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# Endogenous Nitric Oxide Synthesis: Biological Functions and Pathophysiology

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Modern molecular biology has revealed vast numbers of large and complex proteins and genes that regulate body function. By contrast, discoveries over the past ten years indicate that crucial features of neuronal communication, blood vessel modulation and immune response are mediated by a remarkably simple chemical, nitric oxide (NO). Endogenous NO is generated from arginine by a family of three distinct calmodulin-dependent NO synthase (NOS) enzymes. NOS from endothelial cells (eNOS) and neurons (nNOS) are both constitutively expressed enzymes, whose activities are stimulated by increases in intracellular calcium. Immune functions for NO are mediated by a calcium-independent inducible NOS (iNOS). Expression of iNOS protein requires transcriptional activation, which is mediated by specific combinations of cytokines. All three NOS use NADPH as an electron donor and employ five enzyme cofactors to catalyze a five-electron oxidation of arginine to NO with stoichiometric formation of citrulline. The highest levels of NO throughout the body are found in neurons, where NO functions as a unique messenger molecule. In the autonomic nervous system NO functions as a major non-adrenergic non-cholinergic (NANC) neurotransmitter. This NANC pathway plays a particularly important role in producing relaxation of smooth muscle in the cerebral circulation and the gastrointestinal, urogenital and respiratory tracts. Dysregulation of NOS activity in autonomic nerves plays a major role in diverse

pathophysiological conditions including migraine headache, hypertrophic pyloric stenosis and male impotence. In the brain, NO functions as a neuro-modulator and appears to mediate aspects of learning and memory.

Although endogenous NO was originally appreciated as a mediator of smooth muscle relaxation, NO also plays a major role in skeletal muscle. Physiologically, muscle-derived NO regulates skeletal muscle contractility and exercise-induced glucose uptake. nNOS occurs at the plasma membrane of skeletal muscle which facilitates diffusion of NO to the vasculature to regulate muscle perfusion. nNOS protein occurs in the dystrophin complex in skeletal muscle and NO may therefore participate in the pathophysiology of muscular dystrophy.

NO signalling in excitable tissues requires rapid and controlled delivery of NO to specific cellular targets. This tight control of NO signalling is largely regulated at the level of NO biosynthesis. Acute control of nNOS activity is mediated by allosteric enzyme regulation, by posttranslational modification and by subcellular targeting of the enzyme. nNOS protein levels are also dynamically regulated by changes in gene transcription, and this affords long-lasting changes in tissue NO levels. While NO normally functions as a physiological neuronal mediator, excess production of NO mediates brain injury. Overactivation of glutamate receptors associated with cerebral ischemia and other excitotoxic

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processes results in massive release of NO. As a free radical, NO is inherently reactive and mediates cellular toxicity by damaging critical metabolic enzymes and by reacting with superoxide to form an even more potent oxidant, peroxynitrite. Through these mechanisms, NO appears to play a major role in the pathophysiology of stroke, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis.

## INTRODUCTION AND HISTORICAL PERSPECTIVE

Nitric oxide, whose chemical formula is NO, is a gas under ambient conditions. It is distinct from nitrous oxide, the "laughing gas" used as anesthetic and whose formula is N<sub>2</sub>O. Nitric oxide (NO) is notoriously noxious because of its free-radical structure: it possesses an extra electron, making it chemically reactive. Though NO has long been known to occur in bacteria, no one anticipated that such a reactive agent would have vital functions in mammals. Yet, discoveries over the past ten years reveal that NO is one of the major messenger molecules of biology, enabling white blood cells to kill certain pathogens, allowing neurotransmitters to dilate blood vessels and serving as a neuronal messenger molecule.

### Discovery of Endogenous NO as an Immune Mediator

NO is extraordinarily labile, with a half-life of only about 3–5 sec, after which it is converted by oxygen and water into nitrates and nitrites. While humans excrete nitrates, these were generally thought to derive from dietary sources. In 1981, Steven Tannenbaum and associates noted that humans and rats fed diets with low levels of nitrates still excreted substantial amounts of nitrates in the urine.<sup>[1–3]</sup> A clue to one source of nitrate formation came from studies showing increased levels of urinary nitrates in patients with diarrhea and fever.<sup>[4]</sup> Inflammatory processes associated with the diarrhea seemed to

be responsible for the nitrate formation. Indeed, it was noted that injections of bacterial endotoxin or lipopolysaccharide, stimulated nitrate excretion.<sup>[5]</sup>

Determining the source of the nitrate formation and its biological role in inflammatory responses derived primarily from the work of Michael Marletta and Dennis Stuehr and by John Hibbs and associates. Marletta and Stuehr noted that mice with genetically determined deficiency of macrophages had very low levels of nitrate excretion.<sup>[6]</sup> Utilizing isolated cultures of macrophages they found that the combination of lipopolysaccharide and interferon- $\gamma$  stimulated nitrate formation by the macrophages.<sup>[7,8]</sup> They also noted that nitrate formation by macrophages was abolished when arginine was removed from the incubation medium. This indicated that a specific enzyme in macrophages converts arginine into an intermediate chemical that turned out to be NO,<sup>[9]</sup> which in turn is transformed into nitrites and nitrates.

Independently, Hibbs was evaluating the ability of macrophages to kill tumor cells and bacteria. He cultured tumor cells together with macrophages and noted that the tumor killing effects of the macrophages were abolished when arginine was removed from the medium.<sup>[10]</sup> He demonstrated that arginine is converted to both nitrates and to the amino acid citrulline.<sup>[11]</sup> This work provided independent evidence that a specific enzyme of activated macrophages generates NO from arginine (Figure 1). Hibbs also identified the first inhibitor of the NO synthesizing enzyme, when he showed that certain methyl derivatives of arginine blocked the formation of nitrates as well as the tumoricidal activity of macrophages.<sup>[10]</sup>

### Discovery of NO as an Endogenous Messenger Molecule

A completely unrelated line of investigation independently led to the identification of NO as

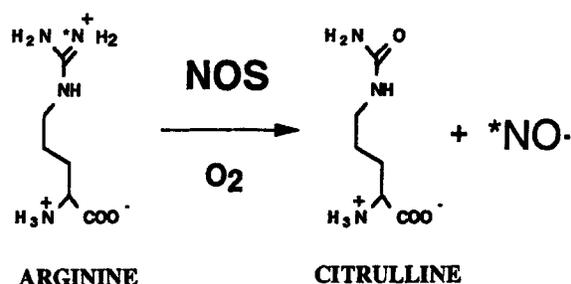


FIGURE 1 NO biosynthesis. NOS forms NO from L-arginine with a stoichiometric generation of L-citrulline. The nitrogen in NO (\*) derives from one of the two equivalents chemically equivalent guanidino-nitrogens (\*) in L-arginine. The oxygen in NO derives from molecular oxygen (O<sub>2</sub>).

a messenger molecule. There are two arms to this story, one relating to mechanisms whereby neurotransmitters dilate blood vessels and the other dealing with drugs that relieve the symptoms of angina.

#### *NO as the Endothelial Derived Relaxing Factor*

For much of this century it was assumed that acetylcholine acted directly on vascular smooth muscle cells to cause vasodilation. However, in 1980, Robert Furchgott demonstrated that acetylcholine acts upon receptors located on endothelial cells to provoke the release of a small molecule that diffuses to and relaxes the adjacent muscle.<sup>[12]</sup> Numerous investigators, including Furchgott, tried to isolate this "endothelial derived relaxing factor," also called EDRF, but were unsuccessful, because EDRF seemed to be extremely labile.<sup>[13,14]</sup> Even without knowing the chemical identity of the factor, researchers were able to show that it acted by stimulating the formation of cyclic GMP,<sup>[15]</sup> a second messenger related to the better known cyclic AMP.

Quite independently other investigators were trying to understand how nitroglycerin exerts its extraordinarily potent and dramatic alleviation of cardiac angina. Nitroglycerin is the active chemical in dynamite, invented by Alfred Nobel. Nitroglycerin's therapeutic effects in angina were sufficiently well known in the late nineteenth

century that in 1885, Nobel, who had cardiac symptoms, wrote a friend "It sounds like the irony of fate that I should be ordered by my doctor to take nitroglycerin internally." The great success of nitroglycerin resulted in numerous derivatives, the organic nitrates, which are still mainstays in anginal therapy. Insight into the molecular mechanism of action of organic nitrates came in the late 1970s, based on research of Ferid Murad and Louis Ignarro, who showed that nitroglycerin and the organic nitrates are themselves inactive, but elicit blood vessel relaxation after they are converted to an active metabolic product, NO.<sup>[16,17]</sup>

Moreover, Murad showed that NO relaxes the muscle by stimulating the formation of cyclic GMP.<sup>[15]</sup> NO augments cGMP levels by binding to iron in the heme that is part of soluble guanylyl cyclase, stimulating the formation of cGMP.<sup>[18,19]</sup>

By 1986 it was predicted that NO or some closely related derivative may account for EDRF activity.<sup>[20-22]</sup> Definitive proof that EDRF is identical to NO was provided by Salvador Moncada and his associates. Using cultured endothelial cells as a source, they monitored the release of EDRF activity, and at the same time, chemically quantitated NO release. They found that the endothelium releases sufficient NO to fully account for relaxation of adjacent muscle cells.<sup>[23]</sup> Moncada's group also found that endothelium synthesizes NO from arginine<sup>[24]</sup> with the stoichiometric formation of citrulline.<sup>[25]</sup> Besides relaxing blood vessels, endothelium-derived NO was also shown to inhibit blood clotting by preventing the aggregation of platelets.<sup>[26-29]</sup>

How important is NO as a normal regulator of blood pressure? Other substances, such as angiotensin and norepinephrine, were previously assumed to be the major determinants of blood pressure. However, physiological studies using inhibitors of NOS demonstrate a primary role for NO in regulating vascular tone. Intravenous administration of NOS inhibitors, such as monomethyl arginine, to animals<sup>[30-32]</sup> or humans<sup>[33,34]</sup> provokes a rapid and marked increase in vascular resistance. NOS inhibitors also cause a more

notable increase in blood pressure than do drugs that influence norepinephrine or angiotensin. Thus, NO may be the principal regulator of basal blood pressure.

#### *NO as a Neuronal Messenger Molecule*

The third major site of NO messenger activities is in neurons. The first hint for a neuronal role for NO dates back to 1977 when Takeo Deguchi noticed that activation of cyclic GMP formation in the brain requires a low molecular weight substance, which he subsequently identified as the amino acid arginine.<sup>[35,36]</sup> Once EDRF was shown to be NO, Moncada reasoned that arginine's role in cyclic GMP formation in the brain likely relates to NO formation.<sup>[37]</sup> Moncada directly demonstrated a NO forming enzyme in brain preparations.<sup>[37]</sup> Independently, John Garthwaite observed the formation of a short-lived substance that had the properties of NO when he stimulated cultured neurons by administering the amino acid glutamate.<sup>[38]</sup> The regulation of NOS by neurotransmitters was first examined in the cerebellum. The cerebellum contains the highest levels of cGMP in the brain with its formation stimulated by glutamate acting on N-methyl-D-aspartate (NMDA) receptors.<sup>[39,40]</sup> By monitoring the conversion of arginine to NO or to citrulline, David Bredt and Solomon Snyder demonstrated in cerebellar slices that NOS activity is enhanced 300% in response to NMDA receptor stimulation.<sup>[41]</sup> The concentration-response relationship for NOS activation was the same as for the stimulation of cGMP levels (Figure 2). The enhanced NOS activity is responsible for the increased levels of cGMP, since methyl-arginine, an inhibitor of NOS, completely prevented the stimulation of cGMP formation in brain.<sup>[41,42]</sup>

#### CHARACTERIZATION OF NO SYNTHASE

Because NO cannot be stored, released or inactivated by conventional regulatory mechanisms,

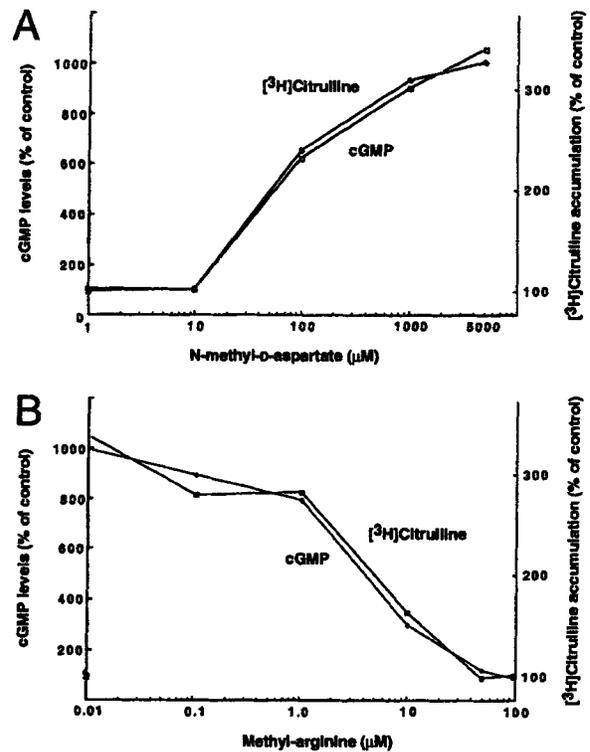


FIGURE 2 Nitric oxide mediates glutamate-linked enhancement of cGMP levels in cerebellum. (A) NMDA treatment of cerebellar slices simultaneously augments both cGMP levels and NOS activity, which is reflected by [3H]-citrulline accumulation. (B) Methyl-arginine, an NOS inhibitor blocks NMDA-stimulated cGMP formation and NOS activation cerebellar slices. All slices in (B) were stimulated with 500 μM NMDA (adapted from Bredt and Snyder<sup>[41]</sup>).

biosynthetic regulation is more important for NO than for other mediators. Indeed, the NO biosynthetic enzyme, NO synthase is one of the most regulated enzymes in biology. Initial efforts to purify the enzyme were unsuccessful because of a rapid loss of enzyme activity upon purification. Observations that calmodulin is required for NOS activity in the brain led to a simple purification of neuronal NOS (nNOS) to homogeneity.<sup>[43]</sup> Using this approach, other groups purified nNOS;<sup>[44,45]</sup> inducible NOS<sup>[46-49]</sup> (iNOS) and endothelial NOS (eNOS).<sup>[50]</sup> Cloning of the cDNA for neuronal,<sup>[51,52]</sup> endothelial,<sup>[53-55]</sup> and inducible<sup>[56-59]</sup> forms of NOS has helped elucidate NOS

function. The structure of NOS reveals numerous regulatory mechanisms.

NOS oxidizes the guanidine group of L-arginine in a process that consumes five electrons and results in the formation of NO with stoichiometric formation of L-citrulline. L-N<sup>ω</sup>-substituted arginine analogs such as L-N<sup>ω</sup>-nitro-arginine (L-NNA) and L-N<sup>ω</sup>-methyl-arginine (L-NMA), function as NOS inhibitors.<sup>[60]</sup> The inhibition of NOS by these substrate analogs can initially be reversed by simultaneous application of excess arginine consistent with their competitive blockade of the active site. However, following prolonged exposure NOS is irreversibly inhibited by some of these agents. The irreversible inactivation of the macrophage enzyme and brain enzymes by L-NMA requires simultaneous incubation with NOS cofactors suggesting "mechanism-based" inhibition.<sup>[61,62]</sup> The time-dependent inactivation of the brain enzyme by L-NNA<sup>[63]</sup> is independent of NOS enzymatic turnover.<sup>[64]</sup>

NOS isoforms display modest differences in their sensitivity to various arginine analogs. L-NNA is a more potent inhibitor of the brain and endothelial enzymes ( $K_i = 200\text{--}500\text{ nM}$ ) and L-N<sup>ω</sup>-aminoarginine (L-NAA) is a more most potent blocker of the inducible enzyme ( $K_i = 1\text{--}5\text{ }\mu\text{M}$ ). Clinically useful inhibitors of NOS will likely need to be isoform specific. Intensive work by numerous pharmaceutical companies has focused on discovery of such selective

antagonists. High throughput drug screening has yielded a highly selective nNOS antagonist<sup>[65]</sup> that appears useful for mitigating neuronal injury in an animal model of cerebral ischemia (see below).

### NO Formation

The conversion of arginine to NO is catalyzed in two independent steps (Figure 3). The first step is a two-electron oxidation of arginine to N<sup>ω</sup>-hydroxyarginine (NHA).<sup>[66]</sup> Although this hydroxylated intermediate is tightly bound to NOS, under certain conditions NHA can be isolated as a product.<sup>[64]</sup> This hydroxylation step resembles a classical P450 type monooxygenation reaction utilizing 1 equivalent of NADPH and 1 equivalent of O<sub>2</sub>.<sup>[66]</sup> The hydroxylation reaction is accelerated by H<sub>4</sub>B, requires calcium and calmodulin as activators, and is blocked by CO.<sup>[64,66,67]</sup>

The steps in the pathway from NHA to NO and citrulline are less clear. Any proposed mechanism should account for experiments which find that this oxidation (i) utilizes 0.5 equivalents of NADPH, (ii) requires O<sub>2</sub> and calcium/calmodulin, (iii) is accelerated by H<sub>4</sub>B, (iv) is inhibited by CO and arginine analogs with a pharmacology similar to that seen in the initial hydroxylation reaction.<sup>[64,66,67]</sup> In one model consistent with these data NOS would use both its reductase and heme domains for successive independent

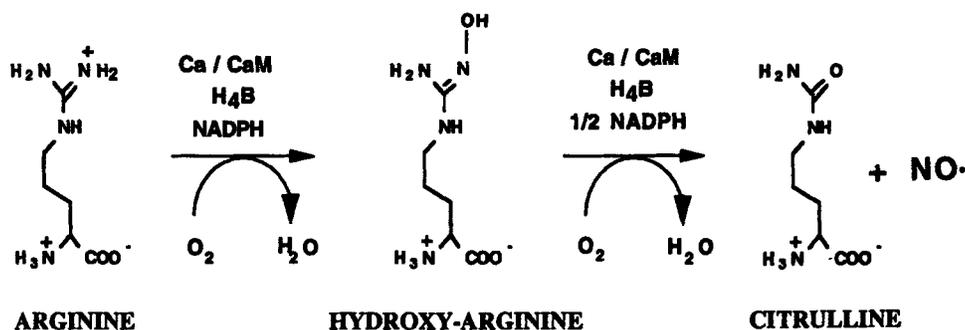


FIGURE 3 Mechanism of NO synthesis. NOS catalyzes a five-electron oxidation of a guanidine nitrogen of L-arginine to generate NO and L-citrulline. L-hydroxyarginine is formed as an intermediate that is tightly bound to the enzyme. Both steps in the reaction are dependent upon calcium and calmodulin and are enhanced by tetrahydrobiopterin.

oxidations of arginine at a common active site with heme directly functioning in the activation of molecular oxygen. For the first hydroxylation both reducing equivalents for oxygen activation derive from NADPH. It has been speculated that NHA and NADPH each provide one electron for the second oxidation step.<sup>[68]</sup> This explains both the 0.5 stoichiometry of NADPH utilization and the unusual five electron chemistry of NO biosynthesis. The crystallographic structure of dimeric iNOS oxygenase domain is consistent with this proposed mechanism. Indeed, these ligand-bound NOS crystal structures suggest that the different protonation state of arginine vs. N-hydroxyl-arginine differentiate the two chemical steps of NO synthesis.<sup>[69]</sup>

### Cloning of nNOS

Isolation of the brain isoform<sup>[43]</sup> permitted its cloning.<sup>[51]</sup> The cDNA predicts a polypeptide of 160 kD and was striking in having 36% identity to CPR in its C-terminal half, the NOS reductase domain, which contains the binding sites of NADPH, FAD and FMN (Figure 4). This homology to CPR is shared by all NOSs cloned to date and reflects the oxidative mechanism of NO

biosynthesis. The sequence of the N-terminal half of NOSs, the heme domains, is not similar to any cloned gene. Although the classic P450 heme binding cysteinyl peptide sequence is absent, the amino acids surrounding cysteine-414 showed some of the expected homology. Indeed mutagenesis and X-ray crystallographic studies have definitively demonstrated cysteine-414 of nNOS as the site for heme coordination.

The reductase domain of NOS shares many functional properties with CPR nNOS catalyzes a rapid NADPH dependent reduction of cytochrome c. In the absence of arginine NOS can transfer electrons from NADPH to O<sub>2</sub> and form O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>.<sup>[70]</sup> The formation of these reactive oxygen intermediates may contribute to glutamate neurotoxicity and neurodegeneration as discussed below. Near the middle of the nNOS cDNA there is an amphipathic alpha helix domain, which conforms to the consensus sequence for calmodulin binding.<sup>[51]</sup> This assignment was confirmed by experiments showing that peptides corresponding to this region bind calmodulin with low nanomolar affinity in a calcium-dependent manner.<sup>[71]</sup> Cloning of nNOS from human cerebellum predicts 94% acid identity with the rat protein.<sup>[52]</sup>

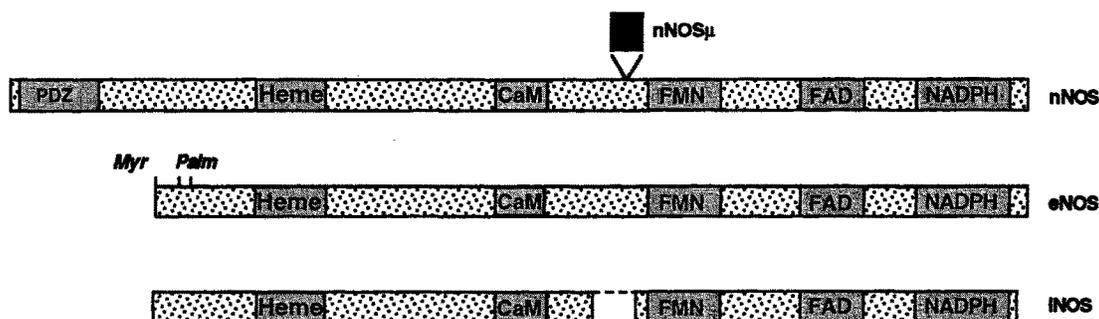


FIGURE 4 Schematic alignment of the cofactor recognition sites within NOS enzymes. Predicted sites for binding heme, calmodulin (CaM), FMN, FAD and NADPH are noted. The N-terminal PDZ domain in nNOS, and the myristoylation (Myr) and palmitoylation (Palm) sites in eNOS are marked. The unique alternatively spliced region in nNOS<sub>μ</sub> from skeletal muscle corresponds to the domain not present in iNOS.

## PHYSIOLOGICAL FUNCTIONS FOR NEURON-DERIVED NO

### Regulation of Intestinal Function

Physiological functions for neuron-derived NO were first demonstrated in the gastrointestinal tract. These studies resolved pharmacological observations that had puzzled physiologists for over twenty years. Following the development of adrenergic blocking agents in the early 1960s it became clear that certain actions of the autonomic nervous system are mediated by non-adrenergic, non-cholinergic (NANC) nerves. This NANC pathway plays a particularly important role in producing relaxation of smooth muscle in the cerebral circulation and the gastrointestinal, urogenital and respiratory tracts.<sup>[72]</sup> Parallel studies by several investigators in the late 1980s demonstrated that the NANC transmitter in several of these pathways is identical to the endothelial-derived relaxing factor (EDRF) described by Furchgott.<sup>[73,74]</sup> Molecular biological studies have helped detail the mechanisms for NO-mediated neurotransmission. In the intestine neuronal NOS (nNOS) is selectively concentrated in axon varicosities of myenteric neurons.<sup>[75]</sup> Adjacent intestinal smooth muscle cells contain an "NO receptor", the soluble guanylyl cyclase. During intestinal peristalsis, myenteric neurons fire action potentials. The resulting calcium influx activates calmodulin, which stimulates nNOS. The NO then diffuses into adjacent smooth muscle cells and augments accumulation of cGMP, which mediates intestinal relaxation. Definitive evidence that neuron-derived NO regulates intestinal motility derives from studies of genetically engineered mutant mice. nNOS knockout mice, which selectively lack the neuronal NOS isoform from conception, display a grossly enlarged stomach that histologically resembles the human disease hypertrophic pyloric stenosis.<sup>[76]</sup> Alterations in NOS may play a causal role in some newborns with this disorder, as recent genetic studies indicate

that nNOS is a susceptibility locus for infantile pyloric stenosis.<sup>[77]</sup>

### Regulation of Blood Flow

Neuron-derived NO also plays a major role in regulation of blood flow. In brain, neuronal activity is associated with an increase in local blood flow and this response is prevented by NOS inhibitors.<sup>[78]</sup> Particularly high levels of nNOS occur in vasodilator nerves that innervate the large cerebral blood vessels.<sup>[79]</sup> Abnormal reactivity of these vessels appears to mediate migraine headache as sumatriptan constricts these large vessels and controls headache.<sup>[80]</sup> Sumatriptan is also effective in treatment of nitroglycerin induced headache, suggesting a role for endogenous NO in migraine.<sup>[81]</sup> Pharmacological manipulations of nNOS may therefore offer an avenue for migraine therapy.

Therapeutic modulation of NO levels for treatment of migraine or other recurring disorders may be complicated by adaptive responses to the therapy. Control of cerebral blood flow involves complex and overlapping physiological pathways regulated by NO and a variety of other vasoactive compounds. Alterations in NO biosynthesis are often compensated by changes in the levels of other mediators. An illustrative example involves the increase in cerebral blood flow that normally occurs in response to hypercapnia. This local vascular reflex to hypercapnia is NO-dependent, as it is acutely blocked by NOS inhibitors.<sup>[78]</sup> Surprisingly, hypercapnic cerebral blood flow responses are intact in nNOS knockout mice, and NOS inhibitors do not block the response in the nNOS knockouts.<sup>[82]</sup> Therefore, the maintenance of hypercapnic blood flow response in the nNOS knockout mice is not due to upregulation of other NOS isoforms but instead is mediated by an NO-independent mechanism. Compensation by such alternative pathways appears to be a common physiological reaction to deficiencies in NO biosynthesis and has been observed in several systems.

### Regulation of Penile Erection

Through regulation of blood flow, neuron-derived NO also mediates penile erection. nNOS is enriched in neurons of the pelvic plexus and is concentrated in their axonal varicosities that ramify in the trabecular smooth muscle of the penis and about the adventitia of the penile arteries.<sup>[83]</sup> Activation of these nerves causes increased blood flow and engorgement of the erectile tissue. NOS inhibitors block penile erection in animal models *in vivo* (Table I)<sup>[84]</sup> and in strips of human cavernosal tissue *in vitro*.<sup>[85]</sup> nNOS mutant mice, however, display normal erectile function.<sup>[86]</sup> Apparently NO derived from other NOS isoforms compensates for the loss of nNOS as NOS inhibitors block penile erection in nNOS mutant mice.

Recent studies demonstrate that abnormalities in NO biosynthesis may also underlie erectile dysfunction. Diabetes mellitus is associated with impaired NOS-dependent erectile function.<sup>[87]</sup> NOS levels in penis are also decreased in aging rats, and this age-related decrease correlates with impaired erectile responses.<sup>[88]</sup> Androgens are essential for penile reflexes in the rat and are essential for normal libido. Similarly, nNOS

TABLE I Effect of NOS inhibition on penile erection in intact rats

Agent	Dose (mg/kg)	% Intracavernous pressure ( $\pm$ SEM)	n
L-Nitroarginine methyl ester	1.0	75 $\pm$ 7	3
	2.5	47 $\pm$ 5	10
	5.0	16 $\pm$ 1	4
	10.0	10 $\pm$ 10.3	3
	40.0	0	5
N-Methyl-L-arginine	10.0	63 $\pm$ 3	11
	20.0	17 $\pm$ 2	2
	40.0	15 $\pm$ 5	4
N-Methyl-D-arginine	40.0	128 $\pm$ 5	2

Penile erection was induced electrically with a Grass S48 square wave stimulator in anesthetized male rats with optimal stimulation parameters. Intracavernous pressures were measured with a 25-gauge needle inserted unilaterally at the base of the penis and connected to a pressure transducer (from Burnett *et al.*<sup>[84]</sup>).

expression in penis is dependent upon active androgens as nNOS levels decrease by 60% one week after castration and are restored to normal levels with testosterone replacement.<sup>[89]</sup> Pharmacological manipulation of NO or NOS expression may therefore offer a viable strategy for treatment for some causes of erectile dysfunction. Indeed, sildenafil (Viagra), an inhibitor of cGMP-specific phosphodiesterase type 5.<sup>[90,91]</sup> enhances male sexual function by promoting the actions of NO in relaxing corpora cavernosum smooth muscle.<sup>[92,93]</sup>

### Functions for NO in the Central Nervous System

Functions for NO in brain remain less certain. Because NO is a uniquely diffusible mediator, it was proposed on theoretical grounds that NO may mediate neuronal plasticity, which underlies aspects of both development and information storage in brain. Evidence for NO involvement in synaptic plasticity has accumulated steadily. At the cellular level, NO signalling appears to be essential for two forms of neuronal plasticity: long-term potentiation (LTP) in the hippocampus<sup>[94]</sup> and long-term depression (LTD) in the cerebellum.<sup>[95]</sup> In these cellular models repeated neuronal stimulation yields long-lasting changes in synaptic strength, and NOS inhibitors prevent these changes. Studies with NOS inhibitors have been controversial because these arginine analogs often have non-specific effects. This controversy may now be resolved by studies of NOS knockout mice. Both endothelial NOS (eNOS) and nNOS activities are found in hippocampus. Mice that lack either eNOS or nNOS have essentially normal LTP whereas mutant mice deficient in both eNOS and nNOS have substantially decreased LTP.<sup>[96]</sup>

### Regulation of Neurotransmitter Release

NO appears to mediate synaptic plasticity by potentiating neurotransmitter release. In several

model systems NOS inhibitors such as nitroarginine block the release of neurotransmitters.<sup>[97-99]</sup> In brain synaptosomes the release of neurotransmitter evoked by stimulation of NMDA receptors is blocked by nitroarginine.<sup>[97,100,101]</sup> Presumably glutamate acts at NMDA receptors on NOS terminals to stimulate the formation of NO, which diffuses to adjacent terminals to enhance neurotransmitter release so that blockade of NO formation inhibits release. In addition to regulating glutamate release, NO can also regulate secretion of hormones and neuropeptides. Regulation of hormone secretion by NO has been most convincingly demonstrated in the hypothalamus.<sup>[102]</sup> An elegant series of experiments by McCann and colleagues has shown that NO directly stimulates release of luteinizing hormone-releasing hormone (LHRH) from hypothalamic explants.<sup>[103]</sup> These findings explain physiological studies showing that NOS inhibitors block mating behavior *in vivo*, as this behavior requires LHRH release.<sup>[104]</sup> NO formation in hypothalamic explants can be stimulated by oxytocin, which also induces mating behavior in an NO-dependent manner *in vivo*.<sup>[104,105]</sup>

### Behavioral Roles for NO

Through regulation of synaptic plasticity and transmitter release, NO mediates complex influences on brain development, memory formation, and behavior. Inhibition of NOS prevents the precise targeting of retinal axons to their proper location in the optic tectum.<sup>[106]</sup> In adult animals, NOS inhibitors hinder motor learning<sup>[107]</sup> and the formation of olfactory memories.<sup>[108]</sup> NOS inhibitors also prevent the long-lasting hyperalgesia that follows tissue injury.<sup>[109]</sup> In rodent experimental models using formalin injection to the paw to induce hyperalgesia, inhibitors of NOS prevent the subsequent augmented response triggered by noxious inputs. nNOS knockout mice, however, display normal sensitization to peripheral tissue damage in this model, and NOS inhibitors do not block sensitization in the

nNOS knockouts.<sup>[110]</sup> Again, the deficiency of nNOS is compensated by an NO-independent pathway. Any potential development of nNOS inhibitors for chronic pain or other neurological disorders must be prepared to tackle this recurring phenomenon of compensation following chronic removal of NOS activity.

### ROLES FOR NO IN SKELETAL MUSCLE

Although endogenous NO was originally appreciated as a mediator of smooth muscle relaxation, more recent studies indicate a role for NO in skeletal muscle. nNOS mRNA is expressed at high levels in human skeletal muscle,<sup>[52]</sup> where nNOS mRNA is alternatively spliced yielding a muscle specific isoform, nNOS $\mu$ . nNOS $\mu$  contains an additional 102 base pair exon, which is inserted between exons 16 and 17 and encodes a 34 amino acid insert between the calmodulin- and FMN-binding domains.<sup>[111]</sup> Regulation of nNOS $\mu$  activity by calcium/calmodulin and other biochemical cofactors is indistinguishable from that of nNOS purified from brain, suggesting that the alternative splicing may instead regulate nNOS interactions with specific skeletal muscle proteins.

### Physiological Functions for NO in Skeletal Muscle

Understanding functions for nNOS in skeletal muscle has been facilitated by the discrete localization of nNOS in myofibers. In rodent muscle, nNOS is specifically enriched beneath the sarcolemma of fast-twitch muscle fibers.<sup>[112]</sup> NOS activity stimulated during muscle membrane depolarization inhibits contractile force in fast-twitch fibers. Depression of contractile force also occurs following induction of iNOS in skeletal muscle. iNOS protein is expressed in diaphragm 12 h following LPS inoculation of rat.<sup>[113]</sup> The decrease in diaphragmatic tone that accompanies sepsis is rapidly reversed by a NOS inhibitor. NO derived from skeletal muscle iNOS therefore appears to mediate a critical

component of the respiratory depression associated with sepsis.

In addition to modulating contractile force, NO also appears to regulate blood flow to skeletal muscle.<sup>[114]</sup> During exercise, blood flow rapidly increases in contracting muscles to accommodate the elevated metabolic demands of the tissue.<sup>[115]</sup> This exercise-induced hyperemia is blunted by NOS inhibitors, suggesting a role for NO in this response.<sup>[116-118]</sup> Because NOS inhibitors do not completely block exercise hyperemia, NO must play a modulatory rather than an absolute role in this response.<sup>[114]</sup>

One critical component of the vascular response to exercise is the attenuation of sympathetic vasoconstriction that occurs in contracting muscle. Because muscle contraction reflexively increases sympathetic drive to skeletal muscle, exercise hyperemia requires that vessels in contracting muscles are less responsive to the sympathetic discharge. Experiments in intact rodent models indicated that this exercise-induced sympatholysis occurs predominately in fast-twitch muscle groups and appears to be mediated by a local metabolite.<sup>[119,120]</sup> Pharmacological studies with NOS inhibitors have shown that NO is responsible for exercise-induced sympatholysis, but the cellular source of the NO has remained unclear.<sup>[121]</sup> This uncertainty is due to the fact that NOS inhibitors do not discriminate between constitutive NOS isoforms, so that the NO could derive either from endothelial NOS (eNOS) in the vasculature or from nNOS in the skeletal myofibers. To identify the source of vasoactive NO in this pathway, Thomas *et al.* have recently evaluated skeletal muscle blood flow responses in mutant mice lacking nNOS.<sup>[122]</sup> The authors found that exercise-induced hyperemia is generally preserved in the nNOS knockouts, but the nNOS knockouts specifically lack contraction-induced suppression of adrenergic vasoconstriction. These experiments definitively demonstrate that skeletal muscle-derived NO mediates sympatholysis. Also, because nNOS is enriched in fast-twitch muscle fibers,<sup>[112]</sup> these results explain

why exercise-induced sympatholysis occurs predominately in fast-twitch fibers.

### NO in Muscular Dystrophy

A striking aspect of nNOS in skeletal muscle is the cellular localization of the synthase at the plasma membrane, or sarcolemma.<sup>[112]</sup> Targeting the synthase to the sarcolemma serves at least two purposes. First, this localization dictates that membrane depolarization and subsequent calcium influx across the sarcolemma regulate calcium/calmodulin-dependent nNOS activity in skeletal muscle. Second, synthesis of NO at the muscle plasma membrane facilitates delivery of NO to surrounding cellular targets such as the vasculature.

Sarcolemmal localization of nNOS is mediated by association of the synthase with a protein complex containing dystrophin, the gene mutated in Duchenne muscular dystrophy.<sup>[123]</sup> Muscle biopsies from patients with Duchenne dystrophy evince a selective loss of nNOS enzyme protein from the sarcolemma, and a similar loss of membrane-associated nNOS occurs in muscles from *mdx* mice, which lack dystrophin (Figure 5). Molecular studies have established that nNOS does not directly bind to dystrophin; instead, nNOS associates with syntrophin, a cytosolic dystrophin binding protein.<sup>[124]</sup> Interaction of nNOS with syntrophin is mediated by PDZ protein motifs near the N-termini of nNOS and syntrophin.

The absence of nNOS in Duchenne dystrophy has been postulated to play a pathological role in the disease.<sup>[123,125]</sup> As evidence for this, nNOS is the only component of the dystrophin complex that is specifically enriched in fast-twitch muscle fibers, and these fibers preferentially degenerate in Duchenne dystrophy.<sup>[126]</sup> However, it is still uncertain how the loss of skeletal muscle nNOS might contribute to pathophysiology. To address this, Thomas *et al.* evaluated skeletal muscle blood flow responses in a mouse model of Duchenne dystrophy, the *mdx* mouse.<sup>[122]</sup> Strikingly, they

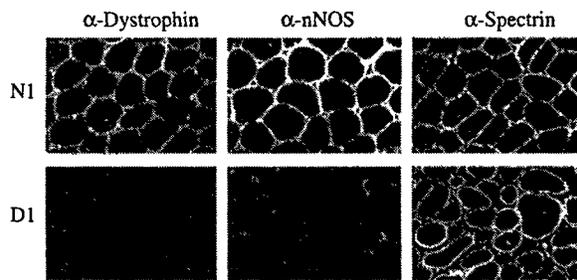


FIGURE 5 nNOS is absent from sarcolemma in Duchenne dystrophy muscle biopsies. Skeletal muscle cryosections of biopsies from normal (N1) or Duchenne muscular dystrophy (D1) skeletal muscle were immunostained with antibodies to dystrophin, nNOS and spectrin. In healthy muscle all three proteins occur at the sarcolemma or plasma membrane of skeletal muscle. The absence of dystrophin in Duchenne muscular dystrophy disrupts nNOS localization to the sarcolemma but has no influence on spectrin, which is not part of the dystrophin complex (from Brenman *et al.*<sup>[123]</sup>).

find that contraction-induced suppression of adrenergic vasoconstriction is absent in the *mdx* mice, which are identical in this regard to nNOS knockout mice. Additionally, the contraction-induced increase in cGMP and inhibition of smooth muscle myosin light chain phosphorylation are compromised in fast-twitch muscles from *mdx* mice.<sup>[127]</sup> Aside from lacking this exercise-induced sympatholytic response, blood flow in the *mdx* mice is normal, and this is similar to what is observed in nNOS knockouts.

Taken together, these studies demonstrate that NO plays a major role in regulating blood flow to exercising muscle by blunting the constrictive response to an otherwise destructive increase in sympathetic tone. Through this mechanism, NO may play a major role in muscle tolerance to heavy exercise. In this regard, it is interesting to note that nNOS protein levels in muscle dramatically increase following exercise.<sup>[128,129]</sup>

This work also suggests that an abnormal vascular response may mediate pathophysiological aspects of Duchenne muscular dystrophy. Although nNOS knockout mice do not themselves show pathological changes characteristic of muscular dystrophy,<sup>[130,131]</sup> this may reflect a

limitation of the mouse model. In humans with Becker muscular dystrophy, which is due to specific mutations of dystrophin, the absence of nNOS, but not other components of the dystrophin complex, correlates closely with the severity of the disease.<sup>[132]</sup> The absence of nNOS in Duchenne and Becker muscular dystrophies subjects the contracting muscle to hypoperfusion and ischemia due to unopposed adrenergic vasoconstriction. As fast-twitch muscle fibers are highly dependent upon nutrient delivery and are not exercise tolerant, this mechanism may help explain the preferential destruction of these fibers in the initial stages of Duchenne dystrophy.<sup>[126]</sup> Manipulating NO levels in muscle may, therefore, represent a possible strategy for treatment of muscular dystrophy.

#### CELLULAR MECHANISMS REGULATING nNOS

NO signaling in excitable tissues requires rapid and controlled delivery of NO to specific cellular targets. Other neurotransmitters are packed in secretory vesicles that are released at synaptic sites. Signal termination is mediated by enzymes and pumps that eliminate the active transmitter from the synapse. Regulation of NO signaling is complicated by the physical properties of NO that prevent storage of NO in lipid-lined vesicles or metabolism of NO by hydrolytic degradatory enzymes. In addition, excessive production of NO is toxic to neurons and other cells. NO signaling must therefore allow for rapid and localized NO production and immediate termination of biosynthesis. This tight control of NO signalling is largely regulated at the level of NO biosynthesis. Indeed, the NOS proteins are amongst the most highly regulated of all neuronal enzymes. Acute control of nNOS activity is mediated by allosteric regulation, by posttranslational modification and by subcellular targeting of the enzyme. nNOS protein levels are also dynamically regulated by changes in gene

transcription, and this affords long-lasting changes in tissue NO levels.

### Regulation of nNOS by Specific Calcium Channels

nNOS activity is primarily regulated by local increases in intracellular calcium, which stimulates nNOS through interaction with calmodulin.<sup>[43]</sup> Distinct cellular calcium influx pathways specifically regulate nNOS in various tissues. In the myenteric nervous system where NO functions as a neurotransmitter, NOS activity is primarily regulated by calcium influx through voltage-dependent calcium channels (Figure 6). Intestinal relaxation mediated by NO is suppressed by the N-type calcium channel antagonist, *w*-conotoxin.<sup>[133]</sup> Vasoactive intestinal polypeptide (VIP) also appears to play an important role in regulating NO synthesis in intestinal neurons.<sup>[134]</sup> In the brain, calcium influx through the N-methyl-D-aspartate (NMDA) type glutamate receptor potently activates nNOS in brain.<sup>[38]</sup> NMDA receptors are also known to play

a critical role in learning and memory; the intimate relationship of NMDA receptors with nNOS helps to explain the role of NO in memory consolidation.

Selective regulation of NOS activity by distinct calcium stores is mediated by targeting nNOS protein to specific intracellular regions. nNOS protein contains an N-terminal PDZ protein motif that mediates subcellular targeting of the enzyme.<sup>[123]</sup> In the brain the PDZ domain of nNOS targets the enzyme to postsynaptic sites by binding to PDZ domains in PSD-95 and PSD-93 proteins.<sup>[124]</sup> Importantly, NMDA receptors also occur at postsynaptic densities through binding to PSD-95.<sup>[135,136]</sup> PSD-95 and related proteins thereby function as molecular scaffolds and physically link nNOS to NMDA receptors (Figure 6). Internalization of peptides that antagonize the interaction of nNOS with PSD-95 block NMDA-coupled increases in NOS activity. Some nNOS protein in brain occurs outside the postsynaptic density and can be regulated by calcium influx through voltage-dependent calcium channels. Molecular mechanisms that link

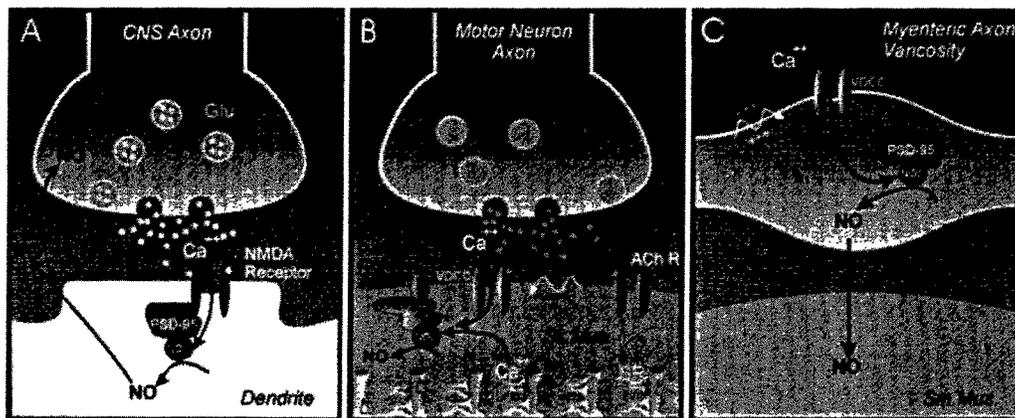


FIGURE 6 Synaptic regulation of neuronal nitric oxide synthase. Protein interactions with nNOS target the synthase to discrete sites in neurons and skeletal muscle. These interactions likely account for differential regulation of nNOS by specific calcium influx pathways. (A) Association with PSD-95 mediates coupling of nNOS to NMDA receptor activity in the CNS. (B) In skeletal muscle (Sk. Mus.) nNOS occurs at the sarcolemma owing to interaction of nNOS with the dystrophin complex. Accordingly, nNOS activity is regulated by calcium influx associated with sarcolemmal depolarization. (C) Myenteric axon varicosities contain both nNOS and PSD-95. Calcium influx through voltage-dependent calcium channels (VDCC) regulates nNOS activity in myenteric neurons and the NO relaxes the adjacent smooth muscle (Sm. Mus.) cells. Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; Glu, glutamate; SR, sarcoplasmic reticulum (adapted from Christopherson and Bredt<sup>[160]</sup>).

nNOS to these voltage-dependent channels are not yet known.

In skeletal muscle nNOS activity is linked to muscle acetylcholine receptors and also to membrane depolarization. nNOS protein in skeletal muscle occurs at the endplate and at the plasma membrane owing to association with the dystrophin glycoprotein complex.<sup>[123]</sup> Again, the nNOS protein is targeted to the cellular domain that regulates the enzyme's activity (Figure 6). Additional protein interactions appear to regulate NOS activity directly. A small molecular weight protein inhibitor of nNOS (PIN) has been described which is enriched in both brain and testes and is highly conserved between species.<sup>[137]</sup> PIN binds specifically to the neuronal isoform of NOS and acts by destabilizing the nNOS dimer, which is the active form of the enzyme.

#### ROLE OF NO IN EXCITOTOXIC PROCESSES IN BRAIN

Inappropriate induction of NOS protein in brain and other tissues clearly mediates injury in diverse disease states. In a similar way, excess stimulation of nNOS at the synapse has the potential to mediate neurotoxicity in brain. Many causes of neuronal injury, including those associated with stroke and certain neurotoxins, are due to excess release of glutamate, which acts at synaptic NMDA receptors to cause neurotoxicity.<sup>[138]</sup> Accordingly, NMDA receptor antagonists are protective in animal models of cerebral ischemia.<sup>[139]</sup> The first experimental evidence that endogenous NO mediates brain injury associated with NMDA receptor activity derived from studies in cultured neurons.<sup>[140]</sup> This work showed that inhibition of NOS attenuates glutamate toxicity in primary neuronal cultures from rat cerebral cortex. Initially this work was controversial, as subsequent studies concerning the role of NO in glutamate toxicity yielded contradictory results. These discrepancies were difficult to resolve due to the use of different neuronal

cell types and culture conditions, which can have large effects on nNOS protein levels. nNOS knockout mice have helped clarify the role of nNOS in glutamate neurotoxicity. Cultured neurons derived from these knockout mice are resistant to glutamate toxicity, establishing that NO derived from nNOS can be toxic.<sup>[141]</sup>

#### NO Toxicity in Stroke

By mediating toxicity associated with excess glutamate release, NO plays a central role in stroke and other neurodegenerative diseases *in vivo*.<sup>[142]</sup> Decisive evidence that neuron-derived NO mediates injury in stroke derives from studies of nNOS knockout mice. Compared to litter-mate controls, nNOS knockouts show similar changes in regional blood flow following focal ischemia but have 38% smaller infarcts (Figure 7).<sup>[143]</sup> In contrast, eNOS deficient mice show decreased blood flow at the periphery of the ischemic region, where NO-mediated excitotoxicity is most prevalent, and suffer an increased infarct size.<sup>[144]</sup> iNOS protein is not present in normal brain, so this isoform does not participate in the acute phase following ischemia. iNOS expression is induced, however, in reactive astrocytes and in infiltrating neutrophils following cerebral ischemia.<sup>[145]</sup> iNOS levels peak within 48 h suggesting that post-ischemic inflammation and iNOS induction may contribute to a late phase of neuronal death. Indeed, mice deficient in iNOS display decreased infarct size in models of cerebral ischemia.<sup>[145]</sup>

Because eNOS activity protects the ischemic brain by maintaining blood flow, initial pharmacological studies showed that non-specific NOS inhibitors, which block both nNOS and eNOS do not effectively protect from injury following stroke. The recent development of specific nNOS antagonists such as ARL17477, 7-nitroindazole and S-methyl-isothioureido-L-norvaline demonstrate that selective blockade of nNOS offers a useful pharmacological strategy for controlling brain injury following stroke in rodent

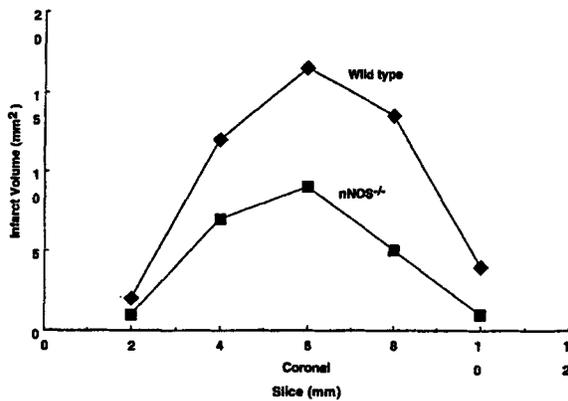


FIGURE 7 Reduced infarct volume in nNOS<sup>-/-</sup> (knock-out) mice. Wild type or NOS<sup>-/-</sup> mice were subjected to middle cerebral artery occlusion for 24 h. Infarction area for each of five coronal sections from rostral to caudal (2–10 mm) shown for wild type and mutants (adapted from Huang *et al.*<sup>[143]</sup>).

models.<sup>[145]</sup> Protective actions by NOS inhibitors are clearly mediated by antagonism of nNOS, as non-specific NOS blockers paradoxically increase infarct volume in nNOS knockouts due to inhibition of eNOS.

### Mechanisms for NO Toxicity

The molecular targets mediating NO toxicity are not yet certain. Unlike the NO pathways that regulate intestinal motility and hippocampal LTP, cGMP is almost certainly not involved in toxicity. Instead, NO neurotoxicity is likely mediated by its free radical character, which makes NO reactive with certain proteins containing heme-iron prosthetic groups, iron-sulfur clusters or reactive thiols.<sup>[146]</sup> Cellular energy depletion is a hallmark of neuronal cell death associated with ischemic injury and NO can attenuate oxidative phosphorylation by inhibiting mitochondrial iron-sulfur cluster enzymes including NADH-ubiquinone oxidoreductase and succinate-coenzyme Q: oxidoreductase. NO can also inhibit glycolysis by reactions with aconitase<sup>[147]</sup> and by competing with oxygen at cytochrome oxidase.<sup>[148]</sup>

In addition to directly reacting with protein prosthetic groups, NO also reacts readily with

superoxide ( $O_2^{\bullet-}$ ) to produce peroxynitrite ( $ONOO^-$ ) (Figure 8), which may mediate much of the NO neurotoxicity.<sup>[149]</sup> Peroxynitrite is a powerful oxidant, but is sufficiently stable to diffuse through a cell to react with a target. Peroxynitrite is particularly efficient at oxidizing iron-sulfur clusters, zinc-fingers, and protein thiols, and these reactions would contribute to cellular energy depletion. Peroxynitrite will also react with superoxide dismutase (SOD) and this combination catalyzes the 3-nitration of protein tyrosine residues, particularly those in cytoskeletal proteins. The accumulation of 3-nitrotyrosine-containing proteins, detected with antisera to 3-nitrotyrosine, is a convenient marker of peroxynitrite formation.<sup>[149]</sup>

Direct evidence that NO and  $O_2^{\bullet-}$  conspire in neuronal toxicity derives from studies of transgenic animals. Cu/Zn SOD is a cytosolic scavenging enzyme that removes reactive  $O_2^{\bullet-}$  and prevents formation of peroxynitrite. Overexpression of Cu/Zn SOD in transgenic mice reduces the infarct volume in the middle cerebral artery (MCA) occlusion model of focal ischemia compared to wild-type mice.<sup>[150]</sup> Cu/Zn SOD overexpressing mice have now been bred with nNOS knockout mice and the resulting double transgenics acquire even less ischemic damage than either single-transgenic parental strain.<sup>[151]</sup>

### NO in Neurodegenerative Diseases

While NO clearly participates in neuronal injury following vascular stroke, the role of NO in human neurodegenerative disease is not as easily understood. The slow progression of these diseases perhaps occurring over fifty years or more, complicates experimental approaches to modeling their pathophysiological mechanism. Histopathological evidence, however, suggests that certain neurodegenerative diseases including Huntington's disease, may also be mediated by NO and glutamate toxicities. NADPH-diaphorase positive neurons in the corpus striatum, which are the NOS neurons, are selectively spared in

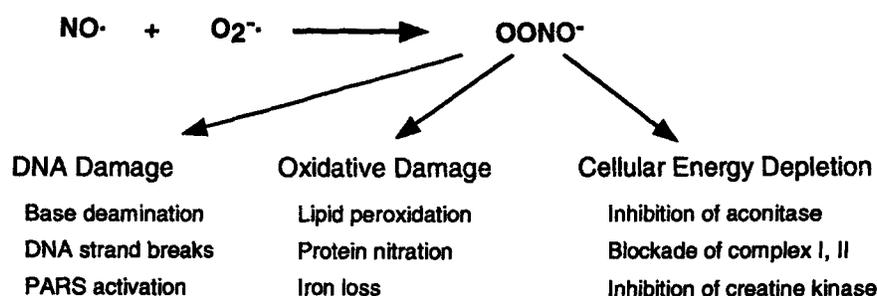


FIGURE 8 Mechanisms for NO neurotoxicity. NO reacts rapidly with superoxide ( $\text{O}_2^{\cdot-}$ ) to form a peroxynitrite ( $\text{OONO}^-$ ), which is a potent oxidant. NO and peroxynitrite mediate cytotoxicity by causing oxidative damage, DNA damage and by depleting cellular energy stores.

Huntington's disease.<sup>[152]</sup> This selective pathology can be replicated in striatal culture models and *in vivo* following lesions with NMDA but not other classes of glutamate agonists.<sup>[153]</sup> The spared NADPH diaphorase neurons are uniquely endowed with high levels of SOD, which may protect the cells from peroxynitrite-mediated NO neurotoxicity.<sup>[154]</sup> Indeed, 3-nitrotyrosine, the footprint of peroxynitrite, is detected in striatal neurons in animals models of Huntington's disease.<sup>[155]</sup>

While stigmata of NO toxicity correlate with Huntington's disease pathology, a more causal role for NO and peroxynitrite toxicity has been established in some forms of Parkinson's disease. MPTP, which contaminated batches of illicit drugs in the 1970s, produces Parkinsonian-like symptoms in humans. MPTP causes pathology by targeting the destruction of nigrostriatal dopaminergic neurons, the same cells that are selectively lost in idiopathic Parkinson's disease. Treatment of experimental animals, including mice and primates, with MPTP replicates this selective toxicity and results in accumulation of 3-nitrotyrosine in the nigrostriatal pathway. Inhibition of NOS prevents both the neurotoxicity of MPTP and the associated formation of 3-nitrotyrosine.<sup>[156]</sup> Definitive evidence that NO and peroxynitrite mediate toxicity in the MPTP model of Parkinson's disease again derives from studies of transgenic mice.<sup>[157]</sup> Both nNOS knockouts and

mice that overexpress Cu/Zn SOD are resistant to MPTP toxicity.

While nNOS inhibitors can prevent acute toxicity associated with MPTP, it remains less clear whether long-term treatment would be therapeutic for slowly developing neurodegenerative disorders. Chronic animal models for these diseases will first need to be established, and then the role of NO can then be rigorously evaluated. The recent identification of a Parkinson's disease gene,<sup>[158]</sup> and the development of a transgenic animal model for Huntington's disease,<sup>[159]</sup> suggest that it will not be long until these issues are decisively addressed.

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