

The NO hypothesis: Possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system

(cerebral blood flow/long-term potentiation/synaptic plasticity/nitric oxide)

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ABSTRACT Several observations suggest that the Ca^{2+} -dependent postsynaptic release of nitric oxide (NO) may be important in the formation and function of the vertebrate nervous system. We explore here the hypothesis that the release of NO and its subsequent diffusion may be critically related to three aspects of nervous system function: (i) synaptic plasticity and long-term potentiation in certain regions of the adult nervous system, (ii) the control of cerebral blood flow in such regions, and (iii) the establishment and activity-dependent refinement of axonal projections during the later stages of development. In this paper, we detail and analyze the basic assumptions underlying this NO hypothesis and describe a computer simulation of a minimal version of the hypothesis. In the simulation, a 3-dimensional volume of neuropil is presented with patterned afferent input; NO is produced, diffuses, and is destroyed; and synaptic strengths are determined by a set of synaptic rules based on the correlation of synaptic depolarization and NO levels. According to the hypothesis, voltage-dependent postsynaptic release of this rapidly diffusing substance links the activities of neurons in a local volume of tissue, regardless of whether the neurons are directly connected by synapses. This property is demonstrated in the simulation, and it is this property that is exploited in the hypothesis to account for certain aspects of long-term potentiation and activity-dependent sharpening of axonal arbors.

Recent experiments indicate that nitric oxide (NO) is produced in the granule cells of the cerebellum in response to glutamate application (1, 2). Other work demonstrates that NO is the endothelial-derived relaxing factor responsible for relaxation of vascular smooth muscle (3). The Ca^{2+} -dependent enzyme nitric oxide synthetase is found in the vertebrate forebrain (4) as well as in the cerebellum (5). Cortical activity is well known to be accompanied by regionally specific changes in blood flow, and therefore we suggest that NO production in a cortical region is one cause of these local vascular responses.

There are reasons to suggest an even more fundamental role for NO in altering synaptic efficacy within various brain regions in the adult as well as in synaptogenesis during development. For example, a body of data has accumulated demonstrating that the synaptic changes of long-term potentiation (LTP) result from the temporal correlation of presynaptic activity and postsynaptic depolarization (6). A number of studies support the notion that LTP is the result of separate presynaptic and postsynaptic components, both of which can contribute to its induction and maintenance (6–11). In development, the segregation and refinement of afferent projections also critically depends upon correlated electrical activity and synaptic changes both within afferent projections and

between the afferents and their targets (12–22). The *N*-methyl-D-aspartate receptor and excitatory transmission in general have been implicated in this segregation (17, 18, 21, 22). A relationship between LTP and activity-driven sharpening of axonal connections has been recently suggested (21, 22). The question arises: what factors could subservise this relationship in neural regions extending beyond an interactive pair of neurons?

An attractive possibility is that the developmental segregation of afferents and synaptic plasticity in the developed nervous system could both be subserved by diffusible paracrine factors that are released into the extracellular space. Arachidonic acid has been suggested as a candidate substance contributing to LTP (23, 24). Recently, Williams *et al.* (23) have suggested that the signal from the postsynaptic site to the presynaptic site in LTP may be NO.

In this paper, we propose that a signal with effects extending well beyond any such pair of synaptic elements is required to account for the observations. We emphasize those properties of NO that make it a particularly attractive candidate for such a signal. We suggest a hypothesis on the possible mode of synaptic action of NO in the adult and developing nervous system. We explore the proposed action of NO by explicit computer modeling of a minimal version of the hypothesis which is applicable to any diffusible substance with similar properties.

BACKGROUND AND THEORY

As illustrated in Fig. 1A, NO is rapidly released from cerebellar granule cells in response to the application of glutamate or related amino acids (1, 2, 25, 26). The glutamate effect is blocked by *N*-methyl-D-aspartate receptor antagonists (1, 25, 26). NO is synthesized by a Ca^{2+} -dependent enzyme that requires Ca^{2+} levels well above those seen in resting neurons (4, 5), so that the rate of NO synthesis within any neural network would be expected to depend on the amount of ongoing excitatory synaptic activity. In biological tissue, NO is oxidized to nitrite and nitrate within a few seconds (27). NO can bind to the heme moiety of soluble guanylate cyclase to initiate the production of cGMP (28), and it is known to activate a protein ADP-ribosyltransferase (29). Because NO is a nonpolar gas like O_2 or CO_2 , it can readily diffuse across cell membranes in an isotropic fashion.

Taken together, these properties of NO prompt the idea that it could serve as a short-lived diffusible substance capable of acting as a signal returning from postsynaptic to both presynaptic and postsynaptic sites within some volume of tissue (Fig. 1B, 23). Such signals could act not only between synapses on the same neuron (heterosynaptic signals, 30), but also among synapses on different neurons (heterocellular signals, 31, 32).

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Abbreviation: LTP, long-term potentiation.

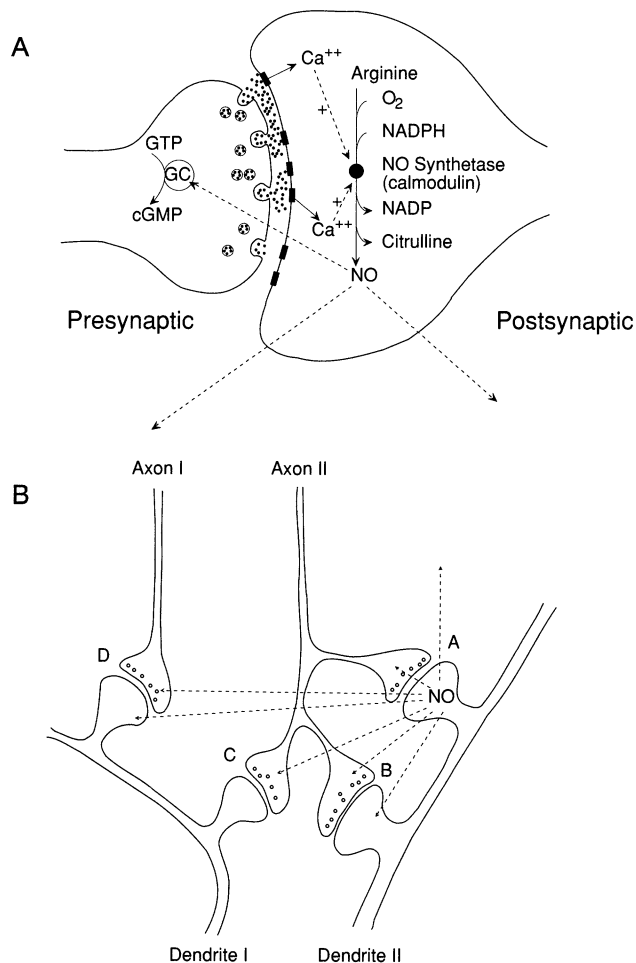


FIG. 1. (A) Schematic diagram of an excitatory synapse illustrating biochemical steps involved in the production, diffusion, and utilization of NO within the neuropil. Glutamate released from presynaptic sites (black dots) binds to *N*-methyl-D-aspartate receptors (filled rectangles) and other receptors within the postsynaptic membrane and induces depolarization and the consequent entry of Ca^{2+} . Ca^{2+} binds to calmodulin, which, in turn, activates nitric oxide synthetase. This enzyme catalyzes the oxidation of arginine, producing NO and citrulline. NO readily diffuses across cell membranes and can activate soluble guanylate cyclase in neighboring presynaptic and postsynaptic sites. (B) Diagram of some neuropil components that illustrates the variety of possible effects of NO. Axon I is inactive and axon II is firing; thus axon II is actively releasing glutamate at synapses A, B, and C. Consistent with the set of synaptic rules used in the simulation, the production of NO at synapse A contributes to the strengthening of synapse A (homosynaptic effect) as well as synapse B (heterosynaptic effect). NO also acts on synapses of dendrites of other neurons (heterocellular effects), tending to strengthen those that fire coordinately (synapse C) and weaken those that do not fire coordinately (synapse D).

The NO Hypothesis. This background provides the basis for the NO hypothesis as it applies to the adult central nervous system. Specifically, the NO hypothesis consists of the following three statements:

(i) Postsynaptic elevation of Ca^{2+} concentration after synaptic activity causes NO production and release from postsynaptic sites in such a fashion that NO alters the synaptic efficacy both of its synapses of origin and of the synapses in the surrounding space. This diffusible signal thus acts within the preestablished neuroanatomy and according to the temporal correlation of presynaptic activity and postsynaptic depolarization in a given volume of tissue. A set of synaptic rules that is consistent with this part of the hypothesis is detailed in the description of the computer

simulation. In particular, we propose that those synapses that fire in a coordinated manner in the same region of neural space are strengthened and those that are not so coordinated are weakened.

(ii) The production of NO reflects the synaptic activity of a local region of neuropil and therefore acts as an indicator of local metabolic demand. Because of its action on vascular smooth muscle, NO controls local capillary blood flow in a need-dependent fashion and, hence, is among the agents that subserve the so-called evoked vascular response (33).

(iii) The effects of NO or any similar signals acting as described in these statements play major roles in the formation and stabilization of synapses during development. This part of the hypothesis, which is not simulated in the current work, is detailed further in the *Discussion*.

The NO hypothesis specifically proposes one mechanism by which NO-mediated alterations in the distribution of synaptic strengths lead to the formation of neuronal groups (34). In any given volume, NO production at a particular time is a function of the distribution of voltages in the population of synapses that occupy that volume. Thus, at any particular time, the distribution of NO in a given volume of tissue reflects the temporal correlations previously present in that volume. In this mechanism, it is the movement of the NO due to diffusion that provides a way by which temporal correlations in neighboring volumes of tissue can be communicated.

Simulation of the NO Hypothesis (part i) in a Minimal Version. The assumptions of the NO hypothesis involve complex spatiotemporal interactions among synapses distributed within a given neuroanatomy. To explore these interactions, we performed a computer simulation of the behavior of neural networks, the connectivity and activity of which are modeled on those present in the central nervous system of vertebrates. The simulation was done on an NCUBE/10 parallel computer using a minimal version of the cortical network simulator (35), modified by P.R.M. to allow explicit simulation of 3-dimensional space, diffusion, and changing network connectivity during a run.

The model consists of three layers of synapses distributed within a 3-dimensional box-shaped volume (Fig. 2). Each synaptic layer contains 16,384 synapses for a total of 49,152 synapses. The volume of the simulated neuropil is divided into 32,768 cubic diffusion compartments with an edge length of $100 \mu\text{M}$, so that the volume is 3.2 mm along each edge. The synaptic layers are situated in a region of this space that is $3.2 \text{ mm} \times 3.2 \text{ mm} \times 0.6 \text{ mm}$ along the x , y , and z axes, respectively (Fig. 2). In the model, all synapses are excitatory, and all are potential sites of NO formation. The afferent axonal input to this synaptic array enters topographically but displays randomized variance in the shapes of its terminal arbors (Fig. 2). Each postsynaptic site receives a single presynaptic connection. The synaptic strengths vary from 0.0 to 1.0 and the initial values are drawn from a Gaussian distribution (mean = 0.43, SD = 0.14).

A specific set of phenomenological rules governing the changes in synaptic strength proposed in the NO hypothesis was adopted in this particular simulation: (i) when the presynaptic ending releases an excitatory neurotransmitter at a time that local NO concentration is high (above some threshold T_1), the subsequent strength of that synapse is increased, (ii) when a presynaptic ending releases an excitatory neurotransmitter at a time that local NO concentration is low (below some threshold T_2), the subsequent strength of that synapse is decreased, and (iii) when an excitatory presynaptic ending fails to release neurotransmitter at a time when the local NO concentration is high (above some threshold T_3), the subsequent strength of that synapse is decreased. To make the synaptic rules biochemically realistic, the three thresholds T_1 , T_2 , and T_3 are not identical. In the simulation described here, $T_2 < T_1 < T_3$.

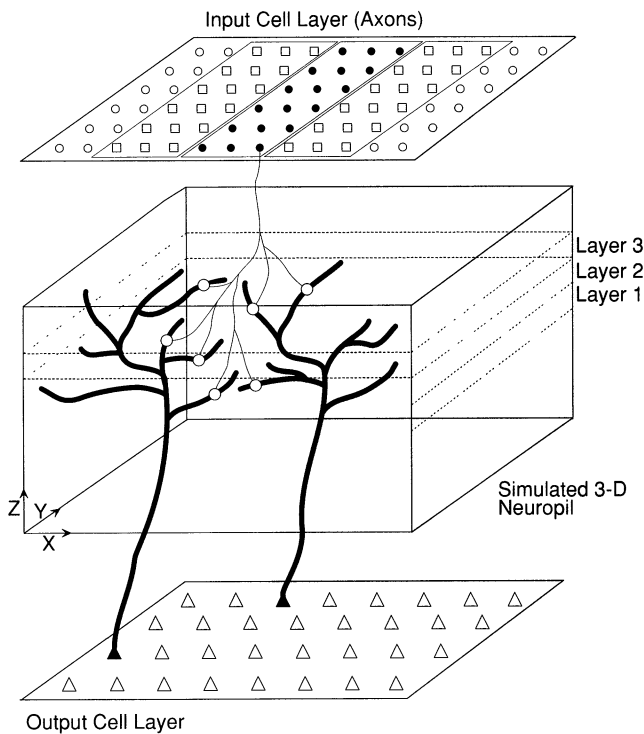


FIG. 2. Schematic diagram of the neural connections in the simulation. Stimuli consist of patterns (one bar or two flanking bars) presented alternately to the input cell layer. This patterned stimulation results in the firing of axons that originate in the input cell layer and terminate in the rectangular block of simulated 3-dimensional (3-D) neuropil. □, Cells active when double bar stimulus is presented; ●, cells active when single central bar stimulus is presented. There are 4096 axons originating in the input cell layer, and 4096 cells in the output cell layer. We have illustrated only one representative axonal arbor and two representative dendritic arbors. The probability for axons to form synapses in each of the three layers within the simulated neuropil is equal. During simulated synaptic activity, the concentration of NO within each diffusion compartment is calculated and used to modulate the strength of the synapses according to the described rules. The probability of firing of each cell in the output layer is determined by summing the voltages at all synapses on its dendritic arbor. For illustrative purposes, this layer is located beneath the neuropil because the NO concentration does not directly affect any properties of cells in the output layer.

During a simulation, an alternating series of patterns of stimuli is presented to the neuropil by simulating activity in the input cell layer that provides the axonal input into the neuropil (Fig. 2). Synaptic strengths and transmembrane potentials are calculated individually for each synapse. Thus, action potentials at any particular synapse will act to depolarize only that particular postsynaptic site, and the degree of depolarization at each postsynaptic site will be a function of the synaptic strength of the particular synapse and the presynaptic firing rate.

Postsynaptic depolarization gives rise to the production of NO at each synapse at a rate proportional to the magnitude of the depolarization above a preset voltage threshold. The active destruction of NO molecules is simulated as a simple exponential decay with a half-life that is fixed in each run (4 sec in the run described in Fig. 3). The concentration of NO at any time in a given region of space is calculated by summing the amount locally produced, subtracting the amount degraded, and calculating changes due to diffusion. Diffusion is assumed to occur isotropically according to the measured diffusion constant of NO (2.60×10^{-5} cm²/sec, ref. 36). The diffusion constant together with the chosen spatial dimensions of the diffusion compartments determines the

length of the time steps in the simulation (0.46 sec per iteration for the simulation presented here).

Postsynaptic linkage occurs at dendritic arbors of the simulated output cell layer (Fig. 2). The dendrites arborize directly (topographically) above their cell of origin and extend to a maximal distance of 900 μ m away from the cell body in both the *x* and *y* directions (Fig. 2). During each time step, the postsynaptic potentials are summed, and those cells in which the total depolarization exceeds a preset threshold are recorded as firing. The spatial distribution of synaptic voltage, synaptic strengths, concentration of NO, and the activity in the output cells are recorded and visually displayed at each time step.

RESULTS

The results presented in this short report do not represent a quantitative analysis (P.R.M. and G.M.E., unpublished observations) of the host of emergent consequences of assuming a rapid diffusive link in a synaptic network. Instead, we use the simulation to emphasize the character and self-consistency of some of the ideas presented in the NO hypothesis.

A representative experiment is illustrated in Fig. 3. Shown is a plane through only one synaptic layer, although all synaptic layers are active and the movement of NO takes place in three dimensions throughout the simulated tissue. Two spatial patterns of activation of the input cell layer were used as stimuli. A single rectangular region of cells (8×14) in the input cell layer was activated for a period of time; this activation was then halted, and two flanking rectangular regions of input cells (both 8×14) were activated for the same period of time (Fig. 3). The exact temporal sequence was: (i) single rectangular region for 1.8 sec, (ii) no input for 0.92 sec, (iii) two flanking rectangular regions for 1.8 sec, and (iv) no input for 0.92 sec. Events *i*, *ii*, *iii*, and *iv* were continually repeated during the simulation.

In Fig. 3 row A, a single rectangular region of activity in the input cell layer (axons) is simulated and the synaptic voltage (voltage), the synaptic strengths (strength), the [NO], and the firing pattern of the output cell layer (cells) are displayed. The spatial distribution of NO (see legend for Fig. 3) shows the highest production of NO in the central region of the pattern of synaptic voltage. Row A represents the activity after 1.8 sec of stimulation. The same basic behavior is seen in Fig. 3 row B for the presentation of the two flanking rectangular regions of activity. In both cases, in the early phases of patterned input to the neuropil, the distribution of synaptic strengths is rather uniform and there are only scattered and weak patterns of firing in the output cell layer.

After 20 to 30 sec of patterned stimulation, however, the spatial distribution of synaptic strengths has organized into a pattern that reflects the interactions of the preexisting anatomy, the temporal correlation present in the input axons, and the 3-dimensional diffusive link mediated by NO. Fig. 3 rows C and D illustrates the state of the network 23.5 and 21.2 sec after stimulation was begun. Due to changes in the distribution of synaptic strengths, the pattern of cell firing in response to the single rectangular region of input activity has coalesced into a single domain composed of strongly firing cells. The pattern of depolarization in the synaptic layer has also organized into a spatially coherent region. The spatial profile of the [NO] shows that a steady state has been reached in response to the strong and continuous pattern of input activity.

The synaptic voltage response to the flanking rectangular regions (Fig. 3 row D) demonstrates that, in this case, the synaptic strengths have also organized into coherent domains that drive the firing of two separate groups of neurons. The model was found to be more sensitive to the relationships between the three concentration thresholds for synaptic change than to the half-life of NO. The half-life of NO was varied from 1.5 sec to 4.4 sec, and the alteration in the spatial

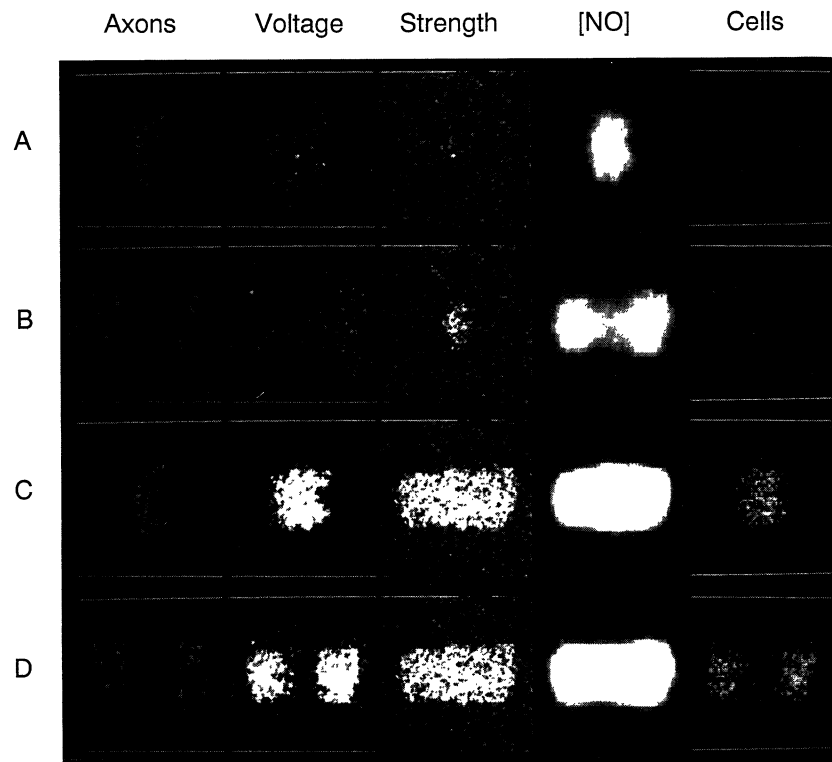


FIG. 3. Development of segregated regions of neuropil as a result of correlated input. Data in each of the five columns is coded as a 128-level gray scale so that the magnitude of each parameter increases with increasing brightness. This figure displays data for a plane passing through only one of the synaptic layers. Axons, pattern of cell firing in the (64×64) input cell layer that provides axons into the simulated neuropil. Voltage, distribution of postsynaptic voltages; Strength, distribution of connection strengths between the input axons and dendrites present; [NO], spatial profile of the NO concentration; Cells, pattern of cell firing in the (64×64) output cell layer. Times after initiation of the stimulus presentation were 1.8 sec, 4.6 sec, 23.5 sec, and 21.2 sec for rows A–D, respectively. Depending on the thresholds for NO-dependent changes in synaptic strength, time required for significant alterations in the distribution of synaptic strengths could be <1 –2 sec.

distribution of synaptic strengths (Fig. 3) was qualitatively the same.

DISCUSSION

Clearly, synaptic strengths can be altered as a result of neural activity in other ways (30, 39). Some advantages afforded by the additional mechanism described here are: (i) the synaptic changes are not confined to the axonal or dendritic arbors of particular cells but act on synapses within a space, and (ii) the mechanism couples blood flow to neural activity.

Functional Consequences of the Hypothesis. The results of these simulations show that, in a network with a diffusive signal like the one hypothesized, patterned neuronal input can give rise to spatially segregated regions of altered synaptic strengths and the consequent spatial grouping of neuronal activity. In a network in which the anatomy is already established, such a diffusive signal allows axon terminals to compete with one another for synaptic strength changes in a manner essentially equivalent to competition for local volumes of tissue (34). Those synapses with activity well correlated with the activity of their spatially proximate neighbors increase their efficacy, and those synapses that are “out of step” with their neighbors decrease their synaptic efficacy. Certain aspects of this particular simulation are reminiscent of reaction diffusion systems (37). The overall mechanism in the nervous system differs from such systems largely, however, because axonal connections allow distant regions of space to affect each other with little time delay—i.e., as though the regions were neighbors.

A number of different agents may serve as a diffusive signal, and a computer model is incapable of establishing which of these substances actually serves such a function. We assume that NO functions as one such signal, and

synaptic rules were chosen so that most of the biochemically reasonable contingencies were included. We realize, however, that a number of other synaptic rules could achieve the same results and elucidating the actual relationship between NO and changes in synaptic efficacy will require a better understanding of the biochemical and physiological details of the actions of NO in a functioning nervous system.

Although it has not been definitely shown that NO is actually synthesized *in vivo* in a functioning central nervous system, a great deal of suggestive evidence supports its presence there (1, 2, 4, 5, 25, 26). A particularly strong case for its presence is provided by the effects of neural activity on local blood flow. The known physiologic function of NO in regulating blood flow in other systems and the dynamic relationship between cerebral blood flow and neural activity that provides the bases of all positron-emission tomography scan studies are readily reconciled by the NO hypothesis. Indeed, the spatial and temporal changes in the concentration of NO that are seen in our simulation are consistent with those that would be expected if the dynamic changes in vascular diameters observed in a functioning brain result in part from the action of NO on vascular smooth muscle in a given neural region.

Developmental Aspects of the Hypothesis. A diffusible signal like NO could have important consequences in shaping neuroanatomy during development. The shape of terminal axonal arbors throughout the vertebrate central nervous system and the anatomic segregation of these arbors into separate layers, slabs, barrels, blobs, and columns have been shown to depend upon the pattern of correlated neuronal activity during development. We suggest that the production of NO (or some alternative short-lived, diffusible signal) in response to correlated afferent activity plays a central role in

the formation, segregation, and shaping of neuropil during the later stages of development. The presence of such a signal provides a means of matching afferent axonal arbors and their recipient dendritic arbors in such a way that a preexisting template is not required and in such a way that correlations present in the afferent input can be reflected in the final anatomy of the network. We briefly describe how this can occur.

Growth cones of axons release neurotransmitter into their surroundings as they migrate toward their targets. We need only assume that, upon entering a volume of tissue characterized by a temporally correlated suprathreshold NO concentration, the growth cone would be induced to branch and form synapses. This action would determine the eventual location and shape of the terminal arbors. Growth cones located far from dendritic targets (e.g., in white matter) or in a dendritic area releasing an out-of-phase return signal would, according to the NO hypothesis, tend not to branch or to form synapses in that region, but would continue to seek an appropriately responding target space.

This behavior would account for the many experimental demonstrations that patterns of neural activity direct the formation and refinement of terminal axonal and dendritic arbors *in vivo* and *in vitro* and that these effects can be disrupted by agents that block *N*-methyl-D-aspartate receptors or appropriately correlated electrical activity (12–22). This conclusion is consistent with glutamate-induced production and release of NO and the disruption of this effect by agents that block glutamatergic transmission (1, 25, 26). The requirement that the formation and stable maintenance of synapses during development depends on temporally correlated high intracellular levels of NO and Ca²⁺ in both presynaptic and postsynaptic sites (Fig. 1a) would provide an epigenetic mechanism to account for the highly specific segregation of multiple axons into a common region of space (e.g., ocular dominance columns, barrel fields, somatotopic maps) as well as the segregation of individual axonal input to specific target neurons (e.g., climbing fiber to Purkinje cell, retinal ganglion cell to relay cells in the lateral geniculate nucleus). This mechanism could also account for the ubiquity of reciprocal excitatory or reentrant connections (34) in the vertebrate forebrain.

Signals from the postsynaptic to the presynaptic neuron mediating such developmental effects have been proposed in other contexts (38, 39). In contrast to the kind of return signal proposed here, however, such retrograde signals have generally been assumed to act in a synapse-specific manner and to be transported to the soma of the presynaptic neuron where they act by affecting patterns of gene expression. Although signals of this kind are known to exist and are important in the autonomic nervous system (39), the suggested roles of NO presented here are distinct from such proposals in two major ways: the NO return signal is distributed within an extended volume of space, and it acts epigenetically within a number of different presynaptic and postsynaptic sites in that space.

Parts of the NO hypothesis can be tested directly by observing whether or not one can disrupt the predicted acute effects of NO (such as the generation of LTP or the regulation of cerebral blood flow) by applying *N*-methylarginine, a specific inhibitor of the NO-synthesizing enzyme (40). Because the inhibitor may disrupt normal protein synthesis and function, the developmental aspect of the hypothesis may not admit a test of this sort. The general idea of a spatially distributed return signal proposed and simulated here is also applicable to other candidate substances and must be analyzed and tested appropriately in each case.

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