# Nitric Oxide in Excitable Tissues: Physiological Roles and Disease

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*Introduction*

The discovery of nitric oxide (NO)[1] as a uniquely diffusible and reactive molecular messenger in the vascular and immune systems motivated searches for NO biosynthesis throughout the body. NO was soon found in abundance in the central and peripheral nervous systems (1–3). Indeed, NO synthase (NOS), the enzyme that produces NO from L-arginine, occurs at higher levels in brain than in any other tissue, which facilitated the initial isolation of an NOS protein and cloning of an NOS cDNA (4). Intensive studies over the past 10 yr have determined that NO mediates diverse physiological functions associated with neurons. In the peripheral nervous system, NO acts much like a classical neurotransmitter in regulating gastrointestinal motility, regional blood flow, and neuroendocrine function. In the brain, NO acts as a neuromodulator to control behavioral activity, influence memory formation, and intensify responses to painful stimuli. Furthermore, NO biosynthesis in excitable tissues is not restricted to neurons. Recent studies have identified skeletal muscle as a major source for NO in the body (5, 6) where NO regulates both metabolism and muscle contractility.

NO biosynthesis in excitable tissues is regulated by increases in intracellular calcium, which activate NOS through the enzyme's dependence upon calmodulin (7). Although small amounts of NO synthesized during neural and skeletal muscle activity mediate physiological functions, excess NO production can mediate tissue injury. For example, large amounts of NO produced during periods of cerebral ischemia mediate neuronal injury in various forms of stroke (8). Similar NO-mediated damage may account for neurodegeneration in other conditions as well, including Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease. NO signaling is also perturbed in various muscle diseases, particularly in Duchenne muscular dystrophy, and these derangements may contribute to the disease processes (9). Therefore, pharmacological regulation of NO synthesis offers an important strategy for treatment of neurodegenerative and muscle diseases.

*Physiological functions for NO in excitable tissues*

*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 more than 20 yr. After the development of adrenergic blocking agents in the early 1960s it became clear that certain actions of the autonomic nervous system are mediated by nonadrenergic, noncholinergic (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. 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 described by Furchgott.

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. Adjacent intestinal smooth muscle cells contain an "NO receptor," the soluble guanylyl cyclase. During intestinal peristalsis, myenteric neurons fire action potentials, and the resulting calcium influx activates calmodulin, which in turn 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 nNOS isoform from conception, display a grossly enlarged stomach that histologically resembles the human disease hypertrophic pyloric stenosis (10). 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 (11).

*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 (12). Particularly high levels of nNOS occur in vasodilator nerves that innervate the large cerebral blood vessels (13). Abnormal reactivity of these vessels appears to mediate migraine headache, as sumatriptan constricts these large vessels and controls headache (14). Sumatriptan is also effective in treatment of nitroglycerin-induced headache, suggesting a role for endogenous NO in migraine. Therefore, pharmacological manipulations of nNOS may offer an avenue for migraine therapy.

Therapeutic modulation of NO levels for treatment of migraine or other recurring disorders may be complicated by

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*Received for publication 17 October 1997.*

1. *Abbreviations used in this paper:* eNOS, endothelial NOS; iNOS, inducible form of NOS; LTP, long-term potentiation; NANC, nonadrenergic, noncholinergic; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase.

J. Clin. Invest.

0021-9738/97/11/2424/06 $2.00
Volume 100, Number 10, November 1997, 2424–2429
http://www.jci.org

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, and alterations in NO biosynthesis are often compensated by changes in the levels of other mediators. One 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 (12). Surprisingly, hypercapnic cerebral blood flow responses are intact in nNOS knockout mice, and NOS inhibitors do not block the response in the nNOS knockout mice (15). 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.

Neuron-derived NO also mediates penile erection through regulation of blood flow. nNOS is enriched in neurons of the pelvic plexus and NOS inhibitors block penile erection in animal models in vivo (16) and in strips of human cavernosal tissue in vitro (17). However, nNOS mutant mice display normal erectile function (18). 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 (19). NOS levels in penis are also decreased in aging rats, and this age-related decrease correlates with impaired erectile responses (20). Androgens are essential for penile reflexes in the rat and are also essential for normal libido. Similarly, nNOS expression in penis is dependent upon active androgens as nNOS levels decrease by 60% 1 wk after castration but are restored to normal levels with testosterone replacement (21). Therefore, pharmacological manipulation of NO or NOS expression may offer a viable strategy for treatment for some causes of erectile dysfunction.

*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 signaling appears to be essential for two forms of neuronal plasticity: long-term potentiation (LTP) in the hippocampus (22) and long-term depression in the cerebellum (23). In these cellular models, repeated neuronal stimulation yields long-lasting changes in synaptic strength. NOS inhibitors prevent these changes. Studies with NOS inhibitors have been controversial because these arginine analogues often have nonspecific 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 (24).

Through regulation of synaptic plasticity, NO mediates complex influences on brain development, memory formation, and behavior. For example, inhibition of NOS prevents the precise targeting of retinal axons to their proper location in the optic tectum (25). In adult animals, NOS inhibitors hinder motor learning (26) and the formation of olfactory memories (27). NOS inhibitors also prevent the long-lasting hyperalgesia that follows tissue injury (28). 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. However, nNOS knockout mice display normal sensitization to peripheral tissue damage in this model, and NOS inhibitors do not block sensitization in the nNOS knockout mice (29). 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 after chronic removal of NOS activity.

*Functions 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 as well. nNOS mRNA is expressed at high levels in human skeletal muscle (5), where it is alternatively spliced, yielding a muscle-specific isoform (nNOS$\mu$) (30). 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 (6). NOS activity stimulated during muscle membrane depolarization inhibits contractile force in fast twitch fibers.

In addition to modulating contractile force, NO derived from sarcolemmal nNOS regulates physiologic functions at the muscle membrane. During muscle development, myocytes fuse to form muscle myotubes, and this membrane fusion is blunted by NOS inhibitors (31). In myocyte/motor neuron cocultures, NOS produced at the postsynaptic muscle membrane functions as a retrograde messenger to regulate myotube innervation (32). In mature muscle fibers, NOS regulates glucose uptake across the sarcolemma. Glucose uptake in skeletal muscle is regulated by both acute exercise and by insulin. NOS inhibitors selectively blunt exercise-induced uptake but have no effect on insulin-stimulated glucose transport (33). Interestingly, chronic exercise increases nNOS protein expression in muscle and this has long-lasting enhancing effects on glucose transport in heavily used muscle (33).

A striking aspect of skeletal muscle nNOS is that the synthase is quantitatively associated with the sarcolemma. nNOS occurs at skeletal muscle sarcolemma owing to its association with the dystrophin complex (9). Detailed biochemical studies have determined the specific protein in the dystrophin complex that binds to nNOS. This work shows that the PDZ protein motif at the $NH_2$ terminus of nNOS binds to a similar PDZ motif near the $NH_2$ terminus of syntrophin (34), a dystrophin-associated protein. Syntrophin directly binds to dystrophin and links nNOS to the dystrophin complex.

Patients with Duchenne muscular dystrophy and *mdx* mice that lack dystrophin evince a loss of nNOS from the sarcolemma. Certain mutations in the rod-like domain of dystrophin that cause Becker's dystrophy result in loss of sarcolemmal nNOS but not other components of the dystrophin complex (34). The specific cause of muscle pathology that results from dystrophin deficiency is unknown. One model proposes that the loss of dystrophin in Duchenne dystrophy disrupts the normal link between the extracellular matrix and the myofiber cytoskeleton (35). This results in sarcolemmal dam-

age and myofiber necrosis. Other evidence suggests that disruptions of intracellular calcium homeostasis and subsequent free radical–induced oxidative damage contribute to muscle pathology in Duchenne dystrophy (36). nNOS represents a possible source for free radical injury in Duchenne dystrophy. On the other hand, because NO also plays a role in myofiber differentiation, the loss of sarcolemmal nNOS signaling may contribute to failed muscle regeneration in Duchenne dystrophy. Future work will determine whether manipulation of skeletal muscle NO levels represents a valuable therapeutic approach to Duchenne dystrophy or other muscle diseases.

*Cellular mechanisms regulating NO actions in excitable tissue*

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, which prevent storage of NO in lipid-lined vesicles or metabolism of NO by hydrolytic degratory enzymes. In addition, excessive production of NO is toxic to neurons and other cells. Therefore, NO signaling must allow for rapid and localized NO production and immediate termination of biosynthesis. This tight control of NO signaling is largely regulated at the level of NO biosynthesis. Indeed, the NOS proteins are among 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 (7). Distinct 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 (Fig. 1 *C*). Intestinal relaxation mediated by NO is suppressed by the N-type calcium channel antagonist, ω-conotoxin (37). In the brain, NO biosynthesis is predominately regulated by calcium influx at the synapse. Glutamate, the major excitatory neurotransmitter in the brain, is the most effective activator of NO biosynthesis in most brain regions. Calcium influx through the *N*-methyl-D-aspartate (NMDA) receptor potently activates nNOS in brain (1). 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 $NH_2$-terminal PDZ protein motif which mediates subcellular targeting of the enzyme (9). 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 (38). Importantly, NMDA receptors also occur at postsynaptic densities through binding to PSD-95 (39, 40). PSD-95 and related proteins thereby function as molecular scaffolds and physically link nNOS to NMDA receptors (Fig. 1 *A*). Internalization of peptides that antagonize the interaction of nNOS with PSD-95 block NMDA-coupled increases in NOS activity (Christopherson, K.S., and D.S.

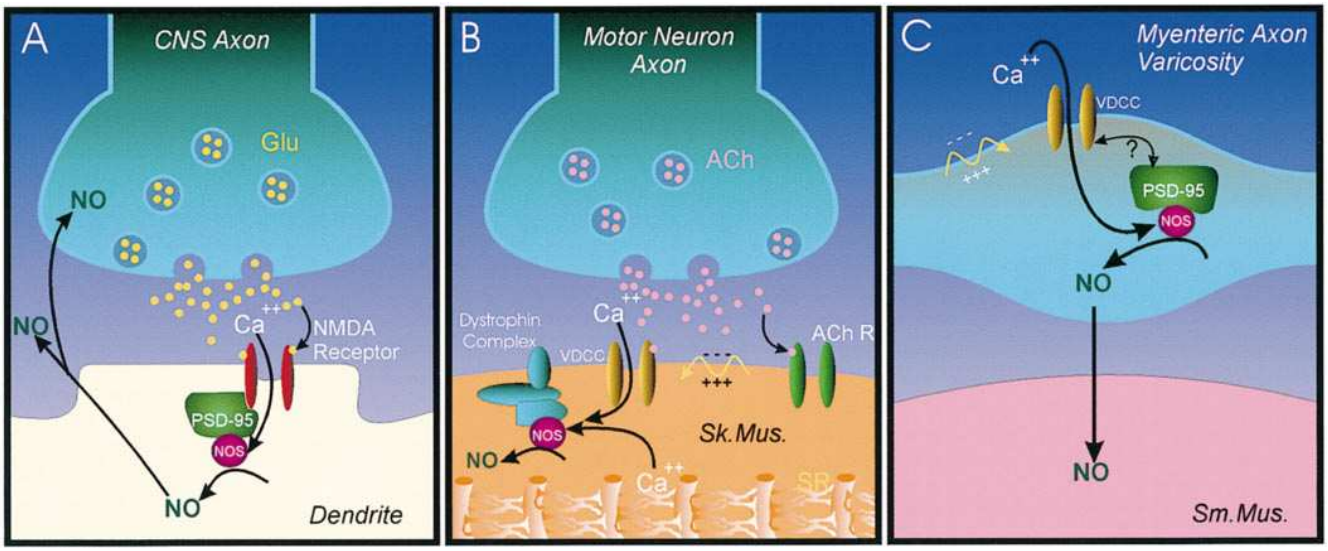


*Figure 1.* Synaptic regulation of nNOS. Protein interactions with nNOS target the synthase to discrete sites in neurons and skeletal muscle. These interactions likely account for differential regulation of nNOS by specific calcium influx pathways. (*A*) Associations with PSD-95 mediates coupling of 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. *ACh*, Acetylcholine; *ACh R*, acetylcholine receptor; *Glu*, glutamate; *SR*, sarcoplasmic reticulum. Illustration by Naba Bora, Medical College of Georgia.

Bredt, manuscript in preparation). 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 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 (9). Again, the nNOS protein is targeted to the cellular domain, which regulates the enzyme's activity (Fig. 1 *B*).

*Transcriptional regulation of NOS in the nervous system.* The calcium-dependent NOS in normal brain was originally termed "constitutive" to distinguish the activity from "inducible" NOS (iNOS) that is present only in cells stimulated with cytokines or similar factors. However, it is now clear that transcription of all three forms of NOS is dynamically regulated in the nervous system. Induction of nNOS appears to be an adaptive response to permit sustained increases in NO biosynthesis. For example, in sensory pathways, NO participates in processing of nociceptive inputs (28). nNOS protein is induced in sensory neurons after tissue damage or peripheral nerve lesion, and this nNOS induction appears to participate in the prolonged nociceptive responses that follow these injuries (41). Dramatic regulation of nNOS also occurs in the central nervous system after certain traumatic neuronal insults. This induction of nNOS in the injured central nervous system can also be a maladaptive response, which mediates neuronal cell death. Avulsion of nerve roots from the ventral spinal cord causes induction of nNOS in motor neurons. The ensuing death of the nNOS positive motor neurons can be prevented with NOS inhibitors, thus demonstrating the toxic role for NO in this response (42).

*Roles of iNOS in brain.* Although traumatic injury is associated with upregulation of nNOS protein levels in neurons, inflammatory processes in brain often cause induction of iNOS in brain astrocytes and microglia. In *Toxoplasma gondii* infection, the induction of iNOS serves as a nonspecific immune response that prevents parasite invasion. Accordingly, iNOS knockout mice are uniquely susceptible to colonization of the central nervous system after peripheral inoculation with *T. gondii* (43). While iNOS induction can protect brain from certain infectious diseases, the excessive levels of NO that result can instead be toxic to neurons. In patients with multiple sclerosis, high levels of iNOS are found in reactive astrocytes in active demyelinating lesions (44). iNOS protein levels are also increased in patients with severe AIDS dementia compared with seropositive individuals displaying no or mild impairment (45). Purified viral gp41 from HIV increases iNOS expression and induces cell death in neuronal cultures, and this cell death is prevented by an NOS inhibitor. Defining the potential role of iNOS inhibitors as therapy in AIDS dementia and other inflammatory brain disorders is an area of active research.

### *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. Accordingly, NMDA receptor antagonists are protective in animal models of cerebral ischemia. The first experimental evidence that endogenous NO mediates brain injury associated with NMDA receptor activity derived from studies in cultured neurons (46). 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 subsequently 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 (47).

*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 (8). Decisive evidence that neuron-derived NO mediates injury in stroke derives from studies of nNOS knockout mice. Compared with littermate controls, nNOS knockout mice show similar changes in regional blood flow after focal ischemia, but have 38% smaller infarcts (48). 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 (49). iNOS protein is not present in normal brain, so this isoform does not participate in the acute phase after ischemia. However, iNOS expression is induced in reactive astrocytes and in infiltrating neutrophils after cerebral ischemia (50). iNOS levels peak within 48 h, suggesting that postischemic 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 (50).

Because eNOS activity protects the ischemic brain by maintaining blood flow, initial pharmacological studies showed that nonspecific NOS inhibitors, which block both nNOS and eNOS, do not effectively protect from injury after stroke. The recent development of specific nNOS antagonists such as ARL17477, 7-nitroindazole, and *S*-methyl-isothioureido-L-norvaline demonstrates that selective blockade of nNOS offers a useful pharmacological strategy for controlling brain injury after stroke in rodent models (50). Protective actions by NOS inhibitors are clearly mediated by antagonism of nNOS, as nonspecific NOS blockers paradoxically increase infarct volume in nNOS knockout mice due to inhibition of eNOS.

*Mechanisms for NO toxicity.* 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 (51). 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 NADH succinate/oxidoreductase. NO can also inhibit glycolysis by reactions with *cis*-aconitase and by competing with oxygen at cytochrome oxidase.

In addition to directly reacting with protein prosthetic groups, NO also reacts readily with superoxide ($O_2^-$) to produce peroxynitrite ($ONOO^-$), which may mediate much of the NO neurotoxicity (52). 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 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 (52).

Direct evidence that NO and $O_2^-$ conspire in neuronal toxicity derives from studies of transgenic animals. Cu/Zn SOD is a cytosolic scavenging enzyme that removes reactive $O_2^-$ and prevents formation of peroxynitrite. Overexpression of Cu/Zn SOD in transgenic mice reduces the infarct volume in the middle cerebral artery occlusion model of focal ischemia compared with wild-type mice (53). Cu/Zn SOD overexpressing mice have now been bred with nNOS knockout mice and the resulting double transgenic mice acquire even less ischemic damage than either single-transgenic parental strain (8).

*NO in neurodegenerative diseases.* Although NO clearly participates in neuronal injury after vascular stroke, the role of NO in human neurodegenerative disease is not as easily understood. The slow progression of these diseases, occurring over 50 yr or more, complicates experimental approaches to modeling their pathophysiological mechanism. However, histopathological evidence suggests that certain neurodegenerative diseases may also be mediated by NO and glutamate toxicities. For example, NADPH-diaphorase positive neurons in the corpus striatum, which are the NOS neurons, are selectively spared in Huntington's disease. This selective pathology can be replicated in striatal culture models and in vivo after lesions with NMDA but not other classes of glutamate agonists. The spared NADPH diaphorase neurons are uniquely endowed with high levels of SOD, which may protect the cells from peroxynitrite-mediated NO neurotoxicity (54). Indeed, 3-nitrotyrosine, the footprint of peroxynitrite, is detected in striatal neurons in animals models of Huntington's disease (55).

Although stigmata of NO toxicity only 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 (56). Definitive evidence that NO and peroxynitrite mediate toxicity in the MPTP model of Parkinson's disease again derives from studies of transgenic mice (57). Both nNOS knockout mice and mice that overexpress Cu/Zn SOD are resistant to MPTP toxicity.

Although 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 be evaluated rigorously. The recent identification of a Parkinson's disease gene (58), and the development of transgenic animal model for Huntington's disease (59), suggests it will not be long until we NO.

## Acknowledgments

This work was supported by grants (to D.S. Bredt) from the National Institutes of Health, the Council for Tobacco Research, the Searle Scholars Program, the EJLB Foundation, the Culpeper Foundation, the Muscular Dystrophy Association, and the Lucille P. Markey Charitable Trust.

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