) reduced spontaneous locomotor activity (Sandi *et al.*, 1995) and hyperlocomotion induced by cocaine (Pudiak and Bozarth, 1993), morphine (Calignano *et al.*, 1993), substance P (Mancuso *et al.*, 1994) or methamphetamine in mice (Ohno and Watanabe, 1995) and rats (Abekawa *et al.*, 1994). The other L-Arg-derived NOS inhibitor, $N^G$-nitro-L-arginine (L-NOARG) suppressed increased locomotion induced by D1 and D2 receptors agonists (Starr and Starr, 1995).

The relatively selective n-NOS inhibitor, 7-NI, reduced locomotion in rats (Connop *et al.*, 1994) and mice (Starr and Starr, 1995. Recently we have shown that central depression induced by 7-NI is associated with a loss of the righting reflex (Dzoljic *et al.*, 1996). The role of endogenous NO in expression of locomotion became of particular interest after identification of n-NOS at the sarcolemma of skeletal muscles (Nakane *et al.*, 1993) and the evidence that NOS inhibitors affected skeletal muscle contraction (Kobzik *et al.*, 1994).

In order to elucidate further the role of NO in motor activity, we examined the effects of new and potent non-L-Arg derived n-NOS inhibitors, 3-Br-7-NI, TRIM and S-Me-TC, on locomotion in mice. These substances are relatively selective inhibitors of n-NOS (Bland-Ward and Moore, 1995; Furfine *et al.*, 1994; Handy *et al.*, 1995). Similar to 7-NI (Moore *et al.*, 1993), TRIM lacks vasopressor activity in the anaesthetized mouse (Handy *et al.*, 1995), while S-Me-TC is the most potent NOS inhibitor described to date with a strong pressor activity (Narayanan *et al.*, 1995). Although the effect of 7-NI on locomotion has already been examined (Connop *et al.*, 1994; Starr and Starr, 1995; Dzoljic *et al.*, 1996), for comparison we included this drug in the study.

**5.3 Methods**

*5.3.1 Animals*

Adult, male Swiss mice (28-35 g) were housed in a room with controlled temperature (21$^0$C), humidity (55%), standard light-dark cycle (light: 07.00-19.00 h) and free access to food and water.

*5.3.2 Procedures*

The animals (n=80) were divided into six groups (n=13-14 each). Each group was treated intraperitoneally (i.p.) with one of the vehicles (saline or dimethyl sulfoxide, DMSO) or one of the NOS inhibitors (7-NI, molecular weight: 160; 3-Br-7-NI, 242; TRIM, 212, and

S-Me-TC, 266). The selected dose for the NOS inhibitors (120 mg/kg) was based on the effects observed in our preliminary experiments and biologically active concentrations of these drugs used by other authors (Bland-Ward and Moore, 1995; Furfine *et al.*, 1994; Connop *et al.*, 1994; Handy *et al.*, 1995; Narayanan *et al.*, 1995).

### *5.3.3 Behavioural Assessment*

Behavioural assesment was carried out in animals placed in plexiglas compartments (40x25x25 cm) and cumulative locomotor activity of mice was measured by a Varimex apparatus (Columbus Instruments, Ohio, USA). After 1 h of habituation the mice were removed, injected with the corresponding drug and rapidly returned to the experimental chamber. The motility of the mice was recorded during 1-h before drug treatment and 3 h following vehicle/drug administration. Only horizontal displacements of the animal across the cage were recorded.

### *5.3.4 Ethical Approval*

The experiments and protocol of this study were approved by the Faculty Commission for experiments, handling and care animals.

### *5.3.5 Drugs*

S-Me-TC (*Alexis*) was dissolved in saline (0.9% NaCl). 7-NI (*Lancaster*), 3-Br-7-NI (*Affiniti*) and TRIM (*RBI*) were dissolved in DMSO (*Merck*).

### *5.3.6 Statistics*

The results were expressed as means±S.E.M. At each time point (1$^{st}$ h, 2$^{nd}$ h and 3$^{rd}$ h after vehicle/drug administration), the significance of the difference between cumulative locomotor scores of the drug-treated groups versus the relevant control group was determined by *Student's* t-test (when two experimental groups were involved: saline v. S-Me-TC) or by one-way analysis of variance (*ANOVA,* when four experimental groups were involved: DMSO v. 3-Br-7-NI, TRIM and 7-NI). ANOVA was followed by multiple comparisons using *Dunnett* test, when F ratios reached significance ($P<0.05$).

## 5.4 Results

### *5.4.1 Control Animals*

Control animals injected with vehicle in an identical volume used to dissolve the drugs (saline 0.1 ml or DMSO 0.025 ml) did not show any behavioural changes. However, in the 1$^{st}$ h before injection they were more active (means±S.E.M, saline: 581±65, DMSO: 687±106) than in the following 3 h after vehicle administration. Additionally, a gradual increase in locomotion was observed in this 3 h period (saline: 1$^{st}$ h: 181±27, 2$^{nd}$ h: 264±32, 3$^{rd}$ h: 340±44 and DMSO: 197±21, 294±37, 365±42, respectively; Fig. 5.1). The higher locomotor scores in the 1$^{st}$ h prior to vehicles administration might be due to the increase in the exploratory behaviour caused by environmental novelty. The decrease in locomotion in the next 3 h after vehicle administration is probably a reflection of anxiety induced by handling and i.p. injection. However, a gradual increase of locomotor activity in the following 3 h after vehicle administration might be due to corresponding decrease of anxiety.

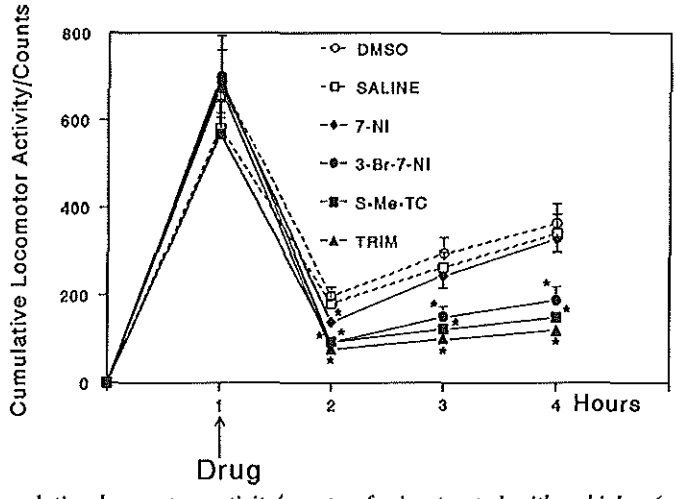


*Figure 5.1 Cumulative locomotor activity/counts of mice treated with vehicles (used as control, DMSO, 0.025 ml, n=14 or saline, 0.1 ml, n=13) or nitric oxide synthase (NOS) inhibitors (120 mg/kg), 7-nitro indazole (7-NI, n=14), 3-bromo-7-nitro indazole (3-Br-7-NI, n=14), S-methyl-L-thiocitrulline (S-Me-TC, n=13), and 1-(2-trifluoro-methylphenyl)imidazole (TRIM, n=14), 1 h before and 3 h after intraperitoneal administration of the vehicle/drug. S-Me-TC was dissolved in saline while 7-NI, 3-Br-7-NI and TRIM were dissolved in DMSO. The vertical lines represent means±S.E.M. and asterisks denote significance at a level of P<0.05 (Student's t-test or ANOVA followed by Dunnett test). Note the prominent decrease in locomotion following administration of NOS inhibitors compared to the control.*

### *5.4.2 Animals Treated with NOS Inhibitors*

These animals looked alert but less active, spending more time on sitting still in the cages, than normal mice treated with vehicle. However, no signs of abnormal posture were observed, except ataxic behaviour caused by TRIM in the 1st h after injection. The impression of quiescence and bradykinesia, following NOS inhibitors, corresponded with reduced motility (Fig. 5.1). The decrease of locomotion caused by S-Me-TC (1th h: 94±7, 2nd h: 121±8, 3rd h: 149±13), 3-Br-7-NI (91±13, 149±22, 188±30) and TRIM (76±12, 99±13, 119±13) lasted for 3 h, while a similar effect of 7-NI (138±13) lasted 1 h (saline v. S-Me-TC, Student's t-test: $P<0.05$; DMSO v. 3-Br-7-NI, TRIM and 7-NI, ANOVA followed by Dunnett test when F ratio reached significance: 1th h- $F=12.4$, 2nd h- $F=12.4$, 3rd h- $F=14.3$, $P<0.05$; Fig. 5.1).

## 5.5 Discussion

Reduced locomotion in mice following administration of NOS inhibitors observed in this study is in accordance with the decrease in the EEG rhythmic slow activity (RSA=theta rhythm, 6-9 Hz) in rats by 7-NI, 3-Br-7-NI and S-Me-TC (Dzoljic *et al.*, 1996). The RSA in the rat is associated with locomotion and other voluntary movements (Depoortere, 1987). The prominent decrease in power of RSA, following administration of NOS inhibitors, might reflect a depression of central neuronal structures involved in the locomotion.

The mode of action of NOS inhibitors in locomotion is not known, but the effect of NO on central neurotransmitters, particularly on DA in basal ganglia could be of importance. It has been shown that NO induces DA release from striatal (Zhu and Luo, 1992) and hippocampal slices (Lonart *et al.*, 1992). Striatal dopaminergic transmission is involved in the control of locomotion (Angulio and McEwen, 1994) and NOS is widely distributed in striatal neurons (Snyder and Bredt, 1991). The idea that NOS inhibitors decrease locomotor activity by reducing dopaminergic transmission is consistent with the fact that these drugs reduce hypermotility induced by dopamine $D_1$ and $D_2$ receptor agonists (Starr and Starr, 1995).

In addition, there is evidence of the involvement of GLU in the control of locomotion (Angulio and McEwen, 1994; Witkin, 1993), probably through the dopaminergic system. GLU release has been reported to be stimulated by NO-donors (Guevara-Guzman *et al.*, 1994) and blocked by NOS inhibitors (Montague *et al.*, 1994). Moreover, both NOS inhibitors and haemoglobin, which binds extracellular NO, reduce the release of striatal DA induced by GLU receptor agonist NMDA (Hanbauer *et al.*, 1992).

Along with DA and GLU, other conventional neurotransmitters (GABA, 5-HT, NA and Ach) were implicated in the regulation of locomotor activity (Angulio and McEwen, 1994; Mancuso *et al.*, 1994). It is known that NO stimulates the release of various neurotransmitters, including NA (Mancuso *et al.*, 1994), GABA, 5-HT and Ach (Guevara-Guzman *et al.*, 1994; Lonart *et al.*, 1992; Prast and Phillippu, 1992). Evidently, decrease in locomotion induced by NOS inhibitors is a complex phenomenon due to derangement of not only dopaminergic transmission, but also of other neurotransmitter systems involved in the control of locomotion.

Additionally, it is of interest to note that there is evidence that NO attenuates anxiety which can further influence locomotor activity. Accordingly, the anxiolytic compound chlordiazepoxide exhibited increased exploration activity in mice, which was reduced by pretreatment with NOS inhibitor, L-NOARG (Quock and Nguyen, 1992). The authors suggested that an anxiogenic component present in L-NOARG might be responsible for the decreased locomotion of animals. However, this explanation should not be generalised, since there is still no evidence that the new NOS inhibitors used in this study may exert a similar anxiogenic effect.

Furthermore, the question arises whether reduction of locomotion is due to the inhibition of central or peripheral n-NOS, or both. Recently it has been found that n-NOS is localized at high levels in fast-twitch fibres at the sarcolemma of skeletal muscle (sarcolemmal n-NOS) (Nakane *et al.*, 1993). Contractile function of skeletal muscle has been augmented by NOS inhibitors, while NO produced near the sarcolemma opposes contractile force (Kobzik *et al.*, 1994). A possible contribution of the inhibited sarcolemmal n-NOS in the locomotion should be considered.

In *conclusion*, the new and potent NOS inhibitors, 3-Br-7-NI, TRIM, S-Me-TC and 7-NI, reduce locomotor activity in mice. The results suggest an importance of NOS activity and NO for locomotion.

## 5.6 References

Abekawa T, Ohmori T, Koyama T. Effect of NO synthase inhibition on behavioral changes induced by a single administration of methamphetamine. *Brain Res* 666:147-150, 1994.

Angulo JA, McEwen BS. Molecular aspects of neuropeptide regulation and function in corpus striatum and nucleus accumben. *Brain Res Rev* 19:1-28, 1994.

Bland-Ward PA, Moore P.K. 7-Nitro indazole derivatives are potent inhibitors of brain, endothelium and inducible isoforms of nitric oxide synthase. *Life Sci* 57:PL 131-135, 1995.

Calignano A, Persico P, Mancuso F, Sorrentino L. Endogenous nitric oxide modulates morphine-induced changes in locomotion and food intake in mice. *Eur J Pharmacol* 231:415-419, 1993.

Connop BP, Rolfe NG, Boegman RJ, Jhamandas K, Beninger RJ. Potentiation of NMDA-mediated toxicity on nigrostriatal neurons by a low dose of 7-nitro indazole, *Neuropharmacol* 33 (1994) 1439-1445, 1994.

Depoortere H. Neocortical rhythmic slow activity during wakefulness and paradoxical sleep in rats, *Neuropsychobiology* 18:160-168, 1987.

Dzoljic E, De Vries R, Dzoljic MR. Effect of new and potent nitric oxide synthase inhibitors on sleep/waking stages and EEG power spectrum in the rat. In: D.G.M. Beersma (Ed.), *Sleep-Wake Research in The Netherlands*, Elinkwijk, Utrecht, Vol. 71, pp. 53-56, 1996.

Dzoljic MR, De Vries R, Van Leeuwen R. Sleep and nitric oxide: effects of 7-nitro indazole, inhibitor of brain nitric oxide synthase. *Brain Res* 718:145-150, 1996.

Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA, Garvey EP. Potent and selective inhibition of human nitric oxide synthase. *J Biol Chem* 269:26677-26683, 1994.

Guevara-Guzman R, Emson PC, Kendrick KM. Modulation of in vivo striatal transmitter release by nitric oxide and cyclic GMP. *J Neurochem* 62:807-810, 1994.

Hanbauer I, Wink D, Osawa Y, Edelman GM, Gally JA. Role of nitric oxide in NMDA-evoked release of [3H]-dopamine from striatal slices. *Neuro Rep* 3:409-412, 1992.

Handy RLC, Wallace P, Gaffen ZA, Whitehead KJ, Moore PK. The antinociceptive effect of 1-(2-trifluoromethylphenyl)imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase *in vitro* in the mouse. *Br J Pharmacol* 116:2349-2350, 1995.

Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372: 504-505, 1994.

Lonart G, Wang J, Johnson KM. Nitric oxide induces neurotransmitter release from hippocampal slices. *Eur J Pharmacol* 220:271-272, 1992.

Mancuso F, Calignano A, Sorrentino L. Endogenous nitric oxide modulates behavioral effects elicited by substance P in rat, *Eur J Pharmacol* 271:329-333, 1994.

Montague PR, Gancayco CD, Winn MJ, Marchase RB, Friedlander MJ. Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. *Science* 263:973-977, 1994.

Moore PK, Babbedge RC, Wallace P, Gaffen Z, Hart SL. 7-Nitro indazole an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* 108:296-297, 1993.

Nakane M, Schmidt HH, Pollock JS, Forstermann U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316:175-180, 1993.

Narayanan K, Spack L, McMillan K, Kilbourn RG, Hayward MA, Masters BS, Griffith OW. S-alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity in vivo. *J Biol Chem* 270:11103-11110, 1995.

Ohno M, Watanabe S. Nitric oxide synthase inhibitors block behavioral sensitization to methamphetamine in mice. *Eur J Pharmacol* 275:39-44, 1995.

Prast H, Philippu A, Nitric oxide releases acetylcholine in the basal forebrain. *Eur J Pharmacol* 216:139-140, **1992.**

Pudiak CM, Bozarth MA. L-NAME and MK-801 attenuate sensitization to the locomotor-stimulating effect of cocaine. *Life Sci* 53:1517-1524, **1993.**

Quock RM, Nguyen E. Possible involvement of nitric oxide in chlordiazepoxide- induced anxiolysis in mice. *Life Sci* 51:255-260, **1992.**

Sandi C, Venero C, Guaza C. Decreased spontaneous motor activity and startle response in nitric oxide synthase inhibitor-treated rats. *Eur J Pharmacol* 277:89-97, **1995.**

Snyder SH, Bredt DS. Nitric oxide as a neuronal messenger. *Trends Pharmacol Sci* 12:125-128, **1991.**

Starr MS, Starr BS. Do NMDA receptor-mediated changes in motor behaviour involve nitric oxide? *Eur J Pharmacol* 272:211-217, **1995.**

Witkin JM. Blockade of the locomotor stimulant effects of cocaine and methamphetamine by glutamate antagonists. *Life Sci* 53:PL405-410, **1993.**

Zhu XZ, Luo LG. Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. *J Neurochem* 59:932-935, **1992.**

# CHAPTER 6

## NITRIC OXIDE AND NEURONAL ACTIVITY
### *Vigilance and EEG Power in Rats:*
### *Effects of Potent Inhibitors of the Neuronal Nitric Oxide Synthase*


### 6.1 Abstract
We examined the effects of potent n-NOS inhibitors, 3-bromo-7-nitro indazole (3-Br-7-NI) and S-methyl-L-thiocitrulline (S-Me-TC) on general behaviour, vigilance stages and electroencephalographic (EEG) power spectra in rats. In addition, we studied the effect of 7-nitro indazole (7-NI) on EEG power spectra in rats during dark and light periods.

3-Br-7-NI induced ptosis and decrease of slow wave sleep and rapid eye movement sleep in the rat. 7-NI and 3-Br-7-NI reduced the EEG power density in all frequency bands in the rat, suggesting a depression of central neuronal activity. This effect of 7-NI was more prominent during the day than during the night, indicating a circadian variation in the NOS response to NOS inhibitor. EEG power was the most reduced in the 7-9 Hz range of the rhythmic slow activity (theta rhythm), which is in accordance with decreased locomotion observed following administration of NOS inhibitors. Although S-Me-TC is the most potent NOS inhibitor in vitro experiments, it had less effect on vigilance and EEG power in the rat than other NOS inhibitors used in this study, probably due to its short lasting and blood pressure raising effect. The present results indicate that nitric oxide exerts an excitatory and circadian dependent effect in the central neuronal structures involved in the regulation of vigilance.


### 6.2 Introduction
NO is a highly reactive molecule produced by the enzyme NOS. Three NOS isoenzymes have been isolated, n-NOS, e-NOS and i-NOS. It seems that in the brain all three isoenzymes are present. The e-NOS is expressed in endothelial cells, but the neuronal distribution in the hippocampal neurons has been also suggested (Lowenstein, 1995). In addition, the NO produced by microvessel endothelial cells in the brain may penetrate in the surrounding tissue, affecting neuronal activity and brain functions. The i-NOS was identified in astrocytes (Simmons and Murphy, 1992), while the n-NOS (also known as a brain NOS) have been found in distinct populations of central neurons (Bredt *et al.*, 1990). Furthermore, n-NOS was detected in the peripheral nervous (autonomic/enteric) system (Rand and Li, 1995) and at high levels in fast-twitch fibres at the sarcolemma (sarcolemmal n-NOS) of skeletal muscle (Nakane *et al.*, 1993).

Concerning the role of NO in vigilance we observed that the relatively weak and non specific inhibitor of NOS, $N^G$-monomethyl-L-arginine (L-NMMA) had a sleep promoting effect in the rat (Dzoljic *et al.*, 1994). A specific inhibitor of the n-NOS, 7-NI, without pressor effect (Moor *et al.*, 1993), induced a prominent central depression, associated with reduced motility, loss of righting reflex and disrupted sleep architecture in rats (Dzoljic *et al.*, 1996). We suggested that mild sedation caused by NOS inhibitors may facilitate sleep, while prominent central depression can lead to the disruption of the sleep pattern. Other authors observed a decrease of slow wave sleep (SWS) in rats following administration of NOS inhibitor $N^G$-nitro-L-arginine methyl acetate (L-NAME) (Kapás *et al.*, 1994). L-NAME is an unspecific NOS inhibitor and potent pressor agent (Rees *et al.*, 1991). An increase in blood pressure may affect the vigilance. A rise in blood pressure induced mechanically or by hypertensive drugs stimulates wakefulness (Baust and Heinemann, 1967; Fevell and Johnson,

1984; Ebenezer, 1994). Evidently, the final effect of NOS inhibitor on vigilance and other central functions can be different, mainly determined by potency and selectivity of the drug.

In order to determine further a role of NO in the CNS, we examined the effect of two relatively new and potent non-L-Arg derived inhibitors of n-NOS, 3-Br-7-NI and S-Me-TC on general behaviour, sleep/waking pattern and EEG power spectra in rats. In addition, we examined the effect of 7-NI on EEG power spectra in rats, during the dark and light periods.

3-Br-7-NI is more potent inhibitor of rat n-NOS than 7-NI but less specific (Bland-Ward and Moore, 1995), while S-Me-TC is the most potent NOS-inhibiting agent described to date (Narayanan and Griffith, 1994) and represents a new class of NOS inhibitors with strong pressor activity (Frey *et al.*, 1994; Narayanan *et al.*, 1995). In contrast to L-Arg-derived NOS inhibitors, the S-Me-TC is not metabolised to L-citrulline, a product that is converted to L-Arg in vivo and may sustain overproduction of NO (Hattori *et al.*, 1994).

## 6.3 Methods

### *6.3.1 Animals*

Experiments were performed on male Wistar rats (300-350 g). The animals were housed in a room with controlled temperature (21±1 $^0$C), humidity (50±1 %) and light (06.00-18.00 h). Rats had a free access to food and water, before and during the experiment.

### *6.3.2 Implantation Procedure*

The animals were anaesthetized with pentobarbital (60 mg/kg, intraperitoneally, i.p.) and implanted (stereotaxically) with epidural stainless steel screw electrode over the parietal cortex (2 mm posterior and 2 mm lateral to bregma) for recording the EEG. Two additional electrodes were inserted into the neck muscle for recording the electromyogram (EMG). A reference electrode (for grounding of animal) was placed over the frontal cortex epidurally. All electrodes were fixed in a socket and secured to the skull with dental cement. After surgery the rats were housed individually in perspex cages (LxWxH: 28x23x30) and allowed 7 days for recovery in a sound-proof and electrically shielded room in which the experiments with EEG/EMG recording were carried out.

### *6.3.3 EEG and EMG Recording*

During the last days of the recovery period, the animals were habituated to the recording cables for 3-4 h daily. The subjects were connected with flexible cables equipped with a swivel connector, permitting a free movement of animals. The EEG and EMG signals were recorded and amplified by a polygraph (Grass 78, Grass Instruments Co., Quincy, Massachusetts, USA), located outside recording room and connected to a 386 microcomputer. Polygraph was calibrated to 100 mV/1 cm. The half-amplitude frequency response was 1-100 Hz for the EEG with a selective 50 Hz filter in each channel. EEG/EMG recording started on the 7th day after operation and consisted of a 4 h session from 10.00-14.00 h. The vehicle of the corresponding NOS inhibitor, used as a control, was administered to the rat on the 7th day after operation. The following day (8th day after operation) the corresponding NOS inhibitor was administered. Vehicles and NOS inhibitors were injected i.p. at the end of the 1st h after attachment to the cables. This time period of 1 h was indicated as an adaptation period. The adaptation time period was used to eliminate the factor of stress due to handling and attachment to the cables in order to ensure the stability of the EEG/EMG

recording. During the adaptation period, the animal was not treated with vehicle or drug and vigilance stages were not evaluated or presented in figures. After animal has been injected by vehicle or drug (on the end of adaptation period), the EEG/EMG registration started for the following 3 h in which the EEG power and stages of vigilance were evaluated.

### *6.3.4 Sleep Scoring*

At the end of the recovery period (7th day after surgery), the animal was connected by a cable to a rotating connector for the recording of the EEG and EMG. Scoring of the sleep and waking stage was based on the visual observation of EEG and EMG patterns. The records were read by an experienced investigator and each 10 s epoch was classified visually being wakefulness, SWS or rapid eye movement (REM) sleep. Stages of vigilance were scored according to previously published criteria (Ursin and Larsen, 1983): wakefulness with low voltage EEG activity; SWS with sleep spindles and 1-4 c/s slow waves; REM sleep with low voltage EEG and low neck muscle tone. The SWS latency or REM sleep latency was defined as the length of time (min) from the drug injection to the first 10 s period of SWS or REM sleep, respectively.

### *6.3.5 EEG Power Spectral Analysis*

EEG spectral analysis was performed by using a Fast-Fourier-Transformation. Signals were recorded with Multi Channel Registration Program (CAID, Dijkzight, Rotterdam). The EEG recording lasted 3 h and was digitized with a sampling frequency of 150 Hz. The EEG data were collapsed into the bins 0.5 to 20.0 Hz. Artifacts epochs were excluded from the analysis. Due to considerable intraindividual variations in the absolute power densities, the power values for each rat were therefore expressed relative to control. In the begin of the self-control condition (7th day after operation) lasting 3 h, the rat was injected with vehicle. The mean-power value for each of the frequency bands during vehicle run (self-control) recording was set to 100% and the corresponding percent deviation of drug run (next day, 8th day after operation) was calculated.

In order to observe the effect of 7-NI in rats during active period (dark period, night) and sleeping period (light period, day), the measurement of EEG power in the vehicle (control, 30 min prior to EEG recording) treated animals started during the dark period (23.00-2.00 h) and continued with the same procedure the next day during the light period (11.00-14.00 h). The same measurement of EEG power was repeated after 5 days, but the animals were treated with 7-NI (30 min prior to EEG recording).

### *6.3.6 Ethical Approval*

The experiments and protocol of this study were approved by the Faculty Commission for experiments, handling and care of animals.

### *6.3.7 Chemicals*

7-NI (*Lancaster*, Mühlheim am Main, Germany) was suspended in arachis oil by sonification. 3-Br-7-NI (*Alexis*, Sissach, Switzerland) was dissolved in dimethyl sulfoxide (DMSO, *Merck*, Hohenbrun, Germany), while the solvent for S-Me-TC (*Bachem*, Bubendorf, Switzerland) was saline. Drug solutions/suspensions were prepared before experiment. The doses of NOS inhibitors and duration of the observation period (3 h) were selected on the basis of several

criteria: duration of the effect of NOS inhibitors on the NOS in vitro (Babbedge *et al.*, 1993), doses of S-Me-TC to increase blood pressure (Narayanan *et al.*, 1995) and duration of effects and doses of NOS inhibitors used in our preliminary sleep experiments in rats. All drugs were administered intraperitoneally (i.p.).

### *6.3.8 Statistical Analysis*

Data were analyzed with statistical software by comparing mean power values in each frequency band, using multiple analysis of variance (MANOVA). Significant differences between treatment were further evaluated with paired *t*-test.

## 6.4 Results

### *6.4.1 Behavioural Effects*

Rats were observed 3 h following drug injection for any unusual behaviour. Behavioural and sleep effects of 7-NI in rats were described earlier (Dzoljic *et al.*, 1996, see introduction). 3-Br-7-NI (30 mg/kg and 75 mg/kg, $n$ = 15) induced signs of peritoneal irritation in rats (writhing, licking the place of drug administration with occasional squalling). Administration of DMSO alone (vehicle, 0.4 ml) induced the same effect. This behaviour lasted a less than 30 s. In animals treated with 3-Br-7-NI (but not with DMSO alone) peritoneal irritation was followed by flat body posture, decreased locomotion and occasional ptosis (2 of 8 animals) and loss of righting reflex (in one case, cut off 10 s). S-Me-TC (30 mg/kg, $n$ = 14) did not affect overt behaviour.

### *6.4.2 Vigilance and EEG power spectrum*

The changes in the EEG power following solvent (at least in the first 30 min) were no noticeable. If in the following time period the rat fell asleep an increase of EEG power occurred.

7-NI (50 mg/kg, $n$ = 5). Similarly to our earlier observations (Dzoljic *et al.*, 1996), 7-NI disrupted the sleep architecture in rats. Normal sleep/wake pattern was replaced by decreased EEG amplitudes and behavioural depression, expressed in decreased locomotion, ptosis and loss of the righting reflex. Since this prominent behavioural depression can not be related to any physiological vigilance stage, the EEG power densities values following administration of 7-NI were expressed relative to the EEG power densities in the control conditions (100%) in which all three vigilance stages (wakefulness, SWS and REM sleep) were present. The EEG power was suppressed by 7-NI in each frequency band and this effect was dose related (15-50 mg/kg, $n$ = 4-5 for each dose, Fig. 6.1).

The maximum decrease in the EEG power was seen in the high theta frequency band (7-9 Hz). This effect of 7-NI (50 mg/kg, $n$ = 5) was more pronounced during the light period (11.00-14.00 h) than during the dark one (23.00-2.00 h, Fig. 6.2).

3-Br-7-NI (30 mg/kg, $n$ = 5) decreased significantly the SWS (Fig. 6.3), while the SWS latency was increased (75 ±, 19 min, versus 23 ± 5.4 min in the control). Since the REM sleep was nearly abolished (Fig. 6.3), the EEG power of REM sleep was not examined.

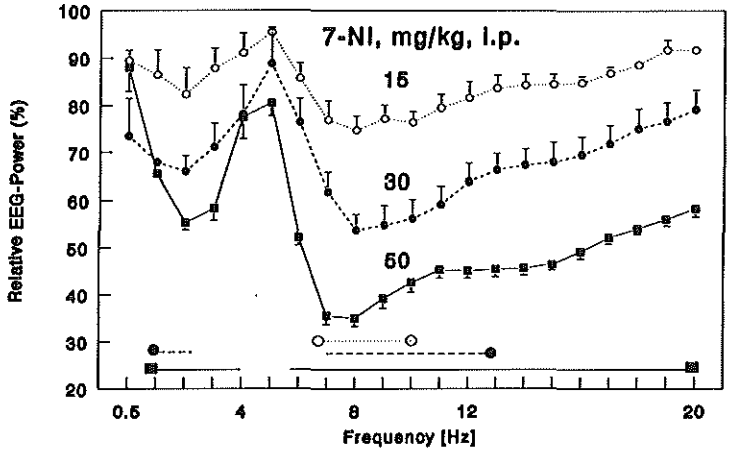


*Fig. 6.1 The EEG power densities in rats (n=4-5 for each dose) during 3 h recording period following intraperitoneal (i.p.) administration of 7-nitro indazole (7-NI). All values are expressed relative to the EEG power densities in the self-control conditions (=100%) during 3 h period. Horizontal lines at the bottom of the graph indicate significant differences from the corresponding control (P<0.05). Note a dose related decrease of the EEG power spectra induced by 7-NI.*

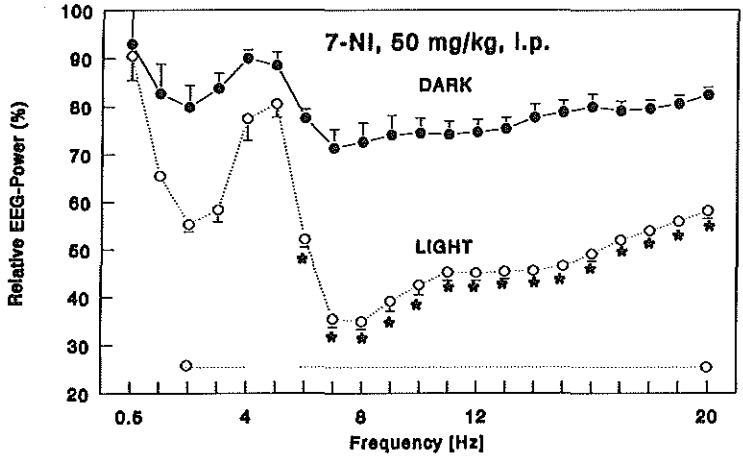


*Fig. 6.2 The EEG power densities in rats (n=5) following administration of 7-nitro indazole (7-NI) during 3 h in the dark period (23.00-2.00 h, solid circle) and in the light period (11.00-14.00 h, open circle). Horizontal line at the bottom of the graph indicates a significant difference between animals treated with 7-NI during light period (open circle) and corresponding control (P<0.05). Asterisks indicate significant difference (P<0.05) between animals treated with 7-NI during the light period in respect to the same animals treated with 7-NI in the dark period. Note a more prominent decrease of the EEG power during the light period compared to the dark period.*

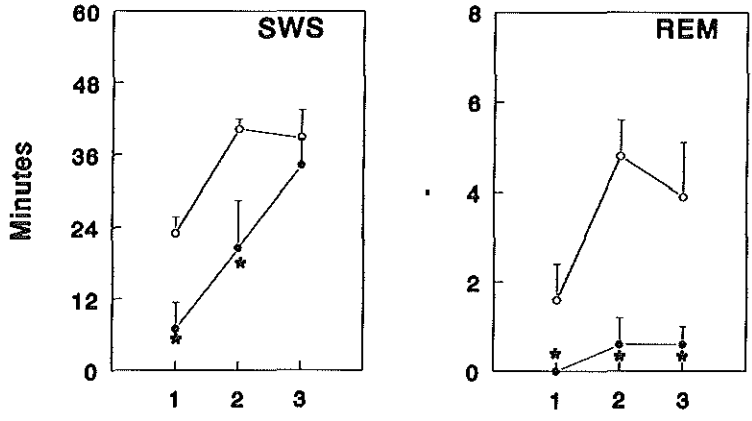


*Fig. 6.3 The sleep pattern in rats (n=5) during 3 h period (in min per h) following i.p. injection of 3-bromo 7-nitro indazole (3-Br- 7-NI, 30 mg/kg, solid circle) or vehicle (dimethyl sulfoxide, open circle). Asterisks indicate significant difference (P<0.05) from the control (t-test after MANOVA). Note the decrease in the SWS in the first two h following drug administration, while REM sleep is nearly abolished.*

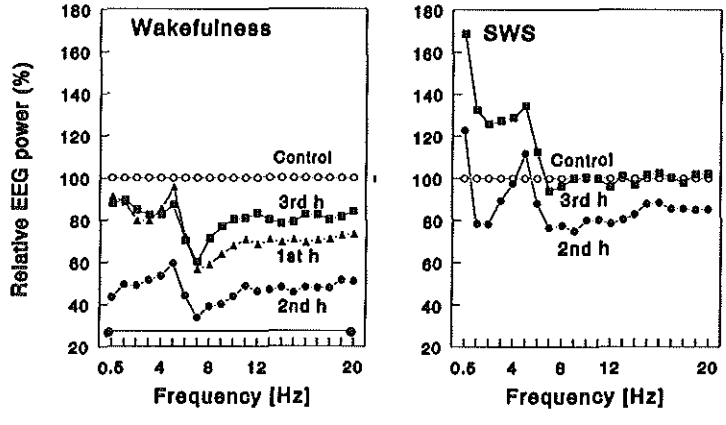


*Fig. 6.4 EEG power densities in rats (n=5) following i.p. administration of 3-bromo 7-nitro indazole (3-Br-7-NI, 30 mg/kg) or vehicle (dimethyl sulfoxide, 0.1 ml). All values are expressed relative to the EEG power densities in the control conditions (=100%). Horizontal line at the bottom of the left figure indicates significant difference from the control (P<0.05) during 2nd h of EEG recording (solid circle). Non-sleeping period, characterized with reduced behavioural activity (see results and discussion), is indicated as Wakefulness. Note a significant decrease of EEG power spectra during wakefulness (particularly in the frequency band 7-9 Hz) with no significant changes in EEG power spectra of SWS.*

In the non-sleeping animals dominated aroused EEG/EMG pattern (low EEG amplitudes and high EMG amplitudes) and depressed behaviour as described above (decreased locomotion and occasional ptosis and loss of righting reflex). The EEG power was decreased in each frequency range during the EEG wakefulness, particularly during the 2nd h following drug administration, but no significant changes in EEG power spectra of the SWS were observed (Fig. 6.4).

S-Me-TC (30 mg/kg, $n$ = 6) had no effect on SWS or REM sleep and the EEG power densities during wakefulness and SWS were decreased but not significantly (Fig. 6.5).

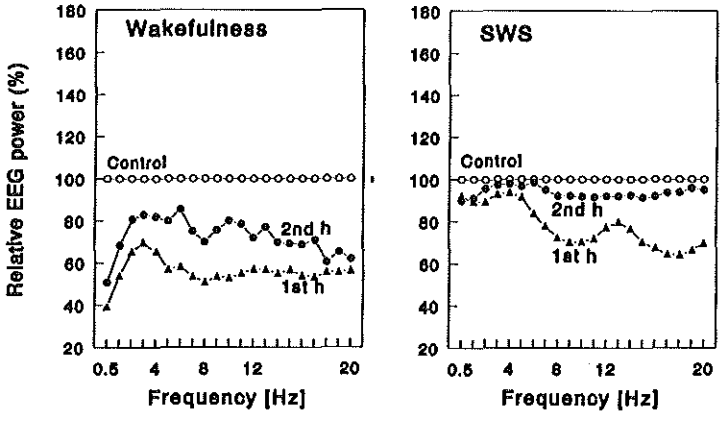


*Fig. 6.5 EEG power densities in rats (n=6) in wakefulness and slow wave sleep (SWS) following i.p. administration of S-methyl-L-thiocitrulline (S-Me-TC, 30 mg/kg, solid circle or triangle) or vehicle (saline, 0.5 ml, i.p., open circle). Note a tendency (not significant) to decrease of EEG power.*

## 6.5 Discussion

The major finding of this study is that NOS inhibitors, 7-NI and 3-Br-7-NI (but not S-Me-TC) decreased the EEG power in rats. In addition, 3-Br-7-NI reduced sleep stages. Concerning sleep stages and EEG power, a non-sleep period following administration of indazole-derived NOS inhibitors (7-NI and 3-Br-7-NI) was characterized by arousal-like EEG/EMG pattern (low EEG amplitudes and high EMG amplitudes) and reduced behavioural activity (decreased locomotion and occasional loss of righting reflex and ptosis). Evidently, indazole-derived NOS inhibitors decreased the sleep but did not increase wakefulness. Thus, an arousal-like EEG/EMG pattern was not associated with awake behaviour, suggesting a dissociation between the EEG/EMG pattern and behaviour. Additionally, the EEG power was suppressed in each frequency range by NOS inhibitors, 7-NI and 3-Br-7-NI. However, this effect was more prominent in the high theta frequency band (7-9 Hz). High frequency of theta rhythm in rats is associated with locomotion and voluntary movements (Depoortere, 1987). Decrease of high theta rhythm is consistent with reduced locomotion, observed in this study following administration of NOS inhibitors. Although, a decrease of the EEG power

was observed during the desynchronization and the behavioural excitation in animals treated with cocaine or d-amphetamine (Ferger *et al.*, 1994), the generalised reduction of the EEG power associated with depressed behaviour (decreased locomotion, Chapter 5; loss of righting reflex and ptosis, Dzoljic *et al.*, 1996) is rather reflection of the central depression than the excitation. Depression of the neuronal activity in various brain regions leads to the decrease of cortical and hippocampal inputs. The reduced input to the hippocampus results in decreased theta activity, whereas the diminished cortical afferent activity causes a decrease of EEG power in other frequency bands. The qualitative changes in the EEG power induced by each of these two indazole-derived NOS inhibitors are similar to the effect of high doses of alcohol (0,75 mg/kg, i.p., Ehlers *et al.*, 1992) or benzodiazepines (3 mg/kg, i.p., Glatt *et al.*, 1983) in rats. Accordingly, a prominent central depression lead to the reduction of locomotor activity and disruption of normal sleep architecture. The central depression induced by indazol-derived NOS inhibitors is consistent with anticonvulsant activity of 7-NI (Van Leeuwen *et al.*, 1995) and other NOS inhibitors used in this study (Chapter 4).

It is of interest to note that depression of the EEG power by 7-NI is less prominent during the dark period (active period of rat) than during the light period. In vehicle treated rats (control) we did not find a significant difference between the EEG power spectra in the light and the dark, probably due to comparison of relatively short time periods of the day and the night (3 h each). In any way, it seems unlikely that differences in the EEG power spectra in the light and in the dark period could be the reason for different effects of NOS inhibitors in the light and in the dark phase. However, it has been reported that NOS activity in the rat brain is higher during the dark phase compared to the light phase (Kapás *et al.*, 1995). The idea of circadian dependence of the NOS activity can be further supported by recent observation that amount of the n-NOS in the rat pineal gland is regulated by environmental lighting condition (Spessert *et al.*, 1995). The elevated activity of NOS in rats during the dark phase with corresponding physiological increase of the NO production, is probably more resistant to the NOS inhibitors during the night than during the day. In addition, these results suggest a possible involvement of the NO in the circadian neuronal activity.

Concerning the difference between NOS inhibitors, it seems clear that 7-NI exerts a more prominent behavioural effect in rats (Dzoljic *et al.*, 1996) than 3-Br-7-NI (this study). This is probably due to the difference in doses used (50 mg/kg of 7-NI, mol weight 160, versus 30 mg/kg 3-Br-7-NI, mol weight 242), but also to the less significant NOS isoform selectivity of 3-Br-7-NI, compared to the 7-NI (Bland-Ward and Moore, 1995). However, it is more interesting that the S-Me-TC, known as the most potent inhibitor of NOS *in vitro* experiments (Narayanan *et al.*, 1995) is less effective in our *in vivo* experiments, compared to other two indazole derivatives. Quantitative differences between 7-NI and S-Me-TC could not be explained by dose differences, since the doses were the same (30 mg/kg) and both drugs have a similar mol weight (242/266). It is more likely that differences between these NOS inhibitors may due to different pharmacokinetic characteristics (absorption, protein binding, penetration in the brain, biotransformation in vivo) or different pharmacodynamic properties of indazole- and thiocitrulline-derivatives. In this respect, S-Me-TC exerts a more potent pressor activity (Narayanan *et al.*, 1995) than indazole-derivatives. The rise in blood pressure induced mechanically (by occlusion of thoracic aorta) or by hypertensive agents (vasopressin) stimulates wakefulness (Baust and Heinemann, 1967; Fevell and Johnson, 1984; Ebenezer, 1994). It has been suggested that cortical arousal can be induced by afferent impulses from

pressoreceptors (Bowes *et al.*, 1981). Evidently, an increase of systemic blood pressure and reduction of local cerebral blood flow (Tanaka, 1991), both due to the inhibition of e-NOS, may significantly reduce an oxygenation and perfusion of the neuronal tissue. This may affect the neuronal reactivity in the CNS and development of the corresponding vigilance stage. Thus, an arousal effect of hypertension following inhibition of e-NOS may oppose a central depression induced by inhibition of n-NOS. Therefore, indazole-derived inhibitors of n-NOS, which do not affect significantly systemic blood pressure may have a qualitatively different effect on vigilance than citrulline-derived S-Me-TC (or L-Arg-derived NOS inhibitors) with prominent pressor activity. Duration of the S-Me-TC-induced hypertension was only 20 min (Narayanan *et al.*, 1995). This indicates that the most potent NOS inhibitor has a relatively short lasting effect, probably due to the reversibility of n-NOS inhibition by S-Me-TC. This idea is supported by experiments *in vitro* in which was demonstrated a reversibility of the n-NOS inhibition induced by S-Me-TC. If mixture of inhibited n-NOS by S-Me-TC was allowed to stand with excess L-Arg for 5 min or longer, the substantial NOS activity was recovered, reaching normal value within 10-15 min (Frey *et al.*, 1994). This may explain a minor changes in the cumulative 3 h EEG power following S-Me-TC. In addition, S-Me-TC, as the most potent NOS inhibitor, more completely blocks the negative feedback mechanism exerted by NO on the NMDA receptors (Izumi *et al.*, 1992). Activation of NMDA receptors and corresponding transmission may lead to the increase of neuronal excitation and arousal.

Regarding the specificity of new NOS inhibitors, an involvement of some additional non-specific effects unrelated to the NOS synthesis, particularly when high doses are used, could not be excluded. Recent experiments of Connop *et al.* (1994) have shown a maximal inhibition of nigral and cerebellar NOS (80/96 %) in rats by 7-NI (20 mg/kg, i.p.). Animals were, sacrificed 30 min after administration of 7-NI. This rises a question whether the effects of higher doses of 7-NI are related to NOS inhibition. NOS inhibition is reversible and our post injection period was 3 h. Therefore, the possibility of different levels of the NOS activity in various time intervals, following different doses of 7-NI could not be ruled out. Accordingly, it could be expected that low dose of 7-NI (15 mg/kg) will induce a shorter duration of the maximal NOS inhibition than high doses of 7-NI (30-50 mg/kg). This may explain a dose related effect of 7-NI, observed in these experiments and many other studies using 7-NI in similar or even high dose range. Although, there is no evidence for non-specific effect of 7-NI or other NOS inhibitors used in this study, the interpretation of the effects of these new drugs and the role of NO in the observed phenomena need an additional verification.

*Concluding*, indazole-derived NOS inhibitors (7-NI, 3-Br-7-NI) induced central depression of neuronal activity with decreased EEG power, leading to disruption of normal sleep architecture and depressed behaviour (reduced locomotion and occasional loss of righting reflex and ptosis). The effect of 7-NI is less prominent during the dark period, indicating a circadian rhythmicity in NOS response to NOS inhibitors. The behavioural depression and reduction of EEG power by citrulline-derived NOS inhibitor S-Me-TC is less prominent. This is probably due to fully reversible and short lasting NOS inhibition by S-Me-TC, prominent increase of systemic blood pressure and reduced negative feed back blockade of NMDA by NO, leading to arousal-inducing effect. We are suggesting that brain

neuronal NO exerts an excitatory and circadian dependent effect in the neuronal structures involved in the regulation of vigilance.

## 6.6 References


Babbedge RC, Bland-Ward PA, Hart SL, Moore PK. Inhibition of rat cerebellar nitric oxide synthase by 7-nitro indazole and related substituted indazoles. *Br J Pharmacol* 110:225-228, 1993.

Baust W, Heinemann H. The role of baroreceptors and blood pressure in the regulation of sleep and wakefulness. *Exp Brain Res* 3:12-24, 1967.

Bland-Ward PA, Moore PK. 7-Nitro indazole derivatives are potent inhibitors of brain, endothelium and inducible isoforms of nitric oxide synthase. Life Sci 57:pp.PL131-135, 1995.

Bowes G, Townsend ER, Bromwley SM, Kozar LF, Phillipson EA (1981) Role of carotid body and of vagal stimuli in the arousal response to airway occlusion in sleeping dog. *Amer Rev Resp Dis* 123:644-647, 1995.

Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347:768-770, 1990.

Connop BP, Rolfe NG, Boegman RJ, Jhamandas K, Beninger RJ. Potentiation of NMDA-mediated toxicity on nigrostriatal neurons by low dose of 7-nitro indazole. *Neuropharmacol* 33:1439-1445, 1994.

Depoortere H. Neocortical rhythmic slow activity during wakefulness and paradoxical sleep in rats. *Neuropsychobiol* 18:160-168, 1987.

Dzoljic MR, De Vries R. Nitric oxide synthase inhibition reduces wakefulness. *Neuropharmacol* 33:1505-1509, 1994.

Dzoljic MR, Van Leeuwen R, Van Vries R. Sleep and nitric oxide: effect of 7-nitro indazole-inhibitor of brain nitric oxide synthase. *Brain Res* 718:145-150, 1996.

Ebenezer IS. The effects of subcutaneous administration of arginine-8-vasopressin on the electroencephalogram of conscious rats are mediated by peripheral vasopressin $V_1$ receptors. *Methods Find Exp Clin Pharmacol* 16:315-321, 1994.

Ehlers CL, Kaneko WM, Wall TL, Chaplin RI. Effects of dizocilpine (MK-801) and ethanol on the EEG and event related potentials (ERPS) in rats. *Neuropharmacol* 31:369-378, 1992.

Ferger B, Krof W, Kuschinsky K. Studies on electroencephalogram (EEG) in rats suggest that moderate doses of cocaine or *d*-amphetamine activate $D_1$ rather than $D_2$ receptors. *Psychopharmacology* 114:297-308, 1994.

Fevell JE, Johnson P. Acute increases in blood pressure cause arousal from sleep in lambs. *Brain Res* 311:259-265, 1984.

Frey C, Narayanan K, McMillan K, Spack L, Gross SS, Masters BS, Griffith OW. L-thiocitrulline. A stereospecific, heme-binding inhibitor of nitric-oxide synthases. *J Biol Chem* 269:26083-26091, 1994.

Glatt A, Duerst T, Mueller B, Demieville H. EEG Evaluation of drug effects in the rat. *Neuropsychobiol* 9:163-166, 1983.

Hattori Y, Campbell EB, Gross SS. Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. Role in the regeneration or arginine for nitric oxide synthesis. *J Biol Chem* 269:9405-9408, 1994.

Izumi Y, Clifford DB, Zorumski CF. Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science* 257:1273-1276, 1992.

Kapás L, Fang J, Krueger JM. Inhibition of nitric oxide synthesis inhibits rat sleep. *Brain Res* 664:189-196, 1994.

Kapás L, Ayers NA, Krueger, JM. Circadian variation in brain nitric oxide synthase activity. *Sleep Res* 24A:106, 1995.

Lowenstein CJ. Nitric oxide synthase: production of a radical messenger. *Transduction Laboratories Insights* 1:8-9, **1995.**

Moore PK, Babbedge RC, Wallace P, Gaffen ZA, Hart SL. 7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* 108:296-297, **1993.**

Nakane M, Schmidt HH, Pollock J, Forstermann U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316:175-180, **1993.**

Narayanan K, Griffith OW. Synthesis of L-thiocitrulline, L-homothiocitrulline, and S-methyl-L-thiocitrulline: A new class of potent nitric oxide synthase inhibitors. *J Med Chem* 37:885-887, **1993.**

Narayanan K, Spack L, McMillan K, Kilbourn RG, Hayward MA, Masters BSS, Griffith OW. S-alkyl-L-thiocitrullines. *J Biol Chem* 270:11103-11110, **1995.**

Rand MJ, Li CG. Nitric oxide in the autonomic and enteric nervous systems. In: Vincent S (ed) Nitric oxide in the nervous system. *Academic Press*, pp 227-279, **1995.**

Rees DD, Palmer RMJ, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 86:3375-3378, **1991.**

Simmons ML, Murphy S. Induction of nitric oxide synthase in glial cells. *J Neurochem* 59:897-905, **1992.**

Spessert R, Layes E, Schollmayer A, Reuss S, Vollrath L. In the pineal gland, but not in the supprachiasmatic nucleus, the amount of constitutive neuronal nitric oxide synthase is regulated by environmental lighting conditions. *Biochem Biophys Res Commun* 212:70-76, **1995.**

Tanaka K, Gotoh F, Gomi S, Takashima S, Mihara B, Shirai T, Nogawa S, Nagata E. Inhibition of nitric oxidase synthesis induced a significant reduction in local cerebral blood flow in the rat. *Neurosci Lett* 127:129-132, **1991.**

Ursin R, Larsen M. Increased sleep following intracerebroventricular injection of the delta sleep-inducing peptide in rats. *Neurosci Lett* 40:145-149, **1983.**

Van Leeuwen R, De Vries R, Dzoljic MR. 7-Nitro indazole, an inhibitor of neuronal nitric oxide synthase attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, **1995.**

# CHAPTER 7

## NITRIC OXIDE AND CENTRALLY-ACTING DRUGS
## *Brain Nitric Oxide Levels: Different Effects of Hypnotic and Analeptic Drugs*


### 7.1 Abstract
Nitric oxide concentrations in the frontal cortex of anaesthetized rat were measured using an electrochemical sensor, before and after intraperitoneal (i.p.) administration of a hypnotic drug (pentobarbital, 20-40 mg/kg) or a convulsant agent (pentylenetetrazol, 50 mg/kg). The concentration of NO was decreased by pentobarbital, while it was increased by pentylenetetrazol. These results indicate that endogenous NO may be involved in the mechanism of action of hypnotic and epileptic drugs. This further suggests that NO concentrations in the human brain may decrease following hypnotic or antiepileptic drug therapy and increase during epileptic attacks or administration of excitatory amphetamine-like drugs. It is concluded that central NO is an important endogenous neuroexcitatory transmitter involved in the seizure susceptibility and activity of some centrally-acting drugs, such as hypnotics and analeptics.


### 7.2 Introduction
Nitric oxide (NO) exerts a significant effect on the neuronal excitability in the brain by releasing three main central excitatory neurotransmitters, ASP, GLU and NA (Guevara-Guzman *et al.*, 1994; Montague *et al.*, 1994). Several studies seem to indicate that NO affects seizure threshold, but the results are still controversial. Both proconvulsant (Osonoe *et al.*, 1994; Van Leeuwen *et al.*, 1995; Chapter 4) and anticonvulsant effects (Starr and Starr, 1993; Maggio *et al.*, 1995) of NO are described. However, concerning the vigilance stages, the results are more consistent, indicating an excitatory effect of NO. It has been reported that a significant decrease in central NO levels occurs during slow wave sleep and that higher NO concentrations are associated with increased neuronal activity, such as in wakefulness or rapid eye movement (Burlet *et al.*, 1995; Williams *et al.*, 1997). Furthermore, Kapàs *et al.* (1995) found that NOS activity in the rat brain is higher during the dark phase (predominately active period in rodents) than in the light phase (predominately sleeping period). Such findings are consistent with our previous results demonstrating a decrease of central NO levels following administration of the NOS inhibitors, which is accompanied with depression of various forms of central neuronal and behavioural activity, such as a decrease in wakefulness (Dzoljic and de Vries, 1994), loss of righting reflex (Dzoljic *et al.*, 1996), inhibition of locomotion (Chapter 5) and reduced EEG power (Chapter 6).

The above account suggests that central depression might be associated with decreased concentration of NO in the brain, while an increase in brain NO levels could be expected during central neuroexcitation. In order to provide data for this hypothesis, we measured brain NO concentrations during drug-induced central depression and excitation.

### 7.3 Methods
#### *7.3.1 Animals and Surgery*
Male Wistar rats (340-360 g, n=20), divided in 4 equal groups, were anaesthetized (Hypnorm$^{R}$, 0.15 ml/kg, i.p.) and artificially ventilated with a frequency of 70/min. Experiments were performed in the laboratory room with constant temperature (21.0±1.0 °C), while the rectal temperature of the animals was maintained at 37.0±0.5 $^{0}$C with a heating pad. The head of the animal was mounted in the stereotaxic head holder and a hole was drilled into

the right frontal region of the skull. After dura was sliced, a sensor for the detection of NO was stereotaxically implanted perpendicularly into the right cortex at coordinates: 2.0 mm lateral, 2.0 mm anterior to bregma and 2.0 mm bellow the dura. A ground electrode was placed subcutaneously in animal's neck. In order to prevent manifest convulsions, the rats which were treated with pentylenetetrazol were injected with d-tubocurarine (0.1 mg/kg, i.p.), 10 min prior to pentylenetetrazol.

### *7.3.2 Detection of NO*

Electrochemical methods using amperometric sensors have frequently been employed to detect quantitative changes in brain NO levels *in vivo* in various species, including the rat (Malinski *et al.*, 1993; Burlet *et al.*, 1995; Williams *et al.*, 1997). In this study, therefore, we also employed a similar experimental approach, using the NO meter (*ISO-NO Mark II*) with an amperometric micro-sensor *(ISO-NOP 200*; both from World Precision Instrument, Inc, Sarasota FL, USA), where oxidation of NO is converted into electric current for the measurement of NO. The *micro-sensor* (tip diameter: 200 μm) is a combination of electrodes, which require no external reference electrode for use. It has a detection limit of 10 nM NO. The micro-sensor electrodes are separated from the external environment by a gas permeable polymeric membrane over the end of a sleeve. The electrodes extend slightly out of the sleeve, stretching the membrane. The integrity of membrane covering the end of the sensor probe was regularly checked by immersing the probe in a strong saline solution (1 M). If the current observed after few minutes in saline was not off scale, the membrane was considered as a normal. The electrode was calibrated daily before each experiment by measuring the current generated by 10 μm NO in aqueous solution, prepared by adding saturated NO solution to 120 ml saline. Aqueous NO solution was prepared under strict inert atmosphere and stored in all-glass flacon with a very small head space. The mean sensitivity of the sensor was 1.1 pA/nM change in NO concentration.

In order to remove tissue particles which may accumulate on the membrane, the tip was cleaned after each use by immersing it in the distilled water for 30 min. After implantation of the sensor into the frontal cortex of the anaesthetized rat and its attachment to the ISO-NO meter, the current measured was recorded on a chart recorder connected to an amplifier. The recording of the current continued until stabilization of current (NO levels), usually over a 1-2 h period. Stabilization of NO levels was defined when during a 15 min period current oscillations were less than 10%.

After NO baseline was reached, animals were injected i.p. with drugs or vehicle. The recording of the current continued for the next 60 min. The NO concentrations for each experiment were calculated by comparing the current measured from experimental amperometric curve with the current obtained after calibration with the NO solution. Due to appreciable interindividual variations in absolute NO concentrations, data were expressed as a percentage of the respective baseline values. On completion of experiments, animals were killed with an overdose of sodium pentobarbital.

### *7.3.3 Statistical Evaluation*

ANOVA followed by Dunnett's multiple comparison test was performed at each time to determine when a significant difference was observed ($P<0.05$).

### *7.3.4 Drugs*

Hypnorm$^{R}$ (fentanyl citrate 0,315 mg/ml and fluanisone 10 mg/ml, Janssen), Narcovet$^{R}$ (pentobarbital sodium 60 mg/ml, Arnhem), pentylenetetrazol (dissolved in 0,9% NaCl, Sigma) and d-tubocurarine (ampoules, Sigma). Fresh drug solutions were prepared before each experiment. Pentobarbital was administered in doses (20-40 mg/kg), which are known to exert the hypnotic and anticonvulsant effects in the rat. We used only one dose of pentylenetetrazol (50 mg/kg), which is known to induce clonic-tonic epileptic convulsions. In preliminary experiments the subconvulsant low dose of pentylenetetrazol (25 mg/kg) was without effect and, therefore, discarded in further study.

### *7.3.5 Ethical Approval*

The experimental protocol of this study was approved by the Faculty Commission for Experiments, Handling and Care of Animals.

## 7.4 Results

NO concentrations in the frontal cerebral cortex of anaesthetized rats during the baseline period at *stabilisation* were in the range of 0.5-4.6 µM (mean±S.E.M. 2.2±1.3 µM). In control animals injected with *vehicle* (0.5 ml saline, n=5), a slow but non-significant decline in the brain NO levels occurred during the 1$^{st}$ h. However, the decline of NO concentrations continued, reaching in our preliminary experiments the significance level in the following hours, until animals died spontaneously. Therefore, the comparison between NO concentrations in control rats (vehicle treated) and drug-treated animals was limited to the first 60 min after drug injection. As shown in Fig. 7.1, treatment with pentobarbital (20 or 40 mg/kg, each group n=5) induced a significant and dose-dependent decrease of the brain NO

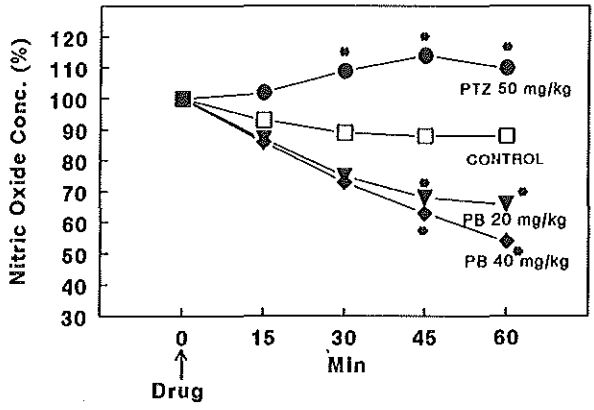


*Figure 7.1 NO concentrations in the frontal cortex of anaesthetized rats, before (t=0) and after i.p. administration of or vehicle (control, 0.5 ml saline) pentobarbital (PB, 20 or 40 mg/kg) or pentylenetetrazol (PTZ, 50 mg/kg); n=5 in each group. NO concentrations, measured by an electrochemical micro-sensor, are expressed as percentage of respective baseline value. Drugs or vehicle were administered (at 0 min) after stabilisation of NO current (see Materials and Methods). Asterisks represents significant differences compared to the control (P<0.05, ANOVA followed by Dunnett's test). Note a dose-dependent decrease of NO concentrations following administration of pentobarbital and an elevation of NO levels induced by pentylenetetrazol.*

concentrations, compared to the control group. In contrast, pentylenetetrazol (50 mg/kg, n=5) increased brain NO levels, reaching significance ($P<0.05$) with respect to the control group after 30 min (Fig. 7.1). When ANOVA reached significance (30 min, $F=13.10$; 45 min, $F=23.94$; 60 min, $F=28.42$), it was followed with Dunnett's test. Significance for pentylenetetrazol was observed at 30 min ($d'=16.94$), while the significance for both drugs, pentylenetetrazol and pentobarbital, reached at 45 min ($d'=17.45$) and 60 min ($d'=16.96$).

**7.5 Discussion**

Baseline concentrations of NO registered in the rat frontal cortex in this study ($\sim 10^{-6}$ M) correlate with NO concentrations found in the rat frontal cortex through the sleep-waking cycle (Burlet *et al.*, 1995) and in the rat parietal cortex during middle cerebral artery occlusion (Malinski *et al.*, 1993; Zhang *et al.*, 1995). However, in these last two studies, the baseline concentrations of NO was lower ($\sim 10^{-8}$ M), compared to the baseline NO levels found in our study. This may be due to different experimental conditions, such as different anaesthetics used (fentanyl and fluanisone in our study versus ketamine and xylazine), different levels of anaesthesia with corresponding difference in the cerebral blood flow (which affects NOS activity) and/or different location of the working electrode (frontal cortex in our study versus parietal cortex). It is of interest to note that a spontaneous decline of NO concentration (not significant in the 1st h of recording), which became more prominent in the following hours, before spontaneous death of animals. Spontaneous decrease of brain NO in control animals is probably due to the applied anaesthesia (Alimoff and Miller, 1993; Tobin *et al.*, 1994) and the subsequent decline of vital functions and central blood flow.

The main finding of this study is that hypnotic and excitatory drugs affect brain NO concentrations in an opposite way. Pentobarbital decreased, while pentylenetetrazol increased the cortical brain NO levels. The mechanism of interactions between these drugs and NO is not clear, but some possibilities can be considered.

It is known that barbiturates inhibit the release of EAA, GLU and ASP (Alifimoff and Miller 1993) and reduce the sensitivity of brain neurons to GLU (Galindo, 1969). It indicates that barbiturates may inhibit NO synthesis by reducing GLU levels and its activity in the brain tissues. This hypothesis is supported by the finding that barbiturates reduce cGMP formation in many brain regions (Kant *et al.*, 1980). It is known that a decrease in synaptic GLU concentration leads to a reduction of NMDA receptor excitability and $Ca^{2+}$ influx in neuronal cells. These changes would reduce brain NOS activity, NO release and, consequently, cGMP formation, known to be regulated by NO (Garthwaite 1991). Furthermore, the corresponding decrease of excitatory effects of NO can modulate vigilance and neuronal excitability (see Introduction). Evidently, the NO system is an additional factor which may contribute (together with barbiturate-induced activation of GABA-inhibitory system) to the mechanism of hypnotic action of pentobarbital and, probably, other sedatives/hypnotics and anticonvulsants.

Related to pentylenetetrazol, it has been shown that its proconvulsant effect is associated with activation of NMDA receptors and an increase in cGMP levels in the brain (Moncada *et al.*, 1991). NMDA receptor activation leads to a stimulation of NOS activity and NO release (Garthwaite, 1991), which may clarify elevated NO concentrations (and cGMP) following administration of pentylenetetrazol. This implicates that activated excitatory NO system may contribute to the pentylenetetrazol-induced neuronal excitability. A possible

involvement of NO pathway in the action of other analeptics, for example amphetamine-like drugs, should be considered. A less prominent effect of pentylenetetrazol on brain NO levels, compared to the opposite effect of pentobarbital is probably due to the use of anaesthetized animals in this study. Anaesthetics may block the glutaminergic transmission (Alimoff and Miller, 1993). Thus, they may indirectly attenuate the pentylenetetrazol-induced activation of NMDA receptors and corresponding NOS activity and NO release. Experiments in unanaesthetized animals may further clarify the role brain NO in the mechanism of action of centrally-acting drugs.

In *conclusion*, the results show that NO concentrations in the frontal cortex of anaesthetized rat are decreased by pentobarbital and increased by pentylenetetrazol. This suggests that brain NO levels contribute to the pharmacologically-modulated excitability of central neurons, induced by hypnotic and analeptic drugs. It further implicates that NO exerts an important effect on the neuronal activity and seizure susceptibility.

### 7.6 References

Alifimoff JK, Miller KW. Mechanism of action of general anaesthetic agents. In: *Principles and Practice of Anaesthesiology*, Rogers MC, Tinker JH, Covino BG, Longnecker DE (Eds), Mosby-Year Book, St Louis, pp 1034-1052, 1993.

Burlet S, Jouvet M, Cespuglio R. Specific voltametric detection of brain nitric oxide throughout the rat sleep-waking cycle. In: *Sleep Res*, Chase MH, Roth T, O'Connor C (Eds), Brain Inform Service, Los Angeles, 24A, p 67, 1995.

Dzoljic MR, De Vries R. Nitric oxide synthase inhibition reduces wakefulness. *Neuropharmacology* 33:1505-1509, 1994.

Dzoljic MR, De Vries R, Van Leeuwen R. Sleep and nitric oxide: effects of 7-nitro indazole, inhibitor of brain nitric oxide synthase. *Brain Res* 718:145-150, 1996.

Galindo A. Effects of procaine, pentobarbital and halothane on synaptic transmission in the central nervous system. *J Pharmacol Exp Ther* 169:185-195, 1969.

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

Guevara-Guzman R, Emson PC, Kendrick KM. Modulation of in vivo striatal transmitter release by nitric oxide and cyclic GMP. *J Neurochem* 62:807-810, 1994.

Kant GJ, Muller TW, Lenox RH, Meyerhoff JL. *In vivo* effects of pentobarbital and halothane anaesthesia on levels of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in rat brain regions and pituitary. *Biochem Pharmacol* 29: 1891-1896, 1980.

Kapàs L, Ayers NA, Kruger JM. Circadian variation in brain nitric oxide synthase activity. In: *Sleep Res*, Chase MH, Roth T, O'Connor C (Eds), Brain Inform Service, Los Angeles, 24A, p106, 1995.

Maggio R, Fumagalli F, Donati E, Barbier P, Racagni G, Corsini GU, Riva M. Inhibition of nitric oxide synthase dramatically potentiates seizures induced by kainic acid and pilocarpine in rats. *Brain Res* 679:184-187, 1995.

Malinski T, Bailey F, Zhang ZG, Chopp M. Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *J Cereb Blood Flow Metab* 13:355-358, 1993.

Moncada S, Palmer RMJ, Higgs EN. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rew* 43:109-142, 1991.

Montague PR, Gancayco CD, Winn MJ, Marchase RB, Friedlander MJ. Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. *Science* 263:973-977, **1994.**

Osonoe K, Mori N, Suzuki K, Osonoe M. Antiepileptic effects of inhibitors of nitric oxide synthase examined in pentylenetetrazol-induced seizures in rats. *Brain Res* 663:338-340, **1994.**

Starr MS, Starr BS. Paradoxical facilitation of pilocarpine-induced seizures in the mouse by MK-801 and the nitric oxide synthase inhibitor L-NAME. *Pharmacol Biochem Behav* 45:321-325, **1993.**

Tobin JR, Martin LD, Breslaw MJ, Traystaman RJ. Selective anaesthetic inhibition of brain nitric oxide synthase. *Anaesthesiology* 81:1264-1269, **1994.**

Van Leeuwen R, De Vries R, Dzoljic MR.7-Nitro indazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, **1995.**

Williams JA, Vincent SR, Reiner RB. Nitric oxide production in rat thalamus changes with behavioral state, local depolarization and brainstem stimulation. *J Neurosci* 17:420-427, **1997.**

Zhang ZG, Chopp M, Bailey F, Malinski T. Nitric oxide changes in rat brain after transient middle cerebral occlusion. *J.Neurol Sci* 128:22-27, **1995.**

# CHAPTER 8

## GENERAL DISCUSSION

### 8.1 *NO and Seizures*

Concerning the role of NO in epilepsy, it has been reported that NOS inhibitors exert both *proconvulsant* (Starr and Starr, 1993; Penix *et al.*, 1994) and anticonvulsant (Osonoe *et al.*, 1994; Van Leewen *et al.*, 1995) effects. This study shows that relatively selective n-NOS inhibitors, 3-Br-7-NI, TRIM and S-Me-TC, attenuate pilocarpine-induced seizures in mice. Since pilocarpine-induced convulsions involve predominantly the limbic system (Turski *et al.*, 1984), this provides evidence for the involvement of NO in limbic epilepsy. The results of this study are also consistent with the finding that L-NAME reduces the severity of seizures induced by the acetylcholinesterase inhibitor, tacrine (Bagetta *et al.*, 1992). The idea of the involvement of NO in the cholinergic model of seizures is supported by data showing an excessive release of EAA in pilocarpine-induced epilepsy (Walton *et al.*, 1990). Additionally, recent studies indicate that NO might increase neuroexcitability by releasing main central excitatory neurotransmitters GLU, NA and ASP (Guevara-Guzman *et al.*, 1994; Montague *et al.*, 1994) as well as inhibiting the uptake of GLU (Lonart and Johnson, 1994; 1995) and NA (Lonart and Johnson, 1995; Miller and Hoffman, 1994). Our results are in accordance with these studies, since a decrease of NO synthesis by NOS inhibitors may reduce brain levels of excitatory neurotransmitters by decreasing their release and facilitating their uptake. This may explain the decrease of the neuronal excitability following administration of NOS inhibitors observed in our study. Therefore, data from these experiments suggest a neuroexcitatory effect of endogenous NO in the brain structures involved in seizure phenomena.

Furthermore, our investigation shows that NOS inhibitors delay the occurrence of convulsions, status epilepticus and mortality. This implicates a significant action of NOS inhibitors on the mechanism of onset and genesis of epilepsy, rather than on the mechanism of maintenance of seizures. In accordance with this are experiments in which L-Arg has been shown to potentiate EAA-induced seizures. Thus, De Sarro *et al.* (1993) have suggested that NO may contribute mainly to the genesis of seizure activity. Concerning the role of cyclic nucleotide levels in the brain, it is implicated that cGMP levels increased by pentylenetetrazol may play a role in seizure onset and/or propagation (Ferrendelli *et al.*, 1980). The fact that NO increases cGMP levels (Garthwaite, 1991; Bagetta *et al.*, 1993) could explain the attenuating effects of NOS inhibitors on the genesis and propagation of seizures.

Our results are in agreement with data that support central anticonvulsant effects of NOS inhibitors. L-NAME antagonizes pentylenetetrazol-induced kindling (Becker *et al.*, 1995) and seizures induced by various excitatory drugs: kainic acid (De Sarro *et al.*, 1991), quinolinic acid (Nakamura *et al.*, 1995), NMDA (De Sarro *et al.*, 1991), pentylenetetrazol (Osonoe *et al.*, 1994), cocaine (Przewlocka *et al.*, 1994) and picrotoxin (Kirkby *et al.*, 1996). L-NOARG protects rats against oxygen- (Zhang *et al.*, 1993) and pentylenetetrazol-induced convulsions (Osonoe *et al.*, 1994). 7-NI attenuates kainate-elicited convulsions in rats (Mülsch *et al.*, 1994) or pilocarpine- (Van Leeuwen *et al.*, 1995) and picrotoxin- (Kirkby *et al.*, 1996) induced seizures in mice.

Although the effect of NOS inhibitors on seizures is predominantly of central origin, the peripheral component should also be taken into account. Recent results have shown that n-NOS is well expressed in the sarcolemma of skeletal muscle (Nakane *et al.*, 1993), while

NOS inhibitors augment contractile force (Kobzik *et al.*, 1994). Therefore, it remains a possibility that motor expression of seizures following administration of NOS inhibitors could be additionally modified by inhibition of sarcolemmal n-NOS. The participation of the sarcolemmal n-NOS in motor seizure phenomena has not been studied yet and it deserves serious consideration.

### *8.2 NO and Locomotion*

Recent data suggest that NO plays an important role in motor activity. The L-Arg-derived NOS-inhibitor, L-NAME, reduces spontaneous locomotor activity (Sandi *et al.*, 1995) and hyperlocomotion induced by cocaine (Pudiak and Bozard, 1993), morphine (Calignano *et al.*, 1993), substance P (Mancuso *et al.*, 1994) and methampetamine (Ohno *et al.*, 1995).

Reduced locomotion following administration of NOS inhibitors observed in this study is in accordance with the decrease in the EEG rhythmic slow activity (RSA=theta rhythm, 6-9-Hz; Dzoljic *et al.*, 1996) as well as the prominent reduction of EEG power in the high theta frequency (7-9 Hz; Chapter 6). The RSA in the rat is associated with locomotion and other voluntary movements (Depoortere, 1987). Therefore, the prominent decrease of power of RSA, following administration of NOS inhibitors might reflect reduced neuroexcitability of brain structures involved in the central control of locomotor activity.

The mode of action of NOS inhibitors in locomotion is not known, but the effect of NO on central neurotransmitters, particularly on DA in basal ganglia could be of importance. Striatal dopaminergic transmission is involved in the control of locomotion (Angulio and McEwen, 1994) and NOS is widely distributed in neurons of the striatum (Snyder and Bredt, 1991). It has been shown that NO induces DA release from striatal (Zhu and Luo, 1992) and hippocampal slices (Lonart *et al.*, 1992). The idea that NOS inhibitors decrease locomotor activity by reducing dopaminergic transmission is consistent with the fact that these drugs reduce hypermotility induced by dopamine $D_1$ and $D_2$ receptor agonists (Starr and Starr, 1995). There is evidence of the involvement of GLU in the control of locomotion (Witkin, 1993; Angulio and McEwen, 1994), probably through the dopaminergic system. Both NOS inhibitors (Montague *et al.*, 1994) and haemoglobin, which binds extracellular NO, reduce the release of striatal DA induced by GLU receptor agonist NMDA (Hanbauer *et al.*, 1992). Other conventional neurotransmitters (GABA, 5-HT, NA and Ach) are implicated in the regulation of locomotor activity (Angulio and McEwen, 1994; Mancuso *et al.*, 1994), while NO stimulates the release of these neurotransmitters (Prast and Phillippu, 1992; Guevara-Guzman *et al.*, 1994; Mancuso *et al.*, 1994). Evidently, a reduction in the locomotor activity induced by NOS inhibitors is a complex phenomenon based on the derangement of various neurotransmitter systems involved in the central control of locomotion.

Additionally, NO may further influence locomotor activity by modifying anxiety. However, these results are controversial. Microinjection of NOS inhibitors into the dorsal central grey has been reported to exert an anxiolytic effect (Guimaraes *et al.*, 1994). In contrast, L-NOARG can reduce exploratory activity increased by the anxiolytic compound chlordiazepoxide (Quock and Nguyen, 1992). From our experiments, there is no evidence that the new NOS inhibitors may affect anxiety.

Taken together, the results of our investigations implicate that brain NOS activity and the corresponding NO release are important regulators of spontaneous locomotor activity.

However, the contribution of the blockade of sarcolemmal n-NOS (Kobzik *et al.*, 1994) in the reduction of locomotor activity following NOS inhibitors should be further examined.

### 8.3 *NO and Vigilance*

Results from previous studies showed that mild sedation caused by NOS inhibitors (L-NMMA and 7-NI) might facilitate sleep, while prominent central depression could lead to disruption of sleep pattern (Dzoljic and De Vries, 1994; Dzoljic *et al.*, 1996). In order to further evaluate the role of NO in the CNS, we examined the effects of new, potent and relatively selective n-NOS inhibitors on the EEG power spectrum, general behaviour and sleep/waking pattern during the dark and light periods.

Data from our experiments show that EEG power was suppressed in each frequency range by NOS inhibitors. Although a decrease in EEG power was observed during desynchronization and behavioural excitation in animals treated with cocaine or d-amphetamine (Ferger *et al.*, 1994), the generalised reduction in EEG power associated with depressed behaviour (loss of righting reflex and ptosis; Dzoljic *et al.*, 1996 and decreased locomotion; Chapter 5) is rather a reflection of CNS depression. Depression of neuronal activity in various brain regions leads to reduced hippocampal and cortical inputs, which in turn may result in decreased theta activity and EEG power, respectively. Additionally, the qualitative changes in EEG power induced by indazole-derived NOS inhibitors used this study are similar to the effects of high doses of alcohol (Ehlers *et al.*, 1992) or benzodiazepines (Glatt *et al.*, 1983) in rats. Accordingly, a prominent decrease of central neuronal activity leads to the reduction of EEG power, disruption of normal sleep architecture (Chapter 6) and decreased locomotor activity (Chapter 5). The central depression induced by NOS inhibitors is consistent with the anticonvulsant activity of 7-NI (Van Leeuwen *et al.*, 1995) and other NOS inhibitors used in this study (Chapter 4).

In addition, the reduction of EEG power by 7-NI is less prominent during the dark phase (active period of rat) than during the light phase (sleeping period). Likewise, it has been reported that NOS activity in the rat brain is higher during the dark phase compared to the light phase (Kapás *et al.*, 1995). The idea of circadian dependence of NOS activity is further supported by the recent observation that the expression of n-NOS in the rat pineal gland is regulated by environmental lighting condition (Spessert *et al.* 1995). The present results indicate that the excitatory effects of NO on central neuronal structures involved in the regulation of vigilance are circadian dependent.

### 8.4 *NO and Centrally-Acting Drugs*

In this study (Chapters 4, 5 and 6), it is suggested that the decrease in brain neuronal activity may be associated with reduced NO levels, while an increase of NO concentrations could be expected during neuroexcitation. In order to provide data for this hypothesis, we measured brain NO levels during pharmacologically-induced central depression and excitation. Our results show that hypnotic and excitatory drugs affected brain NO concentrations in an opposite way; pentobarbital decreased, while pentylenetetrazol increased cortical brain NO levels. The mechanism of interactions between these drugs and NO is not clear, but some possibilities could be considered.

It is known that barbiturates inhibit the release of EAA, GLU and ASP (Alifimof and Miller 1993), reduce the sensitivity of brain neurons to GLU (Galindo, 1968) and decrease

cGMP formation in many brain regions (Kant *et al.*, 1980). Furthermore, the corresponding reduction of brain NO concentrations leads to decreased neuronal excitability (Moncada *et al.*, 1991; Montague *et al.*, 1994; Mülsch *et al.*, 1994). Evidently, this is an additional factor which may contribute (together with barbiturate-induced activation of the GABA-inhibitory system) to the mechanism of hypnotic action of pentobarbital and probably other sedatives/hypnotics and anticonvulsants.

Related to pentylenetetrazol, it has been shown that its proconvulsant effect is associated with activation of NMDA receptors and increase in cGMP levels in the brain (Moncada *et al.*, 1991). Activated NMDA receptors stimulate NOS activity and NO release (Garthwaite, 1991), which may clarify an elevated NO (and cGMP) concentration following administration of pentylenetetrazol. This implicates that the activated excitatory NO system may contribute to the pentylenetetrazol-induced increase of neuroexcitability. Likewise, it was reported that in the kainate elicited convulsions NO formation was increased 6-12 fold in the rat brain (Mülsch *et al.*, 1994). A possible involvement of the NO pathway in the action of other analeptics, for example amphetamine-like drugs, should be considered. Further experiments in conscious animals may more precisely clarify changes in brain NO levels following drug administration.

The fact that NO concentrations in the frontal cortex of the anaesthetized rat were decreased by pentobarbital and increased by pentylenetetrazol suggests that brain NO levels contribute to the pharmacologically-modulated excitability of central neurons. This further implicates a possible decrease of NO concentrations in the human brain following hypnotic or antiepileptic therapy and a corresponding increase in the central NO levels during epileptic attacks or administration of excitatory amphetamine-like drugs.

It is concluded that central NO is an important endogenous neuroexcitatory transmitter involved in seizure susceptibility, locomotion, vigilance stages and activity of some centrally-acting drugs, such as hypnotics and analeptics. Accordingly, interventions in the NO system may offer a new therapeutic approach for the treatment of brain disorders associated with disturbed neuronal excitability.

### 8.5 Implications for Further Research

The main ideas resulting from our experiments are:

- The possible role of NO in skeletal muscles as a modulator of motor seizure phenomena and locomotion should be examined. These experiments could be performed by using more selective NOS inhibitors, which will undoubtedly become available in time. Such compounds may reduce sarcolemmal n-NOS activity and corresponding NO synthesis in skeletal muscles, without affecting those within the CNS.

- New and selective n-NOS inhibitors should be tested as potential antiepileptic drugs and as modulators of vigilance stages in experiments accompanied with EEG recording.

- Experiments performed in conscious animals could better clarify the qualitative aspects of interactions between centrally acting drugs and brain NO concentrations. Knowledge of interactions between NO and different drugs in various models of altered neuronal excitability may contribute to a better understanding of the role of this endogenous gas in

the human brain, particularly during central depression, seizure phenomena and vigilance stages.

## 8.6 References


Alifimof JK, Miller KW. Mechanism of action of general anaesthetic agents. In: *Principles and Practice of Anaesthesiology*, Ed: Rogers MC, Tinker JH, Covino BG, Longnecker DE, Mosby-Year Book, St Louis, pp.1034-1052, 1993.

Angulo JA, McEwen BS. Molecular aspects of neuropeptide regulation and function in corpus striatum and nucleus accumbens. *Brain Res Rev* 19:1-28, 1994.

Bagetta G, Massoud R, Rodino P, Federici G, Nisticò G. Systemic administration of lithium chloride and tacrine increases nitric oxide synthase activity in the hippocampus of rats. *Er J Pharmacol* 237:61-64, 1993.

Becker A, Gecksch G, Scröder H. N-omega-nitro-L-arginine methyl ester interferes with pentylenetetrazol-induced kindling and has no effect on changes in glutamate binding. *Brain Res* 688:230-232, 1995.

Calignano A, Oersico P, Mancuso F, Sorrentino L. Endogenous nitric oxide modulates morphyne-induced changes in locomotion and food intake in mice. *Eur J Pharmacol* 231:415-419, 1993.

Depoortere H. Neocortical rhythmic slow activity during wakefulness and paradoxical sleep in rats. *Neuropsychobiology* 18:160-168, 1987.

De Sarro GB, Di Paola ED, De Sarro A, Vidal MJ. Role of nitric oxide in the genesis of excitatory amino acid-induced seizures from the deep prepiriform cortex. *Fundam Clin Pharmacol* 5:503-511, 1991.

De Sarro GB, Di Paola ED, De Sarro A, Vidal MJ. L-arginine potentiates excitatory amino acid-induced seizures elicited in the deep prepiriform cortex. *Eur J Pharmacol* 12:151-158, 1993.

Dzoljic MR, De Vries R. Nitric oxide synthase inhibition reduces wakefulness. *Neuropharmacology* 33:1505-1509, 1994.

Dzoljic MR, De Vries R, Van Leeuwen. Sleep and nitric oxide: effects of 7-nitro indazole, inhibitor of brain nitric oxide synthase. *Brain Res* 718:145-150, 1996.

Ehlers CL, Kaneko WM, Wall TL, Chaplin RI. Effects of dizocipline (MK-801) and ethanol on the EEG and related potentials (ERPS) in rats. *Neuropharmacol* 31:369-378, 1992.

Ferger B, Krof W, Kuschinsky K. Studies on electroencephalogram (EEG) in rats suggest that moderate doses of cocaine or d-amphetamine activate D1 rather than D2 receptors. *Psychopharmacol* 114:297-308, 1994.

Ferrendelli JA, Blank AC, Gross RA. Relationship between seizure activity and cyclic nucleotide levels in the brain. *Brain Res* 200:93-103, 1980.

Galindo A. Effects of procaine, pentobarbital and halothane on synaptic transmission in the central nervous system. *J Pharmacol Exp Ther* 169:185-195, 1969.

Garthwaite J. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neuro Sci* 14:60-67, 1991.

Glatt A, Duerst T, Mueller B, Demiville H. EEG evaluation of drug effects in the rat. *Neuropsychobiol* 9:163-166, 1983.

Guevara-Guzman R, Emson PC, Kendrick KM. Modulation of in vivo striatal transmitter release by nitric oxide and cyclic GMP. *J Neurochem* 462:807-810, 1994.

Guimaraes FS, de Aguiar JC, Del Bel EA, Ballejo G. Anxiolytic effect of nitric oxide synthase inhibitors microinjected into the dorsal central grey. *Neuro Rep* 5:1929-1932, 1994.

Hanbauer I, Wink D, Osawa Y, Edelman GM, Gally JA. Role of nitric oxide in NMDA-evoked release of [3H]-dopamine from striatal slices. *Neuro Rep* 3:409-412, 1992.

Kant GJ, Muller TW, Lenox RH, Meyerhoff JL. in vivo effects of pentobarbital and halothane anaesthesia on levels of adenosine 3'5'-monophosphate and guanosine 5'5'-monophosphate in rat brain regions and pituitary. *Biochem Pharmacol* 29:1891-1896, **1980.**

Kapàs L, Fang J, Krueger JM. Inhibition of nitric oxide synthesis inhibits rat sleep. *Brain Res* 664:189-196, **1994.**

Kirkby RD, Carroll DM, Grossman AB, Subramaniam S. Factors determining proconvulsant and anticonvulsant effects of inhibitors of nitric oxide synthase in rodents. *Epilepsy Res* 24:91-100, **1996.**

Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature* 372:546-548, **1994.**

Lonart G, Wang J, Johnson KM. Nitric oxide induces neurotransmitter release from hippocampal slices. *Eur J Pharmacol* 220:271-272, **1992.**

Lonart G, Johnson KM. Inhibitory effects of nitric oxide on the uptake of [$^3$H]dopamine and [$^3$H]glutamate by striatal synaptosomes. *J Neurochem* 63:2108-2117, **1994.**

Lonart G, Johnson KM. Characterization of nitric oxide generator-induced hippocampal [$^3$H]-norepinephrine release. II The role of calcium, reverse norepinephrine transport and cyclic 3',5'-guanosine, monophosphate. *J Pharmacol Exp Ther* 275:14-22, **1995.**

Mancuso F, Calignano A, Sorrentino L. Endogenous nitric oxide modulates behavioural effects elicited by substance P in rat. *Eur J Pharmacol* 271:329-333, **1994.**

Miller KJ, Hoffman BJ. Adenosine $A_3$ receptors regulate serotonin transport via nitric oxide and cGMP. *J Biol Chem* 269:27351-27356, **1994.**

Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109-142, **1991.**

Montague PR, Gancayco CD, Winn MJ, Marchase RB, Friedlander MJ. Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. *Science* 263:973-977, **1994.**

Mülsch A, Busse R, Mordvintcev PI, Vanin AF, Nielsen EO, Scheel-Krüger J, Olesen SP. Nitric oxide promotes seizure activity in kainate-treated rats. *Neuro Rep* 5:2325-2328, **1994.**

Nakamura TA, Yamada K, Hasegawa T, Nabeshima T. Possible involvement of nitric oxide in quinolinic acid-induced convulsions in mice. *Pharmacol Biochem Behav* 51:309-312, **1995.**

Nakane M, Schmidt HH, Pollock JS, Forsterman U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* 316:175-180, **1993.**

Ohno M, Watanabe S. Nitric oxide synthase inhibitors block behavioral sensitization to methamphetamine in mice. *Eur J Pharmacol* 275:39-44, **1995.**

Osonoe K, Mori N, Suzuki K, Osonoe M. Antiepileptic effects of inhibitors of nitric oxide synthase examined in pentylenetetrazol-induced seizure in rats. *Brain Res* 663:338-340, **1994.**

Penix LP, Davis W, Subramaniam S. Inhibition of NO synthase increase the severity of kainic acid-induced seizures in rodents. *Epilepsy Res* 18:177-184, **1994.**

Prast H, Philippu A. Nitric Oxide releases acetylcholine in the basal forebrain. *Eur J Pharmacol* 216:139-140, **1992.**

Pudiak CM, Bozarth MA. L-NAME and MK-801 attenuate sensitization to the locomotor-stimulating effect of cocaine. *Life Sci* 51:255-260, **1993.**

Quock RM, Nguyen E. Possible involvement of nitric oxide in chlordiazepoxide-induced anxiolysis in mice. *Life Sci* 51:255-260, **1992.**

Sandi C, Venero C, Guaza C. Decreased spontaneous motor activity and startle response in nitric oxide synthase inhibitor-treated rats. *Eur J Pharmacol* 277:89-97, **1995.**

Spesseret R, Layes E, Schollmayer A, Reuss S, Vollrath L. In the pineal gland, but not in the suprahiasmatic nucleus, the amount of constitutive neuronal nitric oxide synthase is regulated by environmental lighting conditions. *Biochem Biophys Res Commun* 212:70-76, **1995.**

Starr MS, Starr BS. Paradoxical facilitation of pilocarpine-induced seizures in the mouse by MK-801 and the nitric oxide synthesis inhibitor L-NAME. *Pharmacol Biochem Behav* 45:321-325, **1993.**

Starr MS, Starr BS. Do NMDA receptor-mediated changes in motor behaviour involve nitric oxide? *Eur J Pharmacol* 272:211-217, **1995.**

Turski WA, Cavalheiro EA, Bortolotto ZA, Mello LM, Scwarz M, Turski L. Seizures produced by pilocarpine in mice: A behavioral, electroencephalographic and morphological analysis. *Brain Res* 321:237-253, **1984.**

Van Leeuwen R, De Vries R, Dzoljic MR. 7-nitro indazole, an inhibitor of neuronal nitric oxide synthase, attenuates pilocarpine-induced seizures. *Eur J Pharmacol* 287:211-213, **1995.**

Walton NY, Gunawan S, Treiman DM. Brain amino acid concentration changes during status epilepticus induced by lithium and pilocarpine. *Exp Neurol* 108:61-70, **1990.**

Witkin JM. Blockade of the locomotor stimulant effects of cocaine and methamphetamine by glutamate antagonists. *Life Sci* 53:PL405-410, **1993.**

Zhang ZG, Su Y, Oury TD, Piantadosi CA. Cerebral amino acid, norepinephrine and nitric oxide metabolism in CNS oxygen toxicity. *Brain Res* 606:56-62, **1993.**

# CHAPTER 9

## SUMMARY

This thesis deals with effects of relatively selective n-NOS inhibitors on neuronal and behavioural processes in animal models. NOS inhibitors were used as tools for elucidation of the actions of NO in the CNS. A part of this experimental work is also addressed to the measurement of brain NO concentrations during pharmacologically-induced central excitation and depression.

In order to give a more complete picture of this remarkable messenger molecule, the biosynthesis, mechanism of action of NO, distribution of NOS as well as involvement of NO in major physiological and pathophysiological processes and its importance in clinical medicine are discussed in **Chapters 1-3** of this thesis.

Comparison of previous results dealing with NO is hampered because the NOS inhibitors used in earlier studies were rather unselective. In this investigation, we examined the effects of new, potent and relatively selective n-NOS inhibitors: 7-nitro indazole (7-NI), 3-bromo 7-nitro indazole (3-Br-7-NI), 1-(2-trifluoromethylphenyl)imidazole (TRIM) and S-methyl-L-thiocitrulline (S-Me-TC) in seizure phenomena, locomotion, vigilance stages and neuronal activity.

**Chapter 4.** In order to clarify the role of NO in *seizure phenomena,* we examined effects of 3-Br-7-NI, TRIM and S-Me-TC on seizure threshold. The results show that 3-Br-7-NI and TRIM decreased the frequency of status epilepticus and mortality, while TRIM, in addition, significantly reduced the incidence of seizures. The latencies to onset of seizures, status epilepticus and mortality were significantly prolonged by all three NOS inhibitors, while the duration of seizures was reduced by 3-Br-7-NI and TRIM. Concerning differences between NOS inhibitors, several possibilities, such as distinct selectivity for NOS isoenzymes, corresponding changes in cerebral blood flow and modulation of negative feedback of NO on NMDA, were discussed. It is suggested that the main action of S-Me-TC and, probably, of the other two NOS inhibitors is on the mechanism of onset and genesis of epilepsy, rather than on the mechanism of maintenance of seizures.

We concluded that pilocarpine-induced seizures in mice can be attenuated by NOS inhibitors, 3-Br-7-NI, TRIM and, to a lesser extent, S-Me-TC. These data implicate that NO acts as a central endogenously active agent with proconvulsant properties in the motor limbic structures in mice. However, possible involvement of peripheral NO in the modulation of motor seizure phenomena has not been excluded.

**Chapter 5** deals with our attempts to elucidate the role of NO in *locomotion.* In these experiments we also used non-L-Arg-derived and relatively selective n-NOS inhibitors: 7-NI, 3-Br-7-NI, TRIM and S-Me-TC. Our results showed that 7-NI, 3-Br-7-NI, TRIM and S-Me-TC decreased spontaneous locomotor activity in mice. Animals treated with NOS inhibitors looked alert but less active, spending more time sitting in the cage than control mice treated with vehicle. The impression of quiescence and bradykinesia, following NOS

inhibitors, corresponded with decreased locomotor activity. Reduced locomotion following administration of NOS inhibitors observed in these experiments is in agreement with the decrease in the EEG rhythmic slow activity (RSA-theta rhythm, 6-9 Hz) as well as the decrease in EEG power in high theta frequencies (7-9 Hz; Chapter 6). Since the RSA in the rat is associated with locomotion, the prominent decrease in power of RSA, following administration of NOS inhibitors, might reflect a depression of central neuronal structures involved in locomotion.

The mode of action of NOS inhibitors on locomotor activity is still not known. However, we emphasized the possibility that NO can influence neurotransmitters implicated in the regulation of locomotion. Although a contribution of reduced sarcolemmal n-NOS activity in locomotion remains possible, we have concluded that the activity of central NOS and corresponding NO release in the brain are important for spontaneous locomotor activity.

In **Chapter 6**, we described the effects of relatively selective n-NOS inhibitors, 7-NI, 3-Br-7-NI and S-Me-TC on the neuronal activity and vigilance stages. The major finding of these experiments was that NOS inhibitors, 7-NI and 3-Br-7-NI (but not S-Me-TC) decreased EEG power in rats. In addition, 3-Br-7-NI reduced sleep stages. A non-sleep period following administration of indazole-derived NOS inhibitors (7-NI and 3-Br-7-NI) was characterized by an arousal-like EEG/EMG pattern (low EEG with high EMG amplitudes) and reduced behavioural activity (decreased locomotion and occasional loss of righting reflex). Evidently, indazole-derived NOS inhibitors decreased sleep but did not increase wakefulness. Thus, the arousal-like EEG/EMG pattern was not associated with awake behaviour, suggesting a dissociation between the EEG/EMG pattern and behaviour.

EEG power was suppressed in each frequency range by NOS inhibitors, 7-NI and 3-Br-7-NI. Decrease of high theta rhythm is consistent with reduced locomotion, observed in experiments following administration of NOS inhibitors (Chapter 5). The generalised reduction of EEG power associated with depressed behaviour (loss of righting reflex and ptosis) and decreased locomotion (Chapter 5) are a reflection of central depression. Accordingly, a prominent central depression leads to the reduction of locomotor activity and disruption of normal sleep architecture. The central depression induced by indazol-derived NOS inhibitors is consistent with the anticonvulsant activity of NOS inhibitors observed in this study (Chapter 4).

It is of interest to note that the reduction of EEG power by 7-NI was less prominent during the dark phase (active period of the rat) than during the light phase (sleeping period). This is an additional argument that brain NO exerts an excitatory and circadian-dependent effect on neuronal structures involved in the regulation of vigilance.

In **Chapter 7** data related to brain NO levels during pharmacologically induced central depression and excitation, using an electrochemical measurement of NO are given. By using an electrochemical measurement of NO levels in the frontal cortex of the anaesthetized rat, we found that hypnotic and excitatory drugs affected brain NO levels in the opposite way.

The hypnotic drug phenobarbital decreased, while the convulsant drug pentylenetetrazol increased brain NO concentrations. Thus, our results implicate that central depression could be associated with decreased NO levels, while increased NO concentrations could be expected during central excitation. Although the mechanism of interaction between these drugs and NO is not clear, some possibilities were discussed.

The fact that NO concentrations in the frontal cortex of the anaesthetized rat are decreased by pentobarbital and increased by pentylenetetrazol provides further evidence that NO levels in the brain are related to neuronal excitability. It is concluded that NO plays an important role in the regulation of neuronal activity, seizure susceptibility and vigilance stages.

# SAMENVATTING

In dit proefschrift zijn de effecten van relatief selectieve neurale-stikstofoxyde synthese (n-NOS) remmers in neurale- en gedragsprocessen in diermodellen onderzocht. NOS remmers worden gebruikt als een methode voor opheldering van stikstofoxyde (nitric oxide - NO) activiteit in het centraal zenuwstelsel. Daarnaast zijn in een gedeelte van dit experimentele werk de NO concentraties gemeten tijdens farmacologisch geïnduceerde centrale excitatie en depressie.

Om een compleet beeld te geven van dit merkwaardige signaalmolecuul worden de biosynthese, het werkingsmechanisme van NO, de distributie van NOS en de betrokkenheid van NO in de voornaamste fysiologische en pathofysiologische processen, evenals de toepassing als klinisch medicijn en de therapeutische mogelijkheden besproken in de **hoofdstukken 1-3** van dit proefschrift.

Een vergelijking met eerder gedane onderzoeken met betrekking tot NO is moeilijk, daar vaak de gebruikte NOS remmers niet selectief bleken te zijn. Daarom is het onderzoek verricht met nieuwe, effectieve en relatief selectieve n-NOS remmers: 7-nitro indazol (7-NI), 3-bromo-7-nitro indazol (3-Br-7NI), 1-(2-trifuormethylfenyl)imidazol (TRIM) en S-methyl-L-thiocitrulline (S-Me-TC), met betrekking tot toevallen en locomotie, waakzaamheid en neurale activiteit.

**Hoofdstuk 4.** Om de rol van NO bij *epileptische aanvallen* op te helderen hebben we de effecten van 3-BR-7-NI, TRIM en S-Me-TC op de aanvalsdrempel onderzocht.

De resultaten van deze studie geven aan dat 3-Br-7-NI en TRIM de frequentie van status epilepticus en de mortaliteit verlagen, terwijl TRIM tevens de frequentie van de aanvallen verlaagt. De latentietijd voor toevallen, status epilepticus en mortaliteit werd significant verlengd door alle drie NOS remmers, terwijl door 3-Br-7-NI en TRIM ook de duur van de aanvallen werd verminderd. Ten aanzien van de verschillen tussen NOS remmers werden een aantal mogelijke verklaringen besproken, zoals selectiviteit voor NOS iso-enzymen, corresponderende verandering in cerebrale bloeddoorstroming en modulatie van de negatieve 'feedback' van NO op N-methyl-D-aspartaat (NMDA). Gesuggereerd wordt dat S-Me-TC, en mogelijk ook de andere twee NOS remmers, vooral een rol spelen bij het initiëren van een epileptische aanval en niet zo zeer bij het onderhouden van een aanval.

Hieruit concluderen wij dat pilocarpine-geïnduceerde toevallen in muizen kunnen worden verzwakt door NOS remmers, 3-Br-7-NI, TRIM en in mindere mate door S-Me-TC. De resultaten impliceren dat NO werkt als een centraal endogeen actieve stof met proconvulsieve eigenschappen in de motor-limbische structuren in de muis. Mogelijke betrokkenheid van perifeer NO in de modulatie van aanvallen is echter niet uitgesloten.

In **hoofdstuk 5** wordt de rol van NO in *locomotie* behandeld. In deze experimenten hebben we ook non-arginine derivaten en relatief selectieve n-NOS remmers gebruikt: 7-NI, 3-Br-7-NI en S-Me-TC.

Onze resultaten geven aan dat 7-NI, 3-Br-7-NI, TRIM en S-Me-TC een verlaging geven van spontane locomotie in muizen. Dieren behandeld met NOS remmers zijn alert, maar

minder actief en brengen meer tijd zittend in hun kooi door, dan muizen behandeld met vehicle. De indruk van rust en bradykinesie door NOS remmers correspondeerde met een verminderde locomotie. De verminderde locomotie na toediening van NOS remmers, is in overeenstemming met de verlaging van de EEG activiteit (RSA-theta rythm, 6-9 Hz), en met de verlaging van de EEG activiteit in de hoge theta frequentie 7-9 Hz (Hoofdstuk 6). De RSA in de rat hangt samen met locomotie en andere willekeuring bewegingen. De prominente verlaging in sterkte van RSA, na toediening van NOS remmers, kan duiden op een depressie van structuren in het centrale zenuwstelsel, welke betrokken zijn bij bewegingen.

Het werkingsmechanisme van NOS remmers in bewegingsactiviteiten is nog niet bekend. We benadrukken echter de mogelijkheid dat NO neurotransmitters kan beïnvloeden, die betrokken zijn bij de regulatie van locomotie. Ondanks de nog steeds aanwezige mogelijkheid van gereduceerde sarcolemma n-NOS activiteit in locomotie, hebben we geconcludeerd dat de activiteit van centraal NOS en de bijbehorende NO afgifte in de hersenen een belangrijke rol spelen in spontane locomotorische activiteit.

In **hoofdstuk 6** beschrijven we de effecten van relatief selectieve n-NOS remmers, 7-NI, 3-Br-7-NI en S-Me-TC op de *neurale activiteit en slaap-waak stadia.*

Het belangrijkste resultaat van deze experimenten was dat de NOS remmers, 7-NI en 3-Br-7-NI (maar niet S-Me-TC) de EEG activiteit in ratten verlagen. Daarnaast reduceert 3-Br-7-NI de slaap stadia. Na toediening van een indazol-derivaat NOS remmer volgde een niet-slaap periode (7-NI en 3-Br-7-NI), welke gekenmerkt werd door op excitatie gelijkende EEG/EMG patronen (lage EEG- en hoge EMG amplituden) en verminderde gedragsactiviteit (verminderde locomotie en af en toe het verliezen van de oprichtingsreflex). Klaarblijkelijk verminderen indazol-afgeleide NOS remmers de slaap, maar verhogen de wakkere toestand niet. Een op excitatie lijkend EEG/EMG patroon is dus niet gekoppeld aan wakker gedrag, hetgeen een dissociatie tussen het EEG/EMG patroon en het gedrag suggereert.

De EEG activiteit in elk frequentiegebied wordt onderdrukt door de NOS remmers 7-NI en 3-Br-7-NI. De daling van het hoge theta ritme komt overeen met de verminderde locomotie, zoals gezien na toediening van NOS-remmers (hoofdstuk 5). De algemene reductie van de EEG activiteit, geassocieerd met onderdrukt gedrag (verlies van de oprichtingsreflex en uitzakking van het bovenste ooglid) en verminderde locomotie (hoofdstuk 5) zijn kenmerken van centrale depressie. Vergelijkbaar leidt een prominente centrale depressie tot de reductie van bewegingsactiviteit en verstoring van normale slaapactiviteit. De centrale depressie geïnduceerd door indazol-afgeleide NOS remmers is in overeenstemming met de anticonvulsieve activiteit van de NOS remmers welke gebruikt zijn in deze studie (hoofdstuk 4).

Het is van belang te vermelden dat de reductie van de EEG activiteit minder duidelijk was tijdens de donker fase (actieve periode van de rat) dan tijdens de licht fase (slaap periode). Dit vormt een aanvullende bevestiging van de bevinding dat NO een neuro-excitatoire stof is, welke betrokken is bij de regulatie van de slaap/waak stadia.

In **hoofdstuk 7** zijn de resultaten weergegeven van de *NO concentraties* in de hersenen tijdens farmacologisch geïnduceerde centrale depressie en excitatie, gebruik makend van electrochemische metingen van NO.

Door gebruik te maken van electrochemische metingen van NO in de frontale cortex van een verdoofde rat werd gevonden dat hypnotische stoffen en excitatoire stoffen een tegenovergesteld effect op NO concentraties in de hersenen hadden. De hypnotische stof pentobarbital verlaagt, terwijl de convulsante stof pentyleentetrazol de NO concentraties in de hersenen verhoogt. Deze resultaten impliceren dat centrale depressie kan worden geassocieerd met de afname van de NO concentratie, terwijl een toename van de hoeveelheid NO kan worden verwacht tijdens centrale prikkeling. Hoewel het mechanisme van de interactie tussen deze drugs en NO niet opgehelderd is, worden er een aantal mogelijkheden besproken.

Het feit dat de NO concentratie in de frontale cortex van verdoofde ratten verlaagd wordt door pentobarbital en verhoogd wordt door pentyleentetrazol, maakt het aannemelijk dat de NO concentratie in de hersenen gerelateerd is aan de neurale prikkelbaarheid. Geconcludeerd kan worden dat NO een belangrijke rol speelt in de regulatie van neurale activiteit, toevalsgevoeligheid en slaap/waak stadia.

# ACKNOWLEDGEMENTS

Many people have helped me during the course of the investigations described in this thesis and I would like to express my gratitude:

- to Dr. M.R. Dzoljic and Prof. Dr. P.R. Saxena for their guidance and critical remarks,
- to the members of the "promotiecommissie", Prof. Dr. H. Collewijn, Prof. Dr. J. Voogd and Prof. Dr. A.M.L. Coenen, for evaluating the thesis,
- to Dr. R.G. Schoemaker, Dr. F.J. Zijlstra, Dr. H.S. Sharma, Dr. A.H.J. Danser for their useful suggestions,
- to René de Vries for his efficient assistance in the experimental work.
- to Jeanette van Dijk, Corné Tak, Ingrid Garrelds, Caroline van Heijningen, Toos van Kesteren, Antoinette Maassen van den Brink, Peter de Vries, Jan Heiligers, Sue McKay and other members of the Department of Pharmacology for their collegial and collaborative attitude.

I must thank the foundations and companies sponsoring this thesis: Dr Saal van Zwanenbergstichting, Vunizol, Janssen-Cilag, Glaxo-Wellcome and the Erasmus University Rotterdam.

I also wish to thank to Prof. Z.M. Levic and Prof. V.S. Kostic of the Institute of Neurology, Medical Faculty, University of Belgrade, Yugoslavia, for their support during this scientific project.

My special thanks are for my friends, relatives and family for their understanding and love.

# CURRICULUM VITAE AND LIST OF PUBLICATIONS

## CURRICULUM VITAE

- Born in Belgrade (Yugoslavia), August 9, 1959.

- After grammar-school (1978) studied medicine at the *Medical Faculty, University of Belgrade* (1978-1984).

- As a student participated in the summer hospital practice in *Denmark* 1983 (Copenhagen, surgery, head: Prof. T. Drazewiecki; Aarhus, surgery, Prof. Y. Kill) and in *The Netherlands* 1984 (Rotterdam, internal medicine, head: Prof. J.C. Birkenhäger).

- During the study was involved in *several research projects.* The results were presented on several scientific meetings (Congress of Students of Medicine and Stomatology of Yugoslavia, 1983-1984; Congress of Serbian Anti-Cancer Society, 1985; Meeting of Medical Youth Organisation, 1985).

- State Examination for Physicians passed in 1985.

- *Master's degree in neuropharmacology,* at University of Belgrade in 1988. The corresponding experimental work for master's degree entitled: "The influence of enkephalinase inhibitors on the central effects of endogenous opioids", guided by Prof. V.M. Varagic, was done at the Department of Pharmacology at Medical Faculty in Belgrade (head: Prof. M. Krstic) and at the Department of Pharmacology, Faculty of Medicine and Health Sciences, Erasmus University, Rotterdam (head: Prof. I.L. Bonta).

- After working a few years as a physician at University Hospitals in Belgrade (1985-1988) started with specialization in neuropsychiatry (Department of Neuropsychiatry, Medical Faculty, University of Belgrade, head: Prof. Z.M. Levic) and became a *specialist neuropsychiatrist* in 1991.

- *Awarded* with scholarship of Belgrade University (1980) and scholarship of Serbian Academy of Sciences and Arts (1985).

- Employed in the *Institute of Neurology, Medical Faculty, University of Belgrade, Yugoslavia.*

# LIST OF PUBLICATIONS

**Full Papers**

**Dzoljic E**, Varagic VM. Effects of bestatin and phosphoramidon on the hypertensive response to physostigmine in the rat. *Fundam Clin Pharmacol* 1:307-316, **1987.**

**Dzoljic E**, Kaplan CD, Dzoljic MR. Effects of ibogaine on naloxone precipitated withdrawal syndrome in chronic morphine-dependent rats. *Arch In Pharmacodyn* 294:64-70, **1988.**

**Dzoljic E**, Dzoljic MR. Modification of morphine withdrawal in rats by alteration of endogenous opioid system: effects of actinonin and GEMSA. *Drug Develop Res* 18:255-261, **1989.**

Dzoljic MR, Korenhof AM, van der Ent M, **Dzoljic E**, Rupreht J. Central neurotransmitters and opioid withdrawal behaviour: possible clinical consequences. *Yugoslav Physiol Pharmacol Acta* 3:255-259, **1989.**

Dzoljic MR, **Dzoljic E**, van der Ent M. New possibilities for treatment of opiate dependence. *Yugoslav Physiol Pharmacol* Acta 25:31-32, **1989.**

Varagic VM, Stojanovic V, **Dzoljic E**, Prostran M. Blood pressure response to physostigmine as a means of studying the influence of opioids on the central regulation of blood pressure. *Eur J Pharmacol* 183:169-170, **1990.**

Dzoljic MR, **Dzoljic E**, van der Ent M, Ukponmwan OE, Cappendijk SLT, Kaplan CD, van Kappelan WA, Saxena PR. Putative 5-$HT_1$ receptor agonist and morphine withdrawal syndrome - relationship with locomotor activity. *Res Comm Substance Abuse* 13:61-78, **1992.**

**Dzoljic E**, Dzoljic MR. Effects of 5-$HT_1$ agonists on opiate withdrawal syndrome in mice. *Yugoslav Physiol Pharmacol* Acta 31:31-35, **1995.**

**Dzoljic E**, de Vries R, Dzoljic MR. Anticonvulsant activity of new and potent inhibitors of nitric oxide synthase. *Brain Res Bull* 43:191-195, **1997.**

**Dzoljic E**, van Leeuwen R, de Vries R, Dzoljic MR. Vigilance and EEG power in rats: effects of potent inhibitors of the neuronal nitric oxide synthase. *Naunyn-Schmiedebergs Arch Pharmacol* 356:56-61, **1997.**

**Dzoljic E**, de Vries R, Dzoljic MR. New and potent inhibitors of nitric oxide synthase reduce motor activity in mice. *Behav Brain Res* 87:209-212, **1997.**

Brzakovic B, Pokrajac M, **Dzoljic E**, Levic Z, Varagic VM. Cerebrospinal fluid and plasma pharmacokinetics of phenobarbital after intravenous administration to patients with status epilepticus. *Clinical Pharmacokinetics* 14:307-313, **1997.**

Brzakovic B, Pokrajac M, **Dzoljic E**, Levic Z, Varagic V. Urinary excretion of phenobarbital and its metabolite p-hydroxyphenobarbital in convulsing and nonconvulsing patients. *Eur J Drug Metab Pharmacokinetics,* in press.

**Dzoljic E**, de Vries R, Dzoljic MR. Brain nitric oxide levels: different effects of hypnotic and analeptic drugs. Submitted.

**Books**

**Dzoljic E**, de Vries, R, Dzoljic MR. Effects of new and potent nitric oxide synthase inhibitors on sleep/waking stages and EEG power spectrum in the rat. In: *Sleep-Wake Research in The Netherlands,* Beersma DGM (Ed), Elinkwijk, Utrecht, Vol 7, pp. 53-56, **1996.**

Dzoljic M, de Vries R, **Dzoljic E.** An involvement of endogenous nitric oxide (NO) in the mechanism of action of hypnotic and analeptic drugs. Submitted.

Dzoljic DA, **Dzoljic E.** Food, health and cancer. Dzoljic AD, Dzoljic AZ, Dzoljic DA (Eds), Beograd, **1988.**

**Abstracts**

**i. International Congresses**

**Dzoljic E**, Varagic V. The effects of bestatin and phosphoramidon on the hypertensive response to physostigmine in the rat. *Catecholamines and Blood Pressure, Jerusalem, Israel,* **1987.**

**Dzoljic E.** Central Effects of endogenous opioids. *International Pharmacological Association, Amsterdam, The Netherlands,* **1990.** *Eur J Pharmacol* 183:2315, **1990.**

Brzakovic B, Pokrajac M, **Dzoljic E**, Levic Z, Varagic V. Excretion of phenobarbital and p-hydroxyphenobarbital in 24-hour urine of convulsing and nonconvulsing patients. *Second Xenobiotic Metabolism and Toxicity Workshop of Balkan Countries. Ioannina, Greece,* **1995.**

Brzakovic B, Pokrajac M, **Dzoljic E**, Levic Z, Varagic V. Phenobarbital plasma concentrations in convulsing and nonconvulsing patients. *Second European Congress of Epileptology, The Hague, The Netherlands,* **1996.** *Epilepsia* 37:60, **1996.**

**ii. National Congresses/Symposia/Meetings**

**Dzoljic E.** Comparative evaluation of three methods of malignant melanoma treatment. *25th Yugoslav Congress of Students of Medicine and Stomatology,* Plitvice, **1983.**

Tomin R, Radovanovic J, Pralica J, **Dzoljic E.** Controlled prospective randomized study of chemotherapy and immunotherapy in adjuvant treatment of skin malignant melanoma. *6th Yugoslav Congress of Cancerologists*, Skoplje, **1983.**

**Dzoljic E.** Medical complications of alcoholism in women. *26th Yugoslav Congress of Students of Medicine and Stomatology,* Crikvenica, **1984.**

**Dzoljic E.** Application of DTIC in adjuvant therapy of skin malignant melanoma. *26th Yugoslav Congress of Students of Medicine and Stomatology*, Crikvenica, **1984.**

Tomin R, Pralica J, **Dzoljic E**, Putnik M, Jankovic A, Jocovic M. Multiple malignant skin melanoma. *Aspirations and Innovations in Medicine,* Belgrade, **1986.**

**Dzoljic E**, Varagic V. The effects of enkephalinase inhibitors on the hypertensive response to physostigmine in rat. *Meeting of Serbian Pharmacological Society, and Session of the Scientific Council of the Institute for Medical Research,* Belgrade, **1987.**

Varagic V, **Dzoljic E.** The effects of enkephalinase inhibitors on the hypertensive response to physostigmine in rat. *Aspirations and Innovations in Medicine*, Belgrade, **1987.**

Varagic V, Stojanovic V, **Dzoljic E.** The effects of enkephalinase and enkephalinase inhibitors on the central cholinergic mechanism participating in the peripheral adrenergic activation. *Peptide and Amino Acid Transport Mechanism in the Central Nervous System,* Belgrade, **1987.**

**Dzoljic E,** Dzoljic MR. Ibogaine attenuates some of the opiate withdrawal symptoms. *14th Congress of Yugoslav Pharmacological and Physiological Society,* Neum, **1988.**

**Dzoljic E,** Dzoljic MR. The effects of endogenous opioid peptides on the opiate withdrawal syndrome. *Aspirations and Innovations in Medicine,* Belgrade, **1989.**

**Dzoljic E,** Bogdanovic A, Antonijevic N. Lesion of the abducens as the first manifestation of multiple myeloma. *3rd Congress of Yugoslav Neurologists and 1st Congress of Yugoslav Association of Neuroscience,* Belgrade, **1993.**

**Dzoljic E,** Jankovic S, Stojsavljevic N, Drulovic J, Sokic D, Levic Z. Unusual clinical manifestation of vitamin $B_{12}$ deficiency. *3rd Congress of Yugoslav Neurologists and 1st Congress of Yugoslav Association of Neuroscience,* Belgrade, **1993.**

**Dzoljic E,** Jankovic S, Trikic R, Levic Z. Early onset of cerebellar ataxia with preserved muscular reflexes and dominant autosomal heredity. *3rd Congress of Yugoslav Neurologists and 1st Congress of Yugoslav Association of Neuroscience,* Belgrade, **1993.**

**Dzoljic E,** Varagic V, Dzoljic MR, Levic Z. Central effects of endogenous opioid peptides. *3rd Congress of Yugoslav Neurologists and 1st Congress of Yugoslav Association of Neuroscience,* Belgrade, **1993.**

Sokic D, **Dzoljic E,** Mojsilovic B, Pokrajac M, Levic Z. Respiratory function, state of consciousness and toxicity in intravenous application of phenobarbitone. *3rd Congress of Yugoslav Neurologists and 1st Congress of Yugoslav Association of Neuroscience,* Belgrade, **1993.**

Levic Z, Micic J, Drulovic J, Sokic D, Stojsavljevic N, **Dzoljic E,** Jankovic S. Influence of short lasting application of high doses of methylprednisolone on the hypothalamo-hypophyseal-adrenal axis in patients having multiple sclerosis. *3rd Congress of Yugoslav Neurologists and 1st Congress of Yugoslav Association of Neuroscience,* Belgrade, **1993.**

**Dzoljic E,** Petkovic-Medved B, Ribaric-Jankes K. The role of electronistagmography in localization of lesions of sixth cranial nerve. *5th Yugoslav Congress of Clinical Neurophysiology and Electroencephalography, 14th Yugoslav Symposium of Epilepsy,* Belgrade, **1993.**

**Dzoljic E,** Dzoljic MR. 5-$HT_1$-agonists and opioid withdrawal syndrome. *5th Yugoslav Congress of Clinical Neurophysiology and Electroencephalography, 14th Yugoslav Symposium of Epilepsy,* Belgrade, **1993.**

Jovanovic Z, Icanovic H, Vidakovic Z, **Dzoljic E.** Neurophysiological examination in patients with migraine. *Symposium of Yugoslav Headache Society,* Novi Sad, **1994.**

Jovanovic Z, Icanovic H, Vidakovic Z, **Dzoljic E.** Symptomatic headache: etiology and pathogenesis. *Symposium of Yugoslav Headache Society,* Novi Sad, **1994.**

**Dzoljic E,** Dzamic Z, Ribaric-Jankes K. Smooth pursuit test as a neurological diagnostic procedure. *2nd Congress of Yugoslav Association of Neuroscience,* Milocer, **1995.**

**Dzoljic E,** de Vries R, Dzoljic MR. Anticonvulsant effect of new and potent inhibitors of neuronal nitric oxide synthase. *11th Yugoslav Congress of Pharmacology,* Igalo, **1996.**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ach | acetylcholine |
| ACE | angiotensin-converting enzyme |
| ADMA | asymmetric $N^G$-dimethylarginine |
| L-Arg | L-arginine |
| ASP | aspartate |
| BF | basal forebrain |
| 3-Br-7-NI | 3-bromo 7-nitro indazole |
| CBF | cerebral blood flow |
| CNS | central nervous system |
| cGMP | cyclic guanosine 3',5' monophosphate |
| CVR | cerebro-vascular resistance |
| DA | dopamine |
| DMSO | dimethyl sulfoxide |
| EAA | excitatory amino acid |
| EEG | electroencephalogram |
| EMG | electromyogram |
| GABA | gama-aminobutyric acid |
| GLU | glutamate |
| H4B | tetrahydrobiopterin |
| 5-HT | 5-hydroxytryptamine, serotonin |
| L-Arg | L-arginine |
| LPS | lipopolysaharide |
| LTP | long term potentiation |
| LTD | long term depression |
| NA | noradrenaline |
| NANC | non adrenergic non cholinergic |
| NADPH-d | nicotine amide dinucleotide phosphate diaphorase |
| 7-NI | 7-nitro indazole |
| L-NMMA | L-$N^G$-mono-methyl-L-arginine |
| NMDA | N-methyl-D-aspartate |
| NO | nitric oxide |
| L-NOARG | $N^G$-nitro-L-arginine |
| NOS | nitric oxide synthase |
| c-NOS | constitutive nitric oxide synthase |
| e-NOS | endothelial nitric oxide synthase |
| i-NOS | inducible nitric oxide synthase |
| n-NOS | neuronal nitric oxide synthase |
| REM | rapid eye movement |
| RSA | rhythmic slow activity |
| PNS | peripheral nervous system |
| TRIM | 1-(2-trifluoromethylphenyl)imidazole |
| S-Me-TC | S-methyl-L-thiocitrulline |
| SWS | slow wave sleep |