The Generation of Nitric Oxide and Its Roles in Neurotransmission and Neurotoxicity

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Abstract. The N-methyl-D-aspartate (NMDA) receptor plays a key role in synaptic plasticity and is thought to underlie memory, learning and development of the nervous system. The NMDA receptor is a ligand-gated ion channel complex that contains distinct recognition sites for endogenous and exogenous ligands, including glutamate, glycine, Mg$^{2+}$, Zn$^{2+}$, and noncompetitive blockers such as MK-801. In the central nervous system, nitric oxide (NO) is produced in some neurons following activation of excitatory amino acids receptors, particularly those of the NMDA receptor. Nitric oxide is synthesized from L-arginine by the cytoplasmic enzyme nitric oxide synthase (NOS) which is a calcium dependent enzyme, and this pathway is inhibited by the analogues of L-arginine such as NG-monomethyl-L-arginine (L-NMMA) and is augmented by NMDA receptor activation. Activation of the NMDA receptor results in the elevation of intracellular calcium ($[Ca^{2+}]_i$) which in turn activates NOS via the calcium-calmodulin complex. Nitric oxide is not a classical neurotransmitter in the central nervous system since it is not released by exocytosis and does not interact with a receptor protein but rather diffuses rapidly across the membrane and binds with the iron in heme-containing proteins. Nitric oxide can serve as both an oxidizing and reducing agent. It has a strong affinity for heme proteins such as guanylyl cyclase, but there is evidence that NO may have a regulatory role by oxidizing sulfhydryl groups of non-heme proteins such as those on the NMDA receptor. The half-life of NO is 3–7 seconds, but because of its rapid rate of diffusion it can be expected to influence a sphere of cells in a radius of about 100 μm from the site of its generation. One mechanism by which NO is thought to modulate long-term potentiation (LTP) and long-term depression (LTD) by the activation of guanylyl cyclase and the subsequent elevation of cGMP. In fact, elevation of cGMP induced by NMDA is enhanced by L-arginine and inhibited by L-NMMA. It is presumed that NO augments glutamate release, but whether this involves a linkage with cGMP is not known. Centrally, activation of the NMDA receptor is strongly linked with the rapid generation of NO in neurons, but other agonists such as kainate and acetylcholine have been shown to generate NO under selective conditions and regions of the brain. However, there are several lines of evidence suggesting that NO is a neurotoxicant. Stimulation of NMDA receptor results in the release of superoxide anion as well as NO. At least part of NO-induced toxicity results from its reaction with superoxide anion which results in the formation of a strong oxidant, peroxinitrite. (Keio J Med 44 (2): 53–61, June 1995)

Key words: glutamate receptors, nitric oxide, cGMP, LTP/LTD

Characteristics of Excitatory Amino Acid Receptors

Excitatory amino acids (EAAs) such as glutamate and aspartate are the most abundant neurotransmitters in the central nervous system (CNS). Glutamate is the major rapidly acting excitatory neurotransmitter in the mammalian CNS and is in high concentration (approximately 100 mM) in glutamatergic synaptic vesicles. In addition, glutamate has a major metabolic role and is present at concentrations of 10 mM in the cytoplasm of neurons. One metabolic pathway for glutamate involves its decarboxylation to γ-aminobutyric acid. In another reaction
it may undergo transamination with oxaloacetate to form α-ketoglutarate and aspartate. A further aspect of the metabolism of glutamate is its conversion into glutamine by the enzyme glutamine synthetase. The glutamate levels found in rat plasma, cerebrospinal fluid and hippocampal extracellular fluid are 160, 11 and 3 μM respectively. The glutamate is removed by reuptake into both neurons and glia by high-affinity transport system which is an uptake carrier that cotransports two sodium ions into the cell with each glutamate anion, while counter-transports one potassium ion and one hydroxide ion out of the cell.

Receptors for glutamate have been divided into two distinct groups: Ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors. Three classes of iGluRs have been characterized. They are permeable to cations and further classified according to compounds which preferentially activate the receptor: N-methyl-D-aspartate (NMDA), kainate and a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA). Antagonists have been found which are selective for each of these ionotropic receptors: D-2-amino-5-phosphonovalerate and 3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonoate for the NMDA receptor; 6-cyano-7-nitroquinolinoxaline-2, 3-dione and 6,7-nitroquinolinoxaline-2,3-dione for the kainate receptor; and 2-amino-3-[3-(carboxymethoxy)-5-methyl-isoxazole-4-yl]-propionate for the AMPA receptor. A second main class of glutamate receptors is a mGluR which is activated by both trans1-aminocyclopentane-1,3-dicarboxylate and quisqualate. This receptor can be blocked by the selective antagonist L-2-amino-3-phosphono propionate.

NMDA receptor currents behave like classical voltage-dependent channels, which increase their activity with depolarization. Because NMDA receptor activation requires two types of input (binding of agonist such as glutamate, and depolarization to reduce Mg2+ block), the synaptic activation of NMDA receptors shows associative properties. In vivo, glycine is present in the extracellular fluids at sufficient concentration to ensure a high degree of occupancy at its binding site. Thus, synaptic activation of NMDA receptors can be triggered solely by the release of glutamate.

The NMDA receptor has a conductance of approximately 50 pS, a mean open time of 5 ms, and a 10 times higher permeability to calcium over sodium ions. This is in contrast with the kainate receptor which is selectively permeable to sodium over calcium.

The EAAs such as NMDA, glutamate, and kainate have been shown to produce neurodegeneration both in vivo and in vitro. There are two phases of neuronal injury in vitro: An immediate phase, which is often reversible and occurs within a few minutes of glutamate application, is characterized by cell swelling and is dependent on sodium and chloride influx. A delayed phase occurs 24 hours later and is dependent on an increase in intracellular calcium concentration ([Ca2+]i). Using neuronal culture, it has been shown that excitotoxins require the presence of chloride but not calcium for rapid toxicity to occur and that calcium is involved in the delayed toxicity. In fact, in zero calcium buffer a rapid NMDA receptor-mediated toxicity secondary to both the influx of chloride and water in conjunction with the efflux of glutamate occurs in the absence of exogenously added toxicant. Measurements of [Ca2+]i, with the fluorescence dye fura-2 show that a neurotoxic dose of glutamate (50 μM) results in a 10-fold elevation of [Ca2+]i. Some have shown [Ca2+]i remains elevated for longer than 1 hr after glutamate withdrawal, whereas others have shown that [Ca2+]i return to normal even though there is an ensuing cell death. Thus, the role of calcium in cell death is not clear.

The calcium entry that results from the opening of the NMDA channel can induce the membrane translocation and activation of protein kinase C (PKC) which, in turn, mediates the phosphorylation of specific membrane proteins. Sustained increase of neuronal calcium influx which is insensitive to NMDA antagonists and voltage sensitive calcium channel blockers may be triggered by the activation of PKC. A prolonged activation of PKC has been detected in vivo after brain ischemia. Prevention of glutamate- and kainate-induced PKC translocation by gangliosides or down-regulation of PKC by phorbol esters protects neurons from glutamate-induced delayed toxicity. Thus, it is possible that glutamate neurotoxicity may be mediated by a sustained PKC translocation, which results in the unbalancing of [Ca2+]i, homeostasis.

Recently the function of the NMDA receptor and possibly the toxicity that results from activation of the receptor has been linked to nitric oxide (NO) production. Nitric oxide is now thought to be a neurotransmitter in the CNS that is closely linked with the iGluR activation (Fig 1). In the next section, the characteristics of nitric oxide synthase and the roles of NO in the central nervous system will be discussed.

The Localization of Nitric Oxide Synthase

Immunohistochemical staining with antiserum against NOS and in situ hybridization with NOS mRNA revealed that the enzyme was located entirely in neurons and vasculature endothelium of the brain with no staining or hybridization occurring in glia. In these studies the highest amounts of NOS occurred in the granule cells layer of the cerebellum and the olfactory bulb, while only about 2% of the neurons in the cerebral cortex, striatum and hippocampus stained for NOS and these were mostly aspiny neurons. Subsequent studies, however, with polyclonal antibodies against cerebellar NOS...
Fig 1 Schematic diagram of a glutamatergic synapse, showing glutamate synthesis and release from the presynaptic neuron, action of glutamate on postsynaptic ion channels, and removal of glutamate from the extracellular space by uptake. Postsynaptically glutamate can activate excitatory amino acid (EAA) receptors which allow elevation of intracellular calcium levels. Elevated intracellular calcium via NMDA receptor activation can activate NOS results in generation of NO. Glu; glutamate, Gln; glutamine, NMDA, NMDA receptor; K/A, KA/AMPA receptor, mGluR; metabotropic glutamate receptor, VSCC; voltage sensitive calcium channel, NOS; nitric oxide synthase

revealed that the NOS was not exclusively found in neurons, but rather glia stained as well as non-neural cells in the pancreas, lung, kidney, uterus and stomach. However, selective staining was still found in neuronal tissues, and it was often found that cells staining for NOS had adjacent areas which stained heavily for guanylyl cyclase.

While no NOS was detected in glia in the above studies, it now appears that there are a family of enzymes and it is possible that there are other NO generating proteins that were not detected by the above methods. Under various conditions astrocytes show both constitutive and inducible NOS activity. A polyclonal antiserum against NOS different from the above studies showed staining that included Bergmann glia and astrocytes although at a lower level. Indirect evidence comes from the finding that glutamate, norepinephrine and calcium ionophores cause an elevation of cGMP in cerebral astrocytes that is reversed by NOS inhibitors. Furthermore, NOS activity can be induced in microglia, the CNS analog to macrophages, by lipopolysaccharide and in cortical astrocytes in culture. 

Inhibitors and Triggers of Nitric Oxide Synthesis

Nitric oxide synthase inhibitors

Nitric oxide synthase is inhibited by the structural analogues of L-arginine such as N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) and N\textsuperscript{G}-iminomethyl-L-ornithine (L-NIO). Interestingly, 7-nitroindazole which is not a guanidino-substituted L-arginine analogue also inhibits NOS. Inducible NOS is much less sensitive to L-NAME than is the constitutive enzyme. As with the case of the NO generating agents, the arginine analogs are not entirely selective for inhibiting NOS. L-NMMA and L-NIO inhibit the transport of L-arginine whereas L-NAME does not. On the other hand, L-NAME and other alkyl esters of arginine have muscarinic receptor antagonist properties. The lack of specificity of the arginine analogs is often ignored in many experimental protocols.
There are interesting agents that block NO synthesis by interfering with various cofactor binding sites. Carbon monoxide binds to the heme site on NOS to partially inhibit enzyme function. Interestingly, NO binds to the same site and negatively inhibits its own formation and affects expression of NOS mRNA. Diphenyleneiodonium and chemically related compounds are aromatic iodoniums that block NOS by covalently modifying the enzyme. Miconazole inhibit both cytochrome P-450 reductase and NOS probably by interfering with the CA\(^{2+}\)-calmodulin binding site as do fluperazine and calcineurin. With the exception of diphenyleneiodonium which may prove to be a potent and selective inhibitor, it is clear that none of these agents are selective for NOS. Recently, there is an interesting report suggests that glucocorticoids have the downregulating role on NO production via the inhibition of PKC activity particularly under the conditions of stimulated production of NO, such as inflammatory and demyelinating CNS disorders.

**Nitric oxide generating agents**

For many years sydnonimines, sodium nitroprusside, various nitrates such as nitroglycerin (glyceryl trinitrate) and nitrite such as amyl nitrite have been used as vasodilators and anti-anginal agents. The muscle relaxing properties of these agents were thought by some to depend on the formation of nitric oxide. A decade later, NO was shown to be synonymous with endothelium-derived relaxing factor. It is possible that at least some of the effects of the NO generating agents are mediated by S-nitrosothiols.

A sydnonimine, 3-morpholinosydnonimine (SIN-1), produces both NO and superoxide anion in its spontaneous degradation which combines to form peroxynitrite. Some nitric oxide generating compounds such as molsidomine require initial degradation by hepatic enzymes to SIN-1.

Sodium nitroprusside and S-nitroso-N-acetylpenicillamine generate NO spontaneously while neither nitroglycerin nor amyl nitrite is spontaneously decomposed in aqueous solutions to NO. Rather, in the absence of light nitroglycerin interacts with thiol groups to form an intermediate nitrosonium ion (S-NO\(^{+}\)). This is the likely reason that nitroglycerin is neuroprotective while SIN-1 is neurotoxic. The production of an NO\(^{+}\) equivalent (RS-NO) by nitroglycerin provides protection by serving as oxidizing agents while the generation of peroxynitrite by SIN-1 has toxic effect. In fact, when neurons are co-incubated with nitroglycerin plus a reducing agent such as cysteine, nitroglycerin which spontaneously release NO\(^{+}\) becomes neurotoxic as is the case with SIN-1 because nitrosonium could be reduced by reducing agent into nitric oxide then reacts with superoxide to produce peroxynitrite.

**Triggers of nitric oxide synthesis**

Triggers for NO generation in CNS were first shown to be glutamate which resulted in the stimulation of cerebellar granule cells to produce NO. The concentration of NO which has a short half life can be measured indirectly by cGMP elevation or nitrite accumulation. The elevation of cGMP was blocked by NMDA antagonists, and in fact, NMDA receptor activation has been the glutamate receptor best associated with NO generation. In immature cerebellar slices, only the activation of the NMDA receptor is associated with the synthesis of NO. However, in mature cerebellar slices, both quisqualate and AMPA receptor activation result in the formation of NO, again as measured by cGMP elevations and similarly inhibited by hemoglobin and arginine analogues. The release of nitric oxide in response to NMDA is Ca\(^{2+}\)-dependent. Since the action of guanylyl cyclase by sodium nitroprusside is Ca\(^{2+}\)-independent, it seems likely that Ca\(^{2+}\) is necessary for NO synthesis as opposed to the synthesis of cGMP as was previously thought. When the glutamate-induced cGMP elevation was discovered, this effect was presumed a nonspecific consequence of depolarization-induced influx of Ca\(^{2+}\) into the stimulated neuron, and a subsequent activation of guanylyl cyclase by this ion. Thus, the key step in the production of NO is probably the influx of Ca\(^{2+}\) and increased [Ca\(^{2+}\)], levels that are maintained.

In striatal neurons in tissue culture, the non-NMDA agonist, domoate, kainate, AMPA and quisqualate can stimulate the production of NO. If neurons are treated with concanavalin A, the production of NO (as measured by elevations of cGMP) is markedly elevated and is partially blocked by MK-801 suggesting that these non-NMDA agonist were activating a glutamate receptor subtype composed of both kainate and NMDA receptor subunits. In primary cultures of cerebellar granule cells, activation of both the NMDA and kainate receptors are associated with the synthesis of NO as measured by the production of [\(^{3}\)H]citrulline from [\(^{3}\)H]arginine, however, only the production of [\(^{3}\)H]citrulline induced by NMDA was correlated with elevations of cGMP. In vivo, elevations of cerebellar cGMP induced by D-serine, quisqualate, NMDA, kainate, harmaline and pentylentetrazol are blocked by arginine analogues. In primary cultures of astroglial cells derived from cerebr al cortex, norepinephrine and quisqualate result in elevations of NO via the \(\alpha\)-adrenergic and the metabotropic glutamate receptors, respectively.

**The Physiologic and Pathologic Roles of Nitric Oxide**

Nitric oxide is thought to be a neurotransmitter. However NO is not a classical neurotransmitter be-
cause it is not stored in synaptic vesicles or released by exocytosis and is not thought interact with a receptor protein. But it rather diffuses rapidly across the membrane of target neurons where it binds with the iron in heme-containing proteins. The effects of NO have been varied. They include altering synaptic efficiency by mediating such processes as long-term potentiation. Nitric oxide has been linked with some types of glutamate-induced neurotoxicity. It has been proposed that NO might have a role in synaptogenesis during development.

Nitric oxide lowers [Ca\textsuperscript{2+}], in Balb/c 3T3 cells, which lacks guanylyl cyclase so that it is clear that the mechanism is not mediated by elevations of cGMP. Nitric oxide regulates peripheral vascular blood flow and is synonymous with endothelial-derived relaxing factor. Nitric oxide production in various regions of the brain might affect local vascular responses in a similar manner as seen in the periphery such that blood flow is controlled in a need-dependent fashion. Nitric oxide synthase inhibitors block the increase in blood flow in the somatosensory cortex that occurs normally upon stimulation of peripheral nerves.

The following discussion will examine more closely the effects of NO as a neurotransmitter in the CNS, particularly the role of NO in long-term potentiation/long-term depression and explore the toxic effect of NO.

**Long-term potentiation and long-term depression**

NO has been proposed as an intercellular transsynaptic messenger in models of synaptic plasticity such as LTP and LTD, two processes that have been linked with memory formation. Long-term potentiation occurs in the hippocampus after a neuron or group of neurons receive several simultaneous signals. Changes occur so that when the neuron is stimulated at a later time, a greater response occurs, and it is clear that the NMDA receptor with subsequent elevations of [Ca\textsuperscript{2+}], are involved. But it is unclear whether the potentiation is due to an increase in the sensitivity of the receiving neuron (postsynaptic receptor) or to an increase in the amount of neurotransmitter released by the sending neuron (presynaptic). Evidence exists that NO acts as a retrograde transmitter to change the sending neuron and thus affect the amount of transmitter that is released and NO stimulates Ca\textsuperscript{2+}-independent synaptic vesicle release.

Nitric oxide synthase inhibitors block LTP in tissue slice experiments while NO generating agents such as sodium nitroprusside mimics LTP. Furthermore, the effects can be blocked by hemoglobin which is impermeable to the cell thus implicating an effect of NO that extends beyond the generating cell. NOS inhibitors show similar results with the inhibitors able to block the ability of rats to learn spatial tasks.

On the other hand, there is also evidence that NO is affecting the receiving neuron as well. NOS inhibitor, L-NMMA, partially prevented the NMDA-induced elevations of glutamate release and [Ca\textsuperscript{2+}], levels. The effect of NO on glutamate release is unlikely to be secondary to cGMP as the cGMP analogue, dibutyryl cGMP (dBCGMP), does not augment basal or NMDA-induced glutamate release. However, dBCGMP augments the NMDA-induced elevations of [Ca\textsuperscript{2+}], levels. Part of the effect of cGMP might be via positive feedback...
on the NMDA receptor (Fig 2) since dBCGMP increases \[^{[3]}H\]MK-801 specific binding in cultured cerebellar granule cells (unpublished data). The problem with the retrograde messenger theory is that the NO should be made in the appropriate postsynaptic cells which are the pyramidal cells of the hippocampus. However, no NOS has been found in these cells. Furthermore, some laboratories have not found a consistent block of LTP by NOS inhibitors.\(^92\)

Long-term depression is similar but opposite condition to LTP occurring in the cerebellum where long-term firing of parallel and climbing fibers suppresses Purkinje cell firing, the sole output of the cerebellum. Nitric oxide is released following the stimulation of the climbing fibers and LTD can be blocked in a similar manner as LTP by both arginine analogs and hemoglobin.\(^82\)

A combination of sodium nitroprusside and 8-bromo cyclic GMP (a cGMP analog) in conjunction with parallel fiber stimulation can substitute for climbing fiber stimulation in producing LTD in Purkinje cells. Thus, there is likely a role for both NO and one of its effector proteins, guanylyl cyclase in LTD. Quisqualate has also been report to induce LTD in Purkinje cells which is blocked by hemoglobin and arginine analogues.\(^93\)

**Neurotoxicity**

There are several lines of evidence which suggest a role for NO in neurotoxicity. Nitric oxide synthase occurs in discrete neuronal populations in a distribution that is identical to that of neurons staining for NADPH-diaphorase.\(^36,94,95\) Interestingly, it is the neurons containing NOS that are spared in the striatum in Huntington’s disease\(^96\) and in other brain regions after ischemic\(^97\) and excitotoxic (quinolinate) induced damage.\(^98\) In tissue culture, neurons containing NOS are resistant to NMDA-induced neurotoxicity and are usually more sensitive to kainate-induced toxicity.\(^20,99,100\) In cultured cortical neurons some investigators have shown that NOS inhibitors can prevent NMDA-induced toxicity\(^30,31\) while others have noted no association.\(^101,102\)

Ischemic brain damage caused by ligation of cerebral arteries can be markedly reduced by various arginine analog type NOS blockers.\(^103\) At least part of NO-induced toxicity results from its reaction with superoxide anion which results in the formation of peroxynitrite.\(^64\)

Nitric oxide synthase inhibitors or superoxide dismutase (SOD) either provides partial protection against cerebral ischemia in rats that have had their middle cerebral arteries occluded or markedly potentiates the toxicity depending on whether the inhibitor are given before or after the occluding insult.\(^103,104\) The pre-treatment of animals with NOS inhibitors augments the injury while the post-treatment reduces the infarct size.

Some NO generating agents have been shown to be toxic to cultures of cortical neurons while others are neuroprotective. The toxicity of compounds such as SIN-1 are prevented by simultaneously incubating cultures with superoxide dismutase indicating that these compounds further combined with O\(_2^-\) to form ONOO\(^-\) (peroxynitrite) that is apparently the lethal product.\(^64\) On the other hand, NO\(^+\) (nitrosium) generating agents such as nitroglycerin which do not form peroxynitrite but serve as neuroprotective agents probably through the downregulation (oxidation) of the NMDA receptor.\(^105\)

It is possible that other radicals besides NO contribute to some of the toxicity that results from activation of glutamate receptors. For example, several mutations in superoxide dismutase are associated with the autosomal dominant inheritance of familial amyotrophic lateral sclerosis,\(^106\) a neurodegenerative disorder that has been linked with excessive glutamate excitation. In primary cultures of cerebellar granule cells, stimulation of the NMDA receptor results in the release of superoxide radical as assessed with electron paramagnetic resonance studies.\(^107\) The superoxide radicals came from the release of arachidonic acid and could be prevented by a phospholipase A\(_2\) inhibitor. Neither stimulation with kainate or membrane depolarization with KCl produced similar superoxide radicals, and the production of superoxide was not dependent on the NO. Superoxide radicals were dependent on the presence of calcium in the extracellular fluid and could be blocked with MK-801.\(^107\) On the other hand, others have shown that kainate-induced toxicity in cerebellar granule cells can be prevented by inhibitors of xanthine oxidase, a cellular source of superoxide,\(^108\) however NMDA-induced neuronal death was not blocked by superoxide dismutase and xanthine oxidase-induced neuronal death was not potentiated by SIN-1.\(^109\) This indicates that peroxynitrite is not more toxic than superoxide radical in cerebellar granule cells. Nevertheless, there is a possible pathologic link of SOD with NO. Superoxide ion reacts with NO three times faster than it does with the SOD to form peroxynitrite (ONOO\(^-\)) and peroxynitrite reacts with tyrosine residues on SOD.

In tissue culture, inhibitors of NOS block neurotoxicity induced by HIV virus coat protein, gp120, indicating a role for NO in AIDS dementia.\(^110\) Toxicity induced by gp120 requires glutamate and is also blocked by NMDA receptor antagonists as well as superoxide dismutase indicating that toxicity is possibly mediated by peroxynitrite. In some cases, the toxicity of NO might be secondary to cGMP synthesis. High levels of cGMP cause destruction of photoreceptor cells in the retina\(^111\) where NOS has now been demonstrated by immunocytochemistry.\(^34\) Sodium nitroprusside produces a concentration dependent cell death that parallels cGMP formation, and this neurotoxic effect can also be prevented by hemoglobin.\(^30\)
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The Generation of NO and Its Roles in the CNS

60


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