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NITRIC OXIDE SIGNALING IN THE CENTRAL NERVOUS SYSTEM

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INTRODUCTION

Nitric oxide (NO) was first recognized as a messenger molecule in the central nervous system (CNS) in 1988 (52), when it was identified as the unstable intercellular factor that had been hypothesized, a year earlier (53), to mediate the increased cyclic GMP (cGMP) levels that occur on activation of glutamate receptors, particularly those of the NMDA (*N*-methyl-D-aspartate) subtype. The presence of an NO-forming enzyme (NO synthase, or NOS) in the brain was later confirmed (74), and this enzyme was subsequently purified (14) and its cDNA cloned and sequenced (12).

The discovery that NO functions as a signaling molecule in the brain opened a new dimension in our concept of neural communication, one overlaying the classical picture of chemical neurotransmission, where information is passed between neuronal elements at discrete loci (synapses), and in one direction, with a diffusive type of signal that disregards the spatial constraints on neurotransmitter activity normally imposed by membranes, transporters, and inactivating enzymes. In principle, NO could spread out from its site of production to influence many different tissue elements (neuronal, glial, and vascular) that are not necessarily in close anatomical juxtaposition.

During the past few years, much information on the enzymology and molecular characteristics of NO synthesis has accrued, as reviewed in other articles in this volume. Furthermore, data from immunocytochemistry, in situ hybridization, and NADPH diaphorase histochemistry have combined to give

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a reasonably coherent picture of the anatomical locations of NO-generating cells and their processes throughout the CNS (134). The brain contains by far the highest activity of NOS of any tissue so far examined (108), and the widespread distribution of the enzyme therein indicates that NO could be involved in practically all aspects of CNS function. We do not attempt here to review all of the putative roles of NO in the CNS that have accumulated in recent years but instead focus on the cellular and molecular mechanisms of neural NO signaling and signal transduction.

REGULATION OF NO FORMATION

Neurons

Under normal conditions, NOS in the CNS apparently occurs exclusively in neurons (13). The production of NO by intact neurons in response to excitatory stimuli requires Ca^{2+} (52), which reflects the dependence of the activity of neuronal NOS (nNOS) on Ca^{2+} and calmodulin (14, 74). Depending on the location of the enzyme, NOS activation is coupled to one of two main types of physiological stimuli: postsynaptic neurotransmitter receptor stimulation leading to Ca^{2+} influx or mobilization, and action potentials in presynaptic nerves eliciting Ca^{2+} influx through voltage–sensitive Ca^{2+} channels.

Postsynaptically, a major stimulus for NO formation is the activation of receptors for the principal excitatory neurotransmitter glutamate. Of special importance are NMDA receptors whose associated ion channel has a high effective permeability to Ca^{2+}. Other ion channel–coupled glutamate receptors of both the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate subtypes have also been implicated, as have G protein-coupled, or metabotropic, glutamate receptors (94, 123). Evidence also indicates that the action of several other neurotransmitters or neuromodulators may be linked to NO formation; these include 5HT, bradykinin, endothelin, acetylcholine, and noradrenaline (33, 101, 102).

NOS PLASTICITY Although frequently considered constitutive, the NOS may be subject to dramatic up- or down-regulation. Up-regulation was first suggested by in situ hybridization studies of dorsal root ganglia. In normal rats, few neurons in lumbar ganglia express detectable NOS mRNA, but 2 days after transection of the sciatic nerve, mRNA was found in about a third of the neurons. This increase lasted for at least 2 months (133). NADPH diaphorase histochemistry and NOS immunohistochemistry have confirmed expression of the enzyme protein under similar conditions (40, 126). Results obtained using the histochemical technique have further indicated that neurons in the spinal cord (120, 135, 140) and several brain areas (62, 68, 73, 100, 110, 141, 144)

react to injury in a similar fashion. Other factors may also regulate NOS expression; increases in mRNA have been detected in the paraventricular nucleus of the hypothalamus in response to stress (18) and lactation (19), in the pituitary in response to gonadectomy (20), and in the hippocampus as a result of treatment with a combination of lithium and the anticholinesterase drug tacrine (5).

The pineal gland exhibits an example of NOS down-regulation by a physiological stimulus. In this gland, constant light exposure markedly reduced NOS enzymatic activity (by 80% after 8 days). Normal activity was restored when the experimental animals were returned to a light-dark cycle for 2 days, and it appears that noradrenaline (which is released in the gland during darkness) is responsible for the photoneural regulation of NOS in the pineal (111).

Glial Cells

When maintained in tissue culture, astrocytes have been observed to generate NO in a Ca^{2+}-dependent manner in response to bradykinin, noradrenaline, or a metabotropic glutamate receptor agonist (2, 90). Whether they have this neuron-like property in vivo is not clear. Astrocytes and microglial cells in primary culture express a Ca^{2+}-independent, inducible type of NO synthase (iNOS) when exposed to bacterial lipopolysaccharide and/or cytokines (9, 44, 118). This expression is reminiscent of several peripheral cell types, including macrophages, neutrophils, vascular smooth muscle cells, and hepatocytes, in which NO generated by this isoform is believed to participate in immunological defense against invading pathogens (see S Gross & M Wolin, this volume). Molecular cloning shows the sequence of the astroglial iNOS to be similar (>90% homology) to the enzyme in peripheral cells, which suggests that it is the product of the same gene (45). Information on the in vivo regulation and expression of iNOS in the CNS is still sparse, although viral infection (30, 75) and neural injury (136) have been associated with central iNOS expression.

SPREAD OF NO BY DIFFUSION

A key property of NO that sets it apart from other CNS signaling molecules is its extreme diffusibility in both aqueous and lipid environments, which allows a rapid three-dimensional spread of the signal irrespective of the presence of membranes. The important question arises as to how far NO travels before its concentration becomes too low to matter. NO is an unstable free radical species with a half-life that is often said to be as short as a few seconds. Although in vivo the half-life of NO may be much longer (86), we should consider how a fast rate of inactivation would affect the distribution of NO.

In the absence of direct experimental evidence, some insight can be gained by examining a theoretical model based on established diffusion equations (25,

139a). The concentration of NO (C) in an infinite medium at any distance (r) from a point source after time = t can be represented by the following equation:

$$C = \frac{S}{4\pi Dr} erfc \frac{r}{2\sqrt{Dt}},$$

where S is the source strength and D is the diffusion coefficient, which has been measured as 3.3×10^{-5} cm^2/s (81). Taking $S = 21 \times 10^{-18}$ mol/s at the center of a spherical structure of diameter 1 μm (i.e. approximating the dimensions of a dendritic spine or nerve terminal), the NO concentration at the surface will be 1 μM, which corresponds to the concentration measured directly at the surface of a stimulated endothelial cell (81). At steady state, when t in the equation is large, $erfc[r/2\sqrt{(Dt)}] = 1$, and the concentration of NO is inversely proportional to the distance from the source. If we assume that the biologically relevant threshold concentration of NO is 1 nM, a value based on the relaxing effect of NO on aortic smooth muscle (42), the tissue volume in which NO can exert physiological effects becomes equal to a sphere of diameter 1000 μm. Such a volume would enclose about a billion synapses.

This model so far ignores inactivation of NO. If the half-life is short, say 5 s, NO concentrations near to the source would be scarcely affected because diffusion is so fast. Even 20 μm away, the steady-state concentration would be reduced by only 10%. Farther away, significant reductions would be expected such that the distance at which the concentration falls to 1 nM decreases to 170 μm instead of 500 μm. Thus, even with a half-life of a few seconds, NO generated at a single point source should be able to influence function within a sphere with a diameter of approximately 0.3 mm, which is still very large compared with the dimensions of a synapse (the distance from the center of a dendritic spine to the center of an attached presynaptic terminal is approximately 1 μm, and the width of the synaptic cleft is approximately 20 nm).

Given such a large sphere of influence relative to the small size and high packing density of synapses and other neural elements in the CNS, this analysis raises important questions about how any specificity of action can be incorporated into a signaling mechanism that employs NO, a topic discussed below.

NO SIGNAL TRANSDUCTION

Soluble Guanylyl Cyclase and cGMP

Neuronal excitation leads to elevations in cGMP levels in numerous different brain areas including the cerebellum, cerebral cortex, striatum, and hippocampus through Ca^{2+}-dependent NO formation and the subsequent activation of the soluble form of guanylyl cyclase (sGC). This response implies that sGC is a major target for NO in the CNS, just as it is in smooth muscle cells of

blood vessels and other peripheral tissues (e.g. gut, airways) where the resultant rise in cGMP, in these cases, mediates muscle relaxation.

In support of this possibility, the distributions of NOS and NO–stimulated cGMP formation closely correspond to each other in vivo (125). At the cellular level, however, the distributions are usually complementary rather than identical; that is, in a given brain area, the population of cells (mainly neurons) that accumulate cGMP in response to NO are frequently, but not always, distinct from the population of neurons that synthesize NO. This observation reinforces the notion that NO primarily operates as an intercellular messenger. The results also support the hypothesis (50) that, so far as interneuronal signaling is concerned, NO represents a potential line of communication from post- to presynaptic elements, and vice versa, as well as between presynaptic fibers or postsynaptic structures.

sGC exists as a heterodimer made up of α and β subunits of approximately 80 and 70 kDa, respectively. Both subunits are required for enzyme activity. The NO recognition site is an associated heme moiety to which NO binds with high affinity. Enzyme activation may be the result of subsequent conformational change in the enzyme protein (130).

Each subunit can have several isoforms (49). Of the β variety, however, only the β_1 subunit has been identified in rat brain. The widespread distribution of the mRNA for this subunit compared with α_1 has led to the suggestion (43) that β_1 may be a universal component that combines with different α subunits depending on the brain region. In some areas, β_1 and α_1 are both expressed at high levels, which suggests that they are functional partners, but in other areas, α_1 is low relative to β_1, thus indicating the presence of one or more other subunits yet to be identified. The implication that sGC heterogeneity exists is interesting, and a full characterization of this family of proteins, including their anatomical distributions, will be important.

cGMP SIGNAL TRANSDUCTION Unlike in smooth muscle and platelets where NO-stimulated cGMP accumulation is translated into an easily measured endpoint (respectively, relaxation and inhibition of aggregation), the function of cGMP in the CNS is still unclear, although some recent findings are beginning to shed new light on this old problem.

Direct channel gating cGMP-operated channels are well known in retinal photoreceptor cells, where they are responsible for the dark (inward) current carried by Na^+ and Ca^{2+} ions. In the light, phosphodiesterases that hydrolyze cGMP are activated, resulting in channel closure and membrane hyperpolarization. A similar channel is present in olfactory cells but, in this instance, cAMP is thought to be the principal physiological ligand, even though it is less potent than cGMP (143a).

Outside of photoreceptors, two groups independently found evidence for cGMP-operated channels in retinal bipolar cells (92, 117). These channels function much like those in rods, although in this case, receptors for the rod neurotransmitter (glutamate) couple (through a G protein) to cGMP hydrolysis. Shiells & Falk (116) have also suggested that NO may be the stimulator of cGMP synthesis in these cells because the NO donor nitroprusside, but not activators of particulate guanylyl cyclases, could reproduce the effects of cGMP.

Recently, investigators using polymerase chain reaction (PCR) amplification techniques have detected DNA sequences specific for cyclic nucleotide-gated channels in several peripheral tissues including aorta, heart, and kidney. The same study also found a strong signal from the cerebellum (in rabbit), the only brain area apparently examined (6). The aortic cDNA encodes a channel that is highly homologous (94%) to the bovine olfactory channel, less so to the rod channel (57%), and that is gated by cGMP 40-fold more potently than by cAMP. The implication is that an analogous channel exists in the cerebellum.

A similar PCR-based approach has revealed that ganglion cells in the retina express transcripts for a channel more like the one in rods (3). Furthermore, patch-clamp recordings from isolated ganglion cells showed the presence of a functional nonspecific cation channel that was activated by cGMP much more effectively than by cAMP and that could also be activated by NO donors. Thus NO generated in nearby cells (putatively the amacrine cells) may result in depolarization of ganglion cells by activating soluble guanylyl cyclase. The authors (3) of this paper cite unpublished work showing that (unspecified) regions of the CNS also express cGMP-gated channels.

In summary, direct gating of neuronal cation channels by cGMP may be a much more widespread mechanism of NO signal transduction than was originally anticipated.

Protein kinases In smooth muscle, the multiple effects of cGMP (ultimately leading to relaxation) have mostly been ascribed to activation of cGMP-dependent protein kinase (cG-PK) (41). Until recently, cG-PK was believed to have only a limited role in the CNS because it is found in only one type of neuron, the cerebellar Purkinje cell (80). It is now recognized that there are at least two broad classes of this enzyme, cG-PK1 and cG-PKII. Type I, a soluble enzyme composed of two identical subunits (each about 78 kDa), is widely distributed and exists in two alternatively spliced versions, Iα and Iβ. cG-PKIα corresponds to the enzyme in Purkinje cells, where it directs the phosphorylation of a putative phosphatase inhibitor known as the G-substrate. The type II kinase is a monomer (86 kDa) and a particulate enzyme that was originally thought to be present only in intestinal brush border epithelial cells where it regulates Cl^- secretion.

Surprisingly, a cG-PKII has now been cloned and expressed from mouse brain. The protein has a predicted molecular mass of 87 kDa and shows significant homology (about 50% identity overall) to cG-PKI. In transfected COS-1 cells, the kinase was potently activated by cGMP (apparent K_a value, 300 nM) but not by cAMP. Of the tissues examined, cG-PKII is expressed most abundantly in brain and lung, with lesser amounts in kidney and testis (132). Whether it corresponds to the intestinal kinase is unclear at present.

These results clearly bring cG-PK back into focus as a possible transduction mechanism for the NO-cGMP pathway in the CNS, although numerous questions remain regarding the anatomical distribution of this isoform in brain and spinal cord and the identity of its target proteins. One candidate target is DARPP-32, a dopamine- and cAMP-regulated phosphoprotein that, in substantia nigra at least, can also be phosphorylated by cG-PK in response to NO (131). In its phosphorylated form, DARPP-32 is a potent inhibitor of protein phosphatase 1; hence, through this mechanism, NO could maintain enzyme or receptor proteins in their phosphorylated states.

Phosphodiesterases cGMP can either activate or inhibit specific subtypes of cyclic nucleotide phosphodiesterases (PDEs) and thereby effect changes either in its own levels or those of cAMP. Three PDEs are generally considered relevant: a cGMP-inhibited PDE (cGI-PDE) that selectively hydrolyzes cAMP; a cGMP-stimulated PDE (cGS-PDE) that hydrolyzes both cAMP and cGMP; and a cGMP-binding, cGMP-specific PDE (cG-BPDE). Of these, only cGS-PDE appears to be abundant in the brain. Particularly high levels are present in the cerebral cortex, hippocampus, and basal ganglia, whereas low levels are found in cerebellum (121). In hippocampal pyramidal neurons, cGMP suppresses a Ca^{2+} current, apparently through cGS-PDE-induced reduction in cAMP levels (32). Because these neurons also contain soluble guanylyl cyclase (43), NO may produce the same effect, although this remains to be investigated. Roles of the cGS-PDE in other brain regions have not been described.

ADP ribosyl cyclase Cyclic ADP ribose, which is formed enzymatically from NAD^+ by ADP ribosyl cyclase, mobilizes Ca^{2+} from internal stores that are distinct from those sensitive to inositol trisphosphate. The stores sensitive to cyclic ADP ribose are thought to be the ryanodine- and caffeine-sensitive stores that participate in Ca^{2+}-induced Ca^{2+} release (46). Cyclic ADP ribose-induced Ca^{2+} release has been observed in many different tissues, including brain microsomes, but the stimulus for cyclic ADP ribose formation remained unknown until recently. Unexpectedly, studies on sea urchin eggs found that cGMP can evoke intracellular Ca^{2+} release and a rise in cytosolic Ca^{2+}, apparently by stimulating the formation of cyclic ADP ribose (47). These are important findings, with immediate potential relevance for NO/cGMP signal

transduction in the CNS, but whether the mechanism applies to neurons (and other cell types) remains to be seen.

Cyclooxygenases

NO may be important in the regulation of the activity of another type of enzyme, cyclooxygenase (COX). COXs are rate limiting in the synthesis of prostaglandins, thromboxane A2, and prostacyclin from arachidonic acid. COXs comprise two types, inducible and constitutive, known as COX-1 and COX-2 respectively. Like sGC, COXs are heme–containing enzymes that can directly bind NO. The result may be an increase in enzyme activity. McCann and colleagues (103) reported that in hypothalamic fragments, NO mediates the release of PGE_2 that is induced by noradrenaline (acting on α_1 receptors). PGE_2 then stimulates (via cAMP) the release of leuteinizing hormone–releasing hormone. Analogous effects of NO on prostaglandin release occur in various peripheral tissues, including a macrophage cell line in which NO from endogenous or exogenous sources stimulates PGE_2 production via COX-1 and COX-2 in a cGMP-independent manner (109).

Although these results are interesting, they all come from experiments on intact cells, and conclusive evidence for a direct stimulation of COX by NO is lacking. An alternative explanation could be that NO somehow prevents the auto-inactivation of COX (119). The possible relevance of these results to the CNS is that the brain is unique in its expression of inducible COX-2, rather than COX-1, as the predominant isoform of the enzyme under normal conditions (39). In situ hybridization and immunocytochemistry show that COX-2 is present in the forebrain and in discrete populations of neurons elsewhere, with the highest levels being in the granule and pyramidal cells of the hippocampus and in pyramidal cells in the cerebral cortex. Moreover, a rapid but transient (peak after 1–2 h) increase in expression was observed following seizures or synaptic activation of NMDA receptors (143). Future studies must determine if NO regulates COX-2 activity (directly or indirectly) at these locations and, if so, the physiological significance of the resulting production of prostanoids that, like NO, could function as diffusive intercellular messengers.

Other Enzymes

NO can bind to iron-sulfur centers of various enzymes, causing loss of activity (91). These enzymes include *cis*-aconitase (citric acid cycle), NADH-ubiquinone oxidoreductase (mitochondrial complex I), and succinate-ubiquinone oxidoreductase (mitochondrial complex II). NO can also inhibit ribonucleotide reductase, an iron-containing enzyme that is rate limiting for DNA synthesis.

Glycolysis may also be affected as a result of the covalent, NO–stimulated, ADP ribosylation of glyceraldehyde-3-phosphate dehydrogenase. The mechanism appears to involve NO-mediated nitrosylation of a thiol (cysteine) at the

active site, cleavage of bound NAD^+, and then transfer of the resulting ADP-ribose moiety onto the nitrosylated residue. The last step inhibits the enzyme activity (15).

Loss of activity of these enzymes is long lasting and, to produce it, NO needs to be at high levels for prolonged periods of time. Thus these targets should be most relevant to immunological defense, or toxicity, as mediated by the inducible NO synthase.

ACUTE REGULATION OF NEURONAL AND NEUROENDOCRINE FUNCTION BY NO

Neuronal Firing and Ion Channel Modulation

Unlike neurotransmitters, NO or NOS inhibitors applied to individual neurons in vitro frequently do not produce obvious alterations in membrane potential or firing behavior. This result suggests that the actions of NO do not translate directly into acute effects on excitability. Nevertheless, whole-animal studies have provided evidence that the manipulation of central NO levels can markedly affect the sympathetic outflow and, hence, systemic blood pressure (114, 128), alter respiratory rhythm (79), and influence pain thresholds in the spinal cord (84), which suggests that NO is capable of modifying the electrical activity of neurons. We consider some of the possible mechanisms below.

NEURONAL FIRING Two of the clearest examples of the effects of NO on neuronal firing activity have come from studies on invertebrates (molluscs). In one study, the NO donor *S*-nitrosocysteine was perfused onto the buccal ganglion, which is involved in feeding behavior. Increased or decreased firing in different buccal motoneurons occurred, along with associated changes in buccal feeding movements. Inhibitors of NOS had the opposite effects, indicating that NO in this ganglion regulates feeding behavior (89). The other study examined the molluscan equivalent of the olfactory bulb, the procerebral lobe, in which the characteristic electrical activity is an oscillation arising from the interactions between neurons that fire in bursts and nonbursting cells that receive inhibitory signals from the bursting ones (54). Field oscillation frequency was increased by NO donors and decreased by NOS inhibitors. At the single-cell level, NO increased the burst frequency of the bursting cells, thereby increasing the frequency of inhibitory postsynaptic potentials in the nonbursters and eliminating their firing. These results indicate that NO plays an obligatory role in the oscillatory dynamics of neurons in the procerebral lobe and suggest that it participates in odor detection and, possibly, odor learning in this animal.

Modifications in neuronal firing induced by NO have also been observed in mammals. For example, in corticothalamic neurons, NO dampens oscillatory firing behavior (95). In vagal motoneurons, NO donor drugs increase firing rate, and NOS inhibition reduces the excitatory effect of NMDA (129), whereas in the carotid body, NOS inhibitors increase chemoreceptor firing (98, 137). In locus coeruleus neurons, NOS inhibitors augment the amplitude of glutamatergic excitatory postsynaptic potentials (142).

Thus, in different neurons, the net effect of NO on neuronal activity may be either to modulate the firing pattern or to increase or decrease tonic firing rate. However, in most cases whether NO is acting pre- or postsynaptically remains unclear.

ION CHANNEL MODULATION Apart from a direct action mediated through cGMP-gated channels (see above), the NO/cGMP pathway may modulate other ionic conductances, for example, by promoting phosphorylation or dephosphorylation or by modifying cAMP levels. In thalamic neurons, NO donors or 8-bromo-cGMP evoked a small depolarization associated with a fall in input resistance when the membrane potential was maintained at –60 to –90 mV. This change in polarization resulted from a positive shift in the voltage dependence of the hyperpolarization-activated cation conductance, I_h, the net effect being that oscillatory activity was dampened, but tonic firing was unaffected (95). Through this mechanism, NO could play an important role in the regulation of thalamocortical activity.

Ca^{2+} currents can also be influenced, although the effect may differ among neurons. cGMP depresses Ca^{2+} currents in hippocampal neurons (32); NO has a similar action in avian ciliary ganglion neurons (72) and reduces depolarization-induced Ca^{2+} entry into PC12 cells (28). Elsewhere, Ca^{2+} currents may be enhanced, as was first observed in molluscan neurons (96); in rat superior cervical ganglia, for example, intracellularly applied NO donors increase the amplitude of Ca^{2+} currents and also reduce the inhibitory effect of noradrenaline on these same currents (22). In considerations of these different effects on neuronal Ca^{2+} currents, a pertinent observation is that in frog cardiac myocytes, NO donors can increase and decrease the Ca^{2+} current (L-type) depending on their concentration (85).

In smooth muscle, the NO/cGMP pathway activates large Ca^{2+}-dependent K^+ channels ($I_{K(Ca)}$), which leads to membrane hyperpolarization and closure of voltage-sensitive Ca^{2+} channels (105a). In avian ciliary ganglia, however, an NO donor (and L-arginine) inhibited a poorly defined $I_{K(Ca)}$ independently of its action on Ca^{2+} channels (21). Suppression of $I_{K(Ca)}$ postsynaptically will promote repetitive firing, but should it occur presynaptically, increased neurotransmitter release may result.

Neurotransmitter Release and Uptake

The proposal that NO acts presynaptically to modify the release of neurotransmitters (52) has prompted several mainly neurochemical investigations. The effects of NO donors have led investigators to suggest that the release of various transmitters, including excitatory and inhibitory amino acids, catecholamines, and acetylcholine, may be influenced by NO, predominantly in the positive direction. However, crucial controls for the effects of the NO carrier molecules (57) were not usually performed.

More convincing are experiments showing that authentic NO (57), endogenous NO present in unstimulated tissue (16, 99), or NO generated by NMDA receptor activation (29, 60, 88, 122) influences transmitter release.

Indiscriminate stimulation of transmitter release would not, however, be a sensible physiological function for a diffusible molecule such as NO and, unfortunately, relevant physiological investigations are scarce. In cultured hippocampal neurons, one study found that brief exposure to low concentrations of authentic NO (5–10 nM) led to an enduring increase in the frequency of miniature excitatory postsynaptic currents (93)—a result suggesting a presynaptic action on glutamatergic nerve terminals. In contrast, in hippocampal slices, perfusion of 100 nM NO on its own did not affect baseline synaptic transmission (presynaptic fibers stimulated at 0.02 Hz) but, when paired with weak tetanic stimulation (50 Hz for 0.5 s), it induced a long-lasting potentiation of synaptic efficacy, possibly by a presynaptic mechanism (147). On the other hand, perfusion of NO donor drugs, or an inhibitor of cGMP breakdown, reversibly depressed hippocampal synaptic transmission [monitored using low-frequency stimulation (0.033 Hz)], apparently also by a presynaptic mechanism (11). Thus NO may be able to modulate vesicular release of neurotransmitter in either direction, or not at all, depending on the coincident level of presynaptic activity and NO concentration.

A recent study has indicated that NO might affect neurotransmitter uptake (97) because NO donors inhibited the transport of radiolabeled dopamine, serotonin, and glutamate, but not that of noradrenaline, into synaptosome preparations from the striatum. The inhibition had an unexpectedly slow time course and its physiological relevance requires further evaluation.

Neurotransmitter Receptors

Manzoni et al (83) suggested that NO blocked NMDA receptors based on the effects of NO donor drugs on NMDA receptor-mediated currents and proposed that such an action served as a negative feedback mechanism. These effects were not observed in experiments on cerebellar slices (35), but analogous results were obtained from cortical neurons, and Lei et al (76) proposed that the effect was brought about by nitrosation of thiol groups associated with the

receptor. An important criticism of these investigations is the heavy reliance placed on the effects of NO donor drugs that have reactivities not shared by authentic NO and that generate unknown NO concentrations. Many NO donors react with thiols to release NO, and nitrosothiols, for example, can directly transfer NO onto other thiol groups; NO itself, however, cannot directly nitrosate these groups.

Therefore, an essential direction to explore is whether endogenously generated NO inhibits NMDA receptors. Two studies support this possibility, one showing that L-arginine inhibits NMDA-induced increases in intracellular Ca^{2+} levels in cultured striatal neurons, and that this inhibition can be blocked by NOS inhibition or hemoglobin (82), and the other showing that NOS inhibition increases the amplitude of synaptically activated NMDA receptor currents (70). Neither of these studies provide direct evidence for modulation of the receptors by NO, however, and further experiments specifically designed to address the issue are needed.

Neuroendocrine Functions

Several recent reports have indicated that NO regulates hormone release in the hypothalamo-pituitary axis. In explants of the hypothalamus, inhibition of NOS augments release of corticotropin-releasing hormone provoked by elevated K^+ or interleukin-1β, whereas L-arginine and NO donors are inhibitory (24). NOS inhibitors also augment the increased plasma adrenocorticotrophic hormone levels induced by administration of interleukin-1β, vasopressin, and oxytocin in vivo (105), increase plasma oxytocin (but not vasopressin) levels in dehydrated rats (127), augment luteinizing hormone release from dispersed pituitary cells stimulated by gonadotrophin-releasing hormone (20), and potentiate growth hormone release stimulated by growth hormone-releasing hormone in the pituitary (71). In all of these cases, therefore, NO inhibits hormone secretion. Conversely, in other in vitro experiments, NO was proposed to mediate hypothalamic hormone release, including the release of corticotropin-releasing factor induced by interleukin-2 and carbachol, but not by noradrenaline (69); the release of luteinizing hormone-releasing hormone induced by noradrenaline (103) or glutamate (104); and the release of somatostatin induced by growth hormone-releasing factor (1).

NO AND SYNAPTIC PLASTICITY

Synaptic plasticity refers to the capacity of synaptic connections to become selectively and persistently strengthened, or weakened, in response to external stimuli. The phenomenon has long excited neuroscientists attempting to identify a cellular correlate of learning and memory. Long-lasting, activity-dependent changes in synaptic efficacy occur in several brain structures, including

the hippocampus, cortex, and cerebellum, areas that have attracted considerable interest as possible sites for the formation and storage of memory.

The best-studied form of plasticity in the brain is the long-term potentiation (LTP) of synaptic transmission, which is observed at all excitatory synapses in the hippocampal formation. It can be measured as a sustained increase in the magnitude of excitatory postsynaptic potentials (EPSPs) after the delivery of brief trains of high-frequency electrical stimuli to afferent fibers innervating the neurons. LTP recorded at synapses of CA1 pyramidal neurons after the activation of the presynaptic Schaffer collateral-commissural pathway is the most frequently used model for the study of synaptic plasticity, and the type of plasticity in which NO has been most strongly implicated.

Long-Term Potentiation

The molecular and cellular mechanisms that underlie the persistent changes in synaptic strength characterizing LTP must still be resolved. One of the most hotly debated questions concerning LTP, and one that is crucial to understanding the process, concerns the locus (pre- or postsynaptic) at which the modification of synaptic efficacy occurs. For the CA1 region of the hippocampus, a partial answer to this question came from the discovery that the induction of LTP requires postsynaptic NMDA receptor activation and the associated influx of Ca^{2+} (7). However, increasing evidence suggests that at least part of the sustained increase in synaptic strength results from an increased release of the neurotransmitter glutamate from the presynaptic terminal. If induction of LTP is postsynaptic but expression of LTP has at least a component that is presynaptic, then a retrograde trans-synaptic messenger becomes necessary.

NO, being freely diffusible and generated as a result of Ca^{2+} influx associated with NMDA receptor activation, is an attractive candidate for a retrograde messenger (48, 52), but its role in LTP remains controversial.

A first requirement would be that NOS is present in the appropriate postsynaptic neurons and is activated by NMDA receptor stimulation. Until recently, convincing evidence that CA1 pyramidal neurons contained NOS was lacking, although they do stain when the NADPH diaphorase histochemical technique is used (125). A recent immunocytochemical study has shown that CA1 neuronal cell bodies and dendrites in hippocampal sections can be labeled by selective neuronal NOS antibodies provided that unusually gentle fixation techniques are used (138). Surprisingly, immunocytochemistry using antibodies against endothelial NOS has also produced dense staining of the same cells (31). Thus, CA1 neurons probably do contain Ca^{2+}-stimulated NOS. Furthermore, exposure of hippocampal slices to NMDA, or tetanic stimulation of Schaffer collateral fibers, leads to NO formation, as judged by measurements of cGMP accumulation (23, 36).

The second prediction is that NOS inhibitors should block LTP. A number

of LTP studies carried out in the CA1 region of hippocampal slices in vitro have demonstrated this to be so (8, 10, 56, 59, 93, 112). LTP has also been prevented by perfusion of the slices with the NO scavenger hemoglobin (10, 59, 93, 112), which supports a transcellular messenger role for NO because hemoglobin only accesses the extracellular space. However, these studies contain several inconsistencies. One is in the concentration of inhibitor required to block LTP. Whereas Bohme et al (8) reported a complete block of potentiation using a relatively short preincubation of slices with 100 nM L-nitro-arginine, others have required long incubations at 1000- or even 10,000-fold greater concentrations (59, 112). The effects of NOS inhibitors and of hemoglobin may also be the result of inhibition of the depolarization that occurs during the high-frequency stimulus, rather than an effect upon LTP per se (90a). Moreover, the time course of the block of LTP by NOS inhibitors also varies greatly between experiments. Potentiation of synaptic transmission in the CA1 region has been reported to consist of several time-dependent, and possibly mechanistically distinct, phases, of which LTP is a form that persists for over 1 hour. Short-term potentiation (<30 min) and posttetanic potentiation (<5 min) are also observed. It has been reported that NOS inhibitors block all phases of potentiation (8, 93) or, in contrast, leave short-term potentiation intact (59, 112).

A more serious problem is that several groups have been unable to obtain any block of LTP with NOS inhibitors, and a number of confounding variables have come to light. This dilemma has led to the suggestion that NO only participates when a relatively weak stimulus is used for LTP induction (23, 58; but see 56). Possibly the most detailed analysis of experimental differences has been conducted by Williams et al (139), who reported that an effect of NOS inhibitors upon LTP was apparent only in slices from young (5- to 7-week-old) rats and when experiments were performed at a temperature of 24°C. NOS inhibition did not affect LTP when experiments were performed at 30°C in slices from young animals and produced no effects at either temperature in tissue from adult rats. Although some groups reporting positive effects of NOS inhibitors carried out their experiments at low temperatures (59, 112), others used temperatures of 30–32°C (8, 10, 93), which suggests factors other than temperature are also important.

Schuman & Madison (113) used a different experimental protocol to test for the involvement of a diffusible messenger in LTP. They made intracellular recordings from two electrically independent neurons (however, supplied by a common set of afferent fibers) and observed that, in one cell, induction of LTP by means of combined postsynaptic depolarization and low-frequency (1 Hz) stimulation of presynaptic fibers resulted in potentiation at synapses of the second cell, but only if the second cell was nearby, specifically a distance of approximately 150 μm or less, measured at the level of the cell somata.

Potentiation of the second neuron did not occur if LTP was prevented in the first neuron by the inclusion of a NOS inhibitor in the microelectrode. Furthermore, induction of LTP in a population of neurons could overcome the inhibition of LTP imposed on one individual neuron by intracellular application of a NOS inhibitor, suggesting that NO released from noninhibited neurons could compensate for the absence of NO production in the inhibited neuron and result in potentiation. These results provide convincing evidence that NO participates in LTP, at least under certain experimental conditions, and that its diffusible nature allows a previously unsuspected distributed potentiation of synaptic transmission to take place.

Another expectation of the NO hypothesis is that delivery of exogenous NO should, under appropriate circumstances, elicit LTP. One study reported that perfusion of the NO donor sodium nitroprusside caused a depression of synaptic transmission that reverted to a sustained potentiation upon washout (8). In another study, perfusion of a solution containing NO (0.1 μM) had no effect on its own but, when paired with an electrical presynaptic fiber stimulation at a frequency below the threshold for inducing LTP, a potentiation of synaptic responses ensued (147). In contrast, application of several different NO donors during low-frequency stimulation reversibly depressed synaptic transmission in the CA1 region (11).

These apparently disparate findings probably also relate to different experimental conditions and are potentially reconcilable. Because the NO spreads from its source (see above) over a potentially large area, possibly encompassing millions of synapses, it is very unlikely that it acts indiscriminately, especially if one of its roles is to strengthen synapses. A more plausible mechanism would be for NO to modify the behavior of target elements in a manner that depends on their activity, as hypothesized by Gally et al (48). In accordance with this theory, it was found that when presynaptic fibers are stimulated at high frequency (50 Hz) in the presence of NO, synapses become potentiated, but when presynaptic activity is low (0.25 Hz), NO causes synaptic depression (146). Other variables, such as the NO concentration and the duration of exposure to NO, are also likely to be important in determining the outcome.

Taken together, the data support a role for NO in LTP although, given the variable results from in vitro experiments, its precise significance must await in vivo studies. As far as the signal transduction pathway is concerned, high-frequency stimulation results in an accumulation of cGMP in the CA1 region of the hippocampus that is blocked by NOS inhibitors (23). Furthermore, the cGMP analogue 8-Br-cGMP potentiates synaptic responses when paired with a tetanus to the afferent fibers, too weak in itself to induce LTP, and hippocampal slices perfused with selective inhibitors of cG-PK fail to show LTP after a high-frequency stimulus (145). These findings suggest that cGMP mediates NO–dependent LTP.

Long-Term Depression

Implicit in the idea that synapses can be strengthened is a recognition that they may also be depressed. The phenomenon of long-term depression (LTD) can be observed in the hippocampus upon repetitive low-frequency stimulation (1–2 Hz) of presynaptic axons (34), and the induction of LTD reportedly can be blocked by NOS inhibitors and hemoglobin (67), although these results need to be confirmed.

A more widely studied example of LTD is in the cerebellum, where it is thought to be a correlate of cerebellar motor learning (65). It is manifest as a persistent depression of EPSPs recorded at the synapse between parallel fibers and Purkinje cells, the only output neurons of the cerebellar cortex. Purkinje cells have two glutamatergic excitatory inputs via the parallel fibers and climbing fibers; LTD occurs when the two are stimulated simultaneously, and this process requires activation of both AMPA and metabotropic glutamate receptors (78). The expression of LTD appears to be postsynaptic, because quantal analysis has failed to demonstrate any change in presynaptic transmitter release (63), and a decreased sensitivity of postsynaptic AMPA receptors has been reported.

In the absence of climbing-fiber activation, parallel-fiber stimulation results in LTP rather than LTD (106); thus a signal produced by the climbing fibers is needed for the development of LTD. Climbing-fiber activation generates Ca^{2+}-mediated action potentials in Purkinje cell dendrites. The production of these potentials is associated with a large influx of calcium ions, and LTD can be abolished if this process is disrupted (107). The actual mechanism by which the parallel fibers and climbing fibers interact to bring about a reduced sensitivity of postsynaptic AMPA receptors is far from understood, although NO appears to participate under at least some conditions.

Two experimental strategies have been employed to induce LTD in vitro. In one, AMPA receptors and metabotropic receptors are simultaneously activated by exogenous agonists and in the other, parallel fiber-mediated EPSPs are paired with calcium spike firing elicited by depolarization of the postsynaptic cell. Using both these approaches, LTD could be blocked by perfusing the preparations with inhibitors of NOS (26, 27, 66) and also with hemoglobin (66). Shibuki & Okada (115) found further evidence implicating NO in cerebellar LTD. Using an electrochemical NO probe, they demonstrated endogenous NO release following climbing-fiber stimulation. Moreover, exogenous NO or cGMP could substitute for climbing-fiber activation so that, when paired with parallel fiber activity, LTD ensued.

The source of the NO in cerebellar LTD remains uncertain because Purkinje cells do not appear to contain NOS. Since climbing-fiber lesions result in a reduced release of NO, as measured either electrochemically or by biochemical

assessment of cGMP accumulation (115, 124), NO is probably released from climbing fibers themselves or from other cells onto which these fibers synapse. Purkinje cells appear to be the target for NO because dialysis of Purkinje cells with NO donors or in conjunction with parallel fiber stimulation, depresses the parallel fiber-mediated EPSP (27, 61). Hence, the decreased responsiveness of the postsynaptic AMPA receptors may depend upon an increased concentration of cGMP in Purkinje cells and, putatively, a subsequent activation of protein kinase G (61, 66).

In spite of these apparently compelling results implicating NO in LTD, studies using cultured Purkinje cells have shown that LTD obtained by pairing depolarization of the cell with iontophoretic glutamate pulses is unaffected by NOS inhibitors or hemoglobin and that it could not be replicated by the NO donor nitroprusside (55, 77). As with hippocampal LTP, NO-dependent and -independent forms of cerebellar LTD appear to exist.

NEURAL NO AND LOCAL CEREBRAL BLOOD FLOW

Although the role of endothelial-derived NO in the regulation of blood vessel tone is well established (87), the identification of neurons as a source of NO led to the hypothesis that NO may represent the long-sought factor that couples increased local blood flow to neural activity (48). Considerable data suggest that NO participates in the maintenance of resting cerebrovascular tone and, more controversially, that it mediates certain vasodilator responses (38, 64), but the role of NO derived from central neurons, as opposed to endothelial cells or parasympathetic NOS-containing fibers, is still unclear. One finding shows that topical application of NMDA to the rabbit brain in vivo results in a vasodilatation of arterioles on the pial surface that was blocked by NOS inhibitors and reproduced by nitroprusside (37). However, the response to NMDA was also blocked by tetrodotoxin, which inhibits neuronal firing, thereby indicating that the NO signal was not a direct consequence of NMDA receptor activity but may be the result of stimulation of neuronal circuits.

The question was recently explored using a different technique. Akgoren et al (4) electrically stimulated the parallel fibers in the rat cerebellum, which led to a frequency dependent increase in blood flow at the cerebellar surface. This response was reduced by a NOS inhibitor, by an elevated Mg^{2+} concentration (which inhibits synaptic transmission), and most revealingly, by an inhibitor of the AMPA and kainate types of non-NMDA glutamate receptors. These results, together with those showing that activation of AMPA and kainate receptors is associated with NO production in the cerebellum (123) and that parallel fiber-mediated synaptic transmission is blocked by AMPA and kainate antagonists (51) make the first convincing case for the idea that

NO derived from central neurons as a result of synaptic activation of glutamate receptors can influence local blood flow.

CONCLUDING COMMENTS

Recent years have seen an explosion of interest in NO in the CNS, resulting in the implication of this unexpected and atypical messenger molecule in a wide range of neural functions. Because of space limitations, we have focused mainly on the cellular and molecular mechanisms of NO signaling rather than on its broader proposed roles, such as in learning, cognition, and the regulation of sensory, motor, and sexual behavior. We have also left aside discussion of the possible pathological roles of NO, when generated either by excessive glutamate receptor stimulation or as a result of iNOS expression, in CNS disease states.

At the cellular and synaptic level, it is probably unrealistic to define a function of the NO-cGMP signaling pathway in the CNS in the same way that one does with conventional neurotransmitter systems because the NO-cGMP pathway is so different. For a spatially diffuse signal such as NO to have biological meaning, a different set of rules must govern specificity of action. In synapses, for example, the effect of NO appears to depend on the coincident functional state of the participating cellular elements, to the extent that the net result can be an increase, a decrease, or no change in synaptic efficacy. An important task is to understand NO-cGMP signal transduction mechanisms more precisely. However, little is known about the actions of cGMP in the CNS compared with other tissues such as smooth muscle and platelets, although, as discussed above, several promising avenues are opening up. The identification of a potent and selective inhibitor of the NO-stimulated guanylyl cyclase enzyme (J Garthwaite et al, submitted) should greatly assist progress.

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