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Synaptic plasticity: **A role for nitric oxide in LTP**

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Nitric oxide is back in the spotlight with a new series of studies showing that it plays an important role in long-term potentiation, the best-studied type of synaptic plasticity in the central nervous system thought likely to play an important role in learning and memory.

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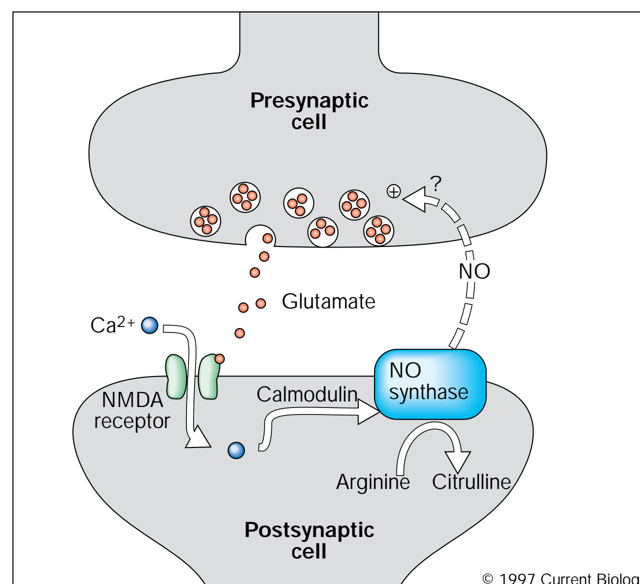
Four years after being named 'molecule of the year' [1], nitric oxide (NO) is still fashionable. A biological role for this highly soluble, membrane-permeant gaseous molecule was first recognized a decade ago when endothelial-dependent relaxation factor, an intercellular messenger mediating vascular relaxation, was identified as NO. Since this debut, NO has been implicated as a messenger molecule in a variety of cellular functions, including synaptic plasticity in the central nervous system.

Specifically, researchers have suggested that NO plays a critical role in the expression of long-term potentiation (LTP). LTP is a form of synaptic plasticity in which synaptic transmission is enhanced for hours or days in response to repetitive stimulation of presynaptic terminals. One consequence of such repetitive stimulation is an influx of Ca^{2+} through postsynaptic *N*-methyl-D-aspartate (NMDA) receptor channels. Although the chain of events leading to increased synaptic strength is unknown, this postsynaptic Ca^{2+} influx is agreed to be a minimal requirement for LTP induction. LTP is under intense study because it is believed to be an important mechanism underlying memory formation in the brain.

A thorny issue in LTP research is the site of its expression. That is, the increase in synaptic strength mediated by LTP may be due to an enhancement of presynaptic transmitter release or postsynaptic transmitter sensitivity, or both. A number of studies favor the idea that there is at least some change in presynaptic properties [2], which begs the question of how events induced postsynaptically — such as an influx of Ca^{2+} in the postsynaptic cell — result in presynaptic changes. The simple answer involves a membrane-permeant retrograde 'messenger': released by the postsynaptic cell, this messenger would diffuse across the synaptic cleft to the presynaptic terminal and trigger the changes associated with LTP (see Fig. 1).

Figure 1



LTP is dependent on Ca^{2+} influx through postsynaptic NMDA receptors. According to the hypothesis that NO acts as a retrograde messenger, the Ca^{2+} binds to calmodulin and activates NO synthase, which converts arginine to NO and citrulline. NO then diffuses to the presynaptic terminal, leading to enhanced transmitter release.

Several candidates for the role of LTP retrograde messenger have been proposed, including NO. Evidence that NO is involved in LTP has been mixed, however. Biologically, NO is synthesized from L-arginine, with the stoichiometric production of citrulline, by a family of enzymes called nitric oxide synthases (NOS). Constitutively expressed forms of the NOS family are regulated by Ca^{2+}/calmodulin, providing a potential link to LTP induction events. In early studies, inhibitors of NOS were applied to hippocampal slices to see whether they inhibited the expression of LTP [3]. NOS inhibitors, such as *N*-nitro-L-arginine and N^G-methyl-L-arginine, were found to block LTP when applied extracellularly or intracellularly to the postsynaptic cell. Hemoglobin, a NO scavenger which is not taken up by cells, also blocked LTP when applied extracellularly. These results provided evidence that NO is involved in LTP of hippocampal synapses, and that it must be released extracellularly for LTP to occur.

Problems have been raised with this neat picture, however. Of particular concern is the expression pattern

of NOS *in vivo*. Studies conflict on whether neuronal NOS (nNOS), the major NOS subtype expressed in the brain, is present in hippocampal pyramidal neurons [4]. Mice with a targeted mutation of the nNOS gene are capable of normal LTP [5], suggesting this NOS subtype is not necessary for the expression of LTP. Furthermore, additional studies show that the blockade of LTP by NOS inhibitors is decidedly dependent on experimental conditions, such as temperature or the stimulation pattern used to induce LTP [2].

At this critical juncture, three studies have come to the rescue of NO [6–8]. All along there has been the suspicion that a subtype of NOS other than nNOS might be involved in LTP. For instance, LTP induced in nNOS mutant mice can be blocked by NOS inhibitors, indicating another NOS subtype is active in these synapses. Indeed, endothelial NOS (eNOS) is well-expressed in hippocampal pyramidal neurons [9]. So Son *et al.* [6] have performed the obvious next step: test LTP in mice with targeted mutations of eNOS and in mice with mutations of both eNOS and nNOS.

Son *et al.* [6] found that, like nNOS mutant mice, eNOS mutant mice express LTP normally. Furthermore, the addition of NOS inhibitors significantly reduces the LTP expressed in eNOS mutant mice, again suggesting some NOS functions in LTP in these mutants — yet another NOS subtype, perhaps? When Son *et al.* [6] tested mice with targeted mutations of both eNOS and nNOS, they found LTP was reduced in magnitude to about 50 % that of wild-type mice, and that this level was not further reduced by NOS inhibitors. From these results, Son *et al.* [6] concluded that NO is indeed involved in LTP, but that the nNOS and eNOS subtypes can compensate for one another.

These conclusions must be evaluated in the light of another recent study, in which Kantor *et al.* [7] exploited an intriguing difference between eNOS and nNOS to distinguish their roles in LTP. Uniquely among the characterized NOS subtypes, eNOS is localized to the cell membrane by the cotranslational addition of the fatty acid myristate to its amino-terminal glycine. This process of myristoylation is critical to the function of eNOS, facilitating the extracellular release of NO [10].

Kantor *et al.* [7] tested the effect of disrupting the myristoylation of eNOS, by incubating hippocampal slices with hydroxymyristic acid (HMA), an inhibitor of the enzyme that performs myristoylation. After a day of incubation in HMA, the slices failed to exhibit LTP. To demonstrate that this effect was specifically due to disrupted eNOS function, Kantor *et al.* [7] constructed a chimeric molecule consisting of the functional domain of eNOS fused to the transmembrane domain of CD8, creating a form of eNOS which localizes to the membrane in the absence of myristoylation. When hippocampal slices were transfected with an adenovirus containing this chimera, LTP was not inhibited by incubation with HMA. This ingeniously designed rescue experiment demonstrates that the inhibition of LTP by HMA is specifically due to the disruption of membrane localization of eNOS.

Kantor *et al.* [7] concluded that membrane-targeted eNOS is necessary for LTP, and that its function cannot be compensated by nNOS. This contradicts the results of Son *et al.* [6], which imply that nNOS and eNOS can be interchanged in LTP function. One possible explanation of this conflict is that mutant animals deprived through development of one NOS subtype can compensate with another subtype, but that adult wild-type animals cannot do so. If this is the case, the results of the above studies suggest eNOS is probably the NOS subtype normally involved in LTP. A further difficulty is that Kantor *et al.* [7] found a complete block of LTP when eNOS function was disrupted, whereas the eNOS/nNOS double mutant mice studied by Son *et al.* [6] showed reduced, but still robust LTP. Given this contradiction, and keeping in mind the reported variable effects of NOS inhibitors on LTP, it seems reasonable to think there may be NOS-dependent and independent components of LTP, expressed under different conditions.

While these studies provide evidence that NO is involved in LTP, little work has been done to address whether NO acts as the LTP retrograde messenger. A recent study [8] may ameliorate this state of affairs. In synapses between cultured hippocampal neurons, an LTP-like phenomenon is observed when a strong tetanic stimulus is applied to the presynaptic neuron. Arancio *et al.* [8] took advantage of this accessible culture system to test the effects of injecting NO agents into the presynaptic or postsynaptic cell. For instance, they found that NOS inhibitors blocked potentiation when injected into the postsynaptic, but not the presynaptic, cell, consistent with the presumed postsynaptic origin of a retrograde messenger.

Arancio *et al.* [8] also found that pairing a weak presynaptic stimulation, which alone does not result in potentiation, with a burst of NO — released from a ‘caged’ NO molecule by photoactivation — induced potentiation of these hippocampal synapses in culture. This potentiation was seen whether the NO burst was applied in the postsynaptic or presynaptic cell. But when the NO burst was applied in the postsynaptic cell, extracellularly applied NO scavengers blocked the potentiation, suggesting the NO must leave the postsynaptic cell to function. When the NO was applied in the presynaptic cell, however, extracellularly applied NO scavengers did not block the potentiation, suggesting NO has its ultimate effect in the presynaptic cell.

This observed behavior fits the profile of the hypothetical LTP retrograde messenger. Caution is required, however, in extrapolating these observations to the LTP seen in brain slices and *in vivo*. First, there is the question of whether the observed potentiation phenomenon applies to LTP. Also, previous studies of the effects of exogenous NO application on slices have produced mixed results; in at least one study [11], pairing release of NO from a caged molecule with weak presynaptic stimulation did not induce LTP in slices. Caveats aside, the case for NO being involved in LTP is looking a great deal brighter. Until recently, it was questionable whether NOS even existed in the very synapses where it was supposed to act. Now, eNOS has been definitively localized to hippocampal pyramidal neurons, and nNOS is probably also present in the same cells. The effects of specifically disrupting nNOS and/or eNOS by molecular and genetic approaches indicates NOS, particularly eNOS, plays an significant role in LTP, at least under some conditions.

Further circumstantial evidence that eNOS generates the retrograde messenger in LTP induction comes from the enzyme's Ca^{2+}/calmodulin regulation and membrane localization. Ca^{2+} influx through postsynaptic NMDA receptors could stimulate eNOS production and extracellular release of NO, which could then diffuse to the presynaptic terminal. How NO would then effect an increase in transmitter release is a matter for speculation. NO has several known effectors, particularly soluble guanylyl cyclase, which produces cGMP, and there is some evidence that cGMP and cGMP-dependent protein kinase are involved in LTP [12]. Other potential NO targets in LTP include ADP-ribosyltransferase and cyclo-oxygenase enzymes.

Although some steps have been made towards confirming that NO is a retrograde messenger in LTP, much remains to be done. Further studies must determine, for example, whether NO really does act presynaptically in LTP, and must elucidate its mode of action. A major problem is the confusion over the basic events of LTP — whether there are presynaptic changes at all — which makes studying the molecules involved a troublesome business. Difficulties may also arise from the possibility, which may be related to the confusion just described, that there are NO-dependent and NO-independent forms of LTP. Sorting through these issues will ensure that researchers of NO and LTP are occupied for a while to come.

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