

Nitric Oxide Acts Directly in the Presynaptic Neuron to Produce Long-Term Potentiation in Cultured Hippocampal Neurons

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Summary

Nitric oxide (NO) has been proposed to act as a retrograde messenger during long-term potentiation (LTP) in the CA1 region of hippocampus, but the inaccessibility of the presynaptic terminal has prevented a definitive test of this hypothesis. Because both sides of the synapse are accessible in cultured hippocampal neurons, we have used this preparation to investigate the role of NO. We examined LTP following intra- or extracellular application of an NO scavenger, an inhibitor of NO synthase, and a membrane-impermeant NO donor that releases NO only upon photolysis with UV light. Our results indicate that NO is produced in the postsynaptic neuron, travels through the extracellular space, and acts directly in the presynaptic neuron to produce long-term potentiation, supporting the hypothesis that NO acts as a retrograde messenger during LTP.

Introduction

Chemical neurotransmission has traditionally been thought to occur only in an anterograde direction, from the presynaptic to the postsynaptic neuron. However, evidence from a number of systems suggests that signaling can also occur in a retrograde direction, from the postsynaptic to the presynaptic neuron (Murphey and Davis, 1994). Such retrograde signaling has been postulated for long-term potentiation (LTP) in the CA1 region of the hippocampus, where the induction of LTP usually requires activation of postsynaptic NMDA receptors but the expression of LTP appears to involve, in part, a presynaptic increase in transmitter release (Bliss and Collingridge, 1993; Hawkins et al., 1993). Nitric oxide (NO), a soluble gas that can diffuse through cell membranes, has been suggested to act as a retrograde messenger during LTP (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992), but the role of NO has been controversial (Bliss and Collingridge, 1993; Hawkins, 1996). For example, inhibitors of NO synthase have blocked LTP under some experimental circumstances but not under others. Recently, it was shown that LTP is reduced in mice with

targeted mutations of the neuronal and endothelial isoforms of NO synthase (Son et al., 1996 [this issue of *Cell*]). This result supports the involvement of NO in LTP, but does not address the question of whether NO acts as a retrograde messenger.

One of the difficulties in testing the retrograde message hypothesis has been lack of access to the presynaptic terminal in hippocampal slices. The hippocampal cell culture system provides a good model for studying presynaptic mechanisms (Bekkers and Stevens, 1990; Malgaroli and Tsien, 1992) because LTP can be induced while delivering drugs intracellularly to either the pre- or postsynaptic cell. We have previously demonstrated that LTP can be induced reliably in monosynaptically connected pairs of cultured hippocampal neurons by high frequency trains of depolarization (tetani) of the presynaptic neuron during temporary removal of Mg^{2+} from the bath (Arancio et al., 1995). Like LTP in slices, LTP in culture requires Ca^{2+} influx through postsynaptic NMDA receptor channels, which are normally blocked by extracellular Mg^{2+} . However, the time course of the potentiation in culture differs somewhat from that in slices in that there is relatively less PTP and STP immediately after the tetani. Brief application of NO to the cultures produces a long-lasting increase in the frequency of spontaneous miniature synaptic currents, suggesting that NO could act presynaptically to enhance transmitter release during LTP (O'Dell et al., 1991). To examine the role of NO in LTP of evoked transmitter release, we have studied potentiation of synaptic transmission between pairs of hippocampal neurons in culture following intra- or extracellular application of (i) an NO scavenger, (ii) an inhibitor of NO synthase, and (iii) a membrane-impermeant NO donor, CNO-4, that releases NO only upon photolysis with UV light (Makings and Tsien, 1994; Lev-Ram et al., 1995). Our results indicate that NO is synthesized in the postsynaptic cell, travels through the extracellular space, and acts directly in the presynaptic neuron to enhance transmitter release during LTP.

Results

To test for the possible involvement of NO in LTP of evoked synaptic currents in culture, we first applied oxymyoglobin (MbO_2 , 10 μM), a membrane impermeant scavenger of NO, to the bath. MbO_2 blocked induction of LTP by tetanic stimulation (Figure 1). By contrast, tetanic stimulation induced significant potentiation following bath application of metmyoglobin ($Mbmet$), a compound with a much lower affinity for NO than MbO_2 (Gorbunov et al., 1995). MbO_2 did not have any effect on the baseline excitatory postsynaptic current (EPSC) compared to control experiments with no tetanic stimulation, where there was a slight rundown of the EPSC due to the whole-cell ruptured patch recording (Arancio et al., 1995). A two way ANOVA with one repeated measure (test time) revealed that the four training procedures in Figure 1D produced significantly different amounts of potentiation ($F[3,56] = 9.47$, $p < .01$), and there was

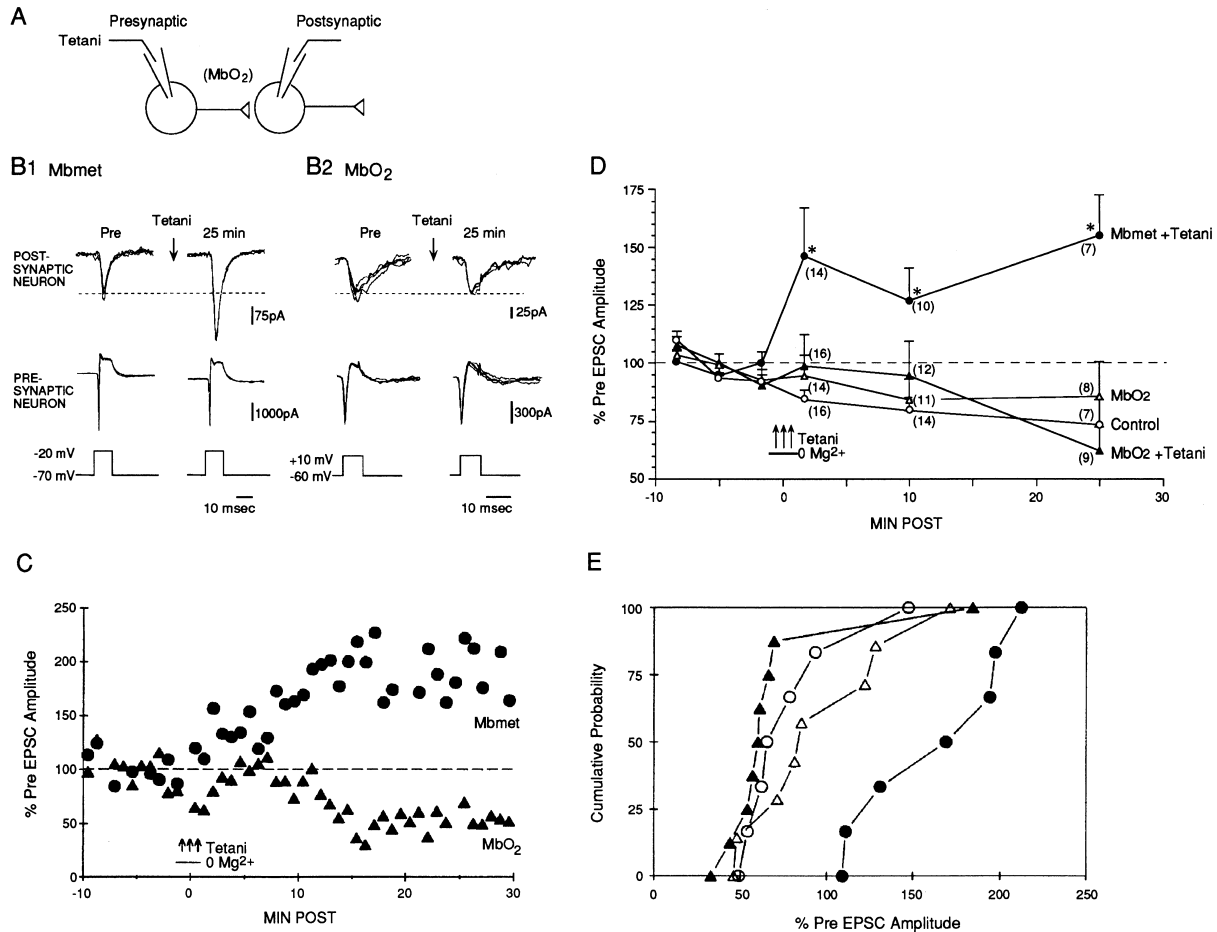


Figure 1. Extracellular Oxymyoglobin (MbO₂), but Not Metmyoglobin (Mbm), Blocks Tetanus-Induced LTP

(A) Experimental arrangement.

(B₁) Example of potentiation by tetanic stimulation with Mbmet in the bath. EPSCs were produced in the postsynaptic neuron by step depolarization that elicited an inward current in the presynaptic neuron once every 50 s. The current in the presynaptic neuron has had leakage subtracted. Both recordings are a.c. coupled. Sample traces are shown before (Pre) and 25 min after tetanic stimulation of the presynaptic neuron (three 50 Hz, 2 s trains of depolarizations at 20 s intervals) during brief perfusion with Mg²⁺-free solution. Four successive traces are superimposed at each time period. The dashed line shows the average Pre value.

(B₂) Example of block of potentiation by extracellular MbO₂.

(C) Trial-by-trial results from two experiments like the ones shown in (B). EPSC amplitude has been normalized to the average value during the 10 min before training (Pre) in each experiment. Tetanic stimulation (3 arrows) occurred at time zero. The horizontal bar shows the time during which the bath solution was changed to one with 0 Mg²⁺.

(D) Average potentiation by tetanic stimulation with Mbmet in the bath (closed circles) and block of potentiation by MbO₂ in the bath (closed triangles). MbO₂ by itself (open triangles) did not cause any effect on EPSC amplitude compared to test-alone control (open circles). Each point represents the average of four successive trials, and the numbers in parentheses indicate the n at that point. Individual experiments were terminated when the electrode seal was lost or the input resistance changed in either the pre- or postsynaptic cell. The points indicate the geometric means, the error bars indicate SEM, and asterisks indicate a significant difference from the Pre level (dashed line). Average Pre values were 51 pA, 47 pA, 50 pA, and 112 pA, not significantly different by a one-way analysis of variance (ANOVA).

(E) Cumulative probability distributions of the potentiation 25 min after training in the four groups shown in (D). Symbols the same as in (D). Each point represents an individual experiment.

no significant interaction. Subsequent analysis showed that tetanic stimulation with Mbmet in the bath produced significantly greater potentiation than each of the other training procedures ($p < .01$ in each case), which were not significantly different from each other. The results of individual experiments 25 min after training showed the same pattern (Figure 1E). These results are similar to data previously obtained in experiments on hippocampal slices, where hemoglobin blocks tetanus-induced LTP (O'Dell et al., 1991; Schuman and Madison,

1991; Haley et al., 1992), and suggest that NO must travel across the extracellular space to cause long-lasting enhancement of the EPSC.

We next wished to take advantage of the culture system by injecting various substances intracellularly into either neuron through the patch pipette. Because the two cell bodies are usually less than 100 μm apart, even fairly large molecules injected into the cell bodies should diffuse to the synapses within minutes (Popov and Poo, 1992). In a previous study (Arancio et al., 1995), we

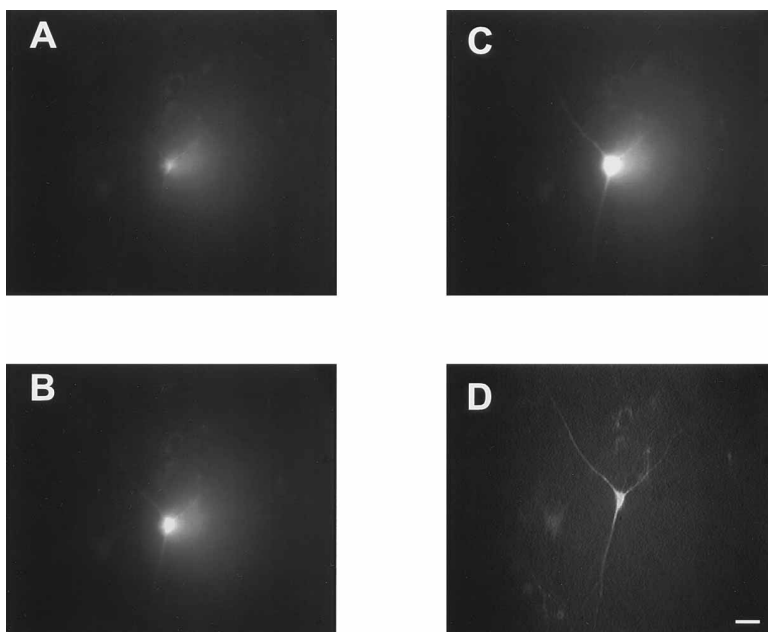


Figure 2. Diffusion of Myoglobin to Distal Processes of a Cultured Hippocampal Neuron

Fluorescein-labeled myoglobin was included in the pipette solution during ruptured patch whole-cell recording, and the neuron was imaged with a CCD attached to an epifluorescence microscope 1 min (a), 5 min (b), 12 min (c), and 12.5 min (d) after breaking into the cell. The pipette, which is out of focus in (a)–(c) was gently removed between (c) and (d). Scale bar = 30 μm .

obtained three lines of evidence supporting this prediction: (i) Lucifer Yellow observed under a fluorescent microscope reached distal processes, (ii) BAPTA injected into the presynaptic cell blocked synaptic transmission, and (iii) cGMP injected into the presynaptic cell produced activity-dependent potentiation of synaptic transmission. All three effects occurred within 10 min of the start of injection. As an additional test, we included fluorescein-labeled myoglobin (which has a larger molecular weight than Lucifer Yellow or BAPTA) in the patch pipette. As shown in Figure 2, myoglobin reached the distal processes of the neuron within 12 min of breaking into the cell. The neurons were then fixed and immunostained for synaptophysin (a marker of presynaptic terminals), which revealed that myoglobin had reached synaptic areas of the injected neuron (Figure 3). These results encouraged us to use the intracellular injection technique to examine the role of NO in LTP.

To test whether NO must travel through either the pre- or postsynaptic cell, we injected MbO₂ into the pre- or postsynaptic neuron through the patch pipette. Injection of MbO₂ (50 μM) into either the pre- or postsynaptic neuron blocked potentiation of the EPSC by tetanic stimulation in 0 Mg²⁺. In interleaved experiments without myoglobin, tetanic stimulation in 0 Mg²⁺ induced significant potentiation (Figure 4). The EPSC was still potentiated 45 min after tetanic stimulation in two experiments that lasted that long (\bar{X} = 44% increase). In some experiments Mbmet was injected into either the pre- or postsynaptic neuron, and tetanic stimulation produced similar potentiation (Figure 4E). Intracellular injection of MbO₂ alone into either the presynaptic cell or the postsynaptic cell had no effect on the baseline EPSC amplitude. A two-way ANOVA with one repeated measure (test time) revealed that the five training procedures in Figure 4D produced significantly different amounts of potentiation ($F[4,53]$ = 7.20, p < .01), and there was no significant interaction. Subsequent analysis showed that tetanic stimulation produced significantly greater

potentiation than each of the other training procedures (p < .01 each case), which were not significantly different from each other.

These data are consistent with the hypothesis that NO travels from the postsynaptic neuron, through the extracellular space, to the presynaptic neuron during LTP. However, other routes are possible. Furthermore, because MbO₂ can bind other molecules in addition to NO (for instance CO), these experiments do not uniquely identify the retrograde messenger (Stevens and Wang, 1993; Zhuo et al., 1993). We therefore used an inhibitor of NO-synthase, N^G-monomethyl-L-arginine (L-NMMA). Similarly to previous experiments in hippocampal slices (O'Dell et al., 1991; Schuman and Madison, 1991), injection of L-NMMA (0.5 μM) into the postsynaptic neuron blocked potentiation of the EPSC by tetanic stimulation in 0 Mg²⁺. By contrast, following injection of L-NMMA into the presynaptic neuron, tetanic stimulation induced significant potentiation (Figure 5). The EPSC was still potentiated 45 min after tetanic stimulation in 4 experiments that lasted that long (\bar{X} = 23% increase). In control experiments, injection of L-NMMA into either the post- or presynaptic cell had no effect on the baseline EPSC amplitude. A two-way ANOVA revealed that the four training procedures in Figure 5D produced significantly different amounts of potentiation ($F[3,26]$ = 5.53, p < .01), and there was no significant interaction. Subsequent analysis showed that tetanic stimulation with presynaptic L-NMMA produced significantly greater potentiation than each of the other training procedures (p < .05 in each case), which were not significantly different from each other. At a higher concentration (100 μM), L-NMMA in either the presynaptic (n = 5) or postsynaptic (n = 5) neuron blocked potentiation (data not shown), possibly due to nonspecific actions of the inhibitor (which has an IC₅₀ in the low micromolar range in isolated preparations; East and Garthwaite, 1990), or to a leak of the inhibitor outside the injected cell.

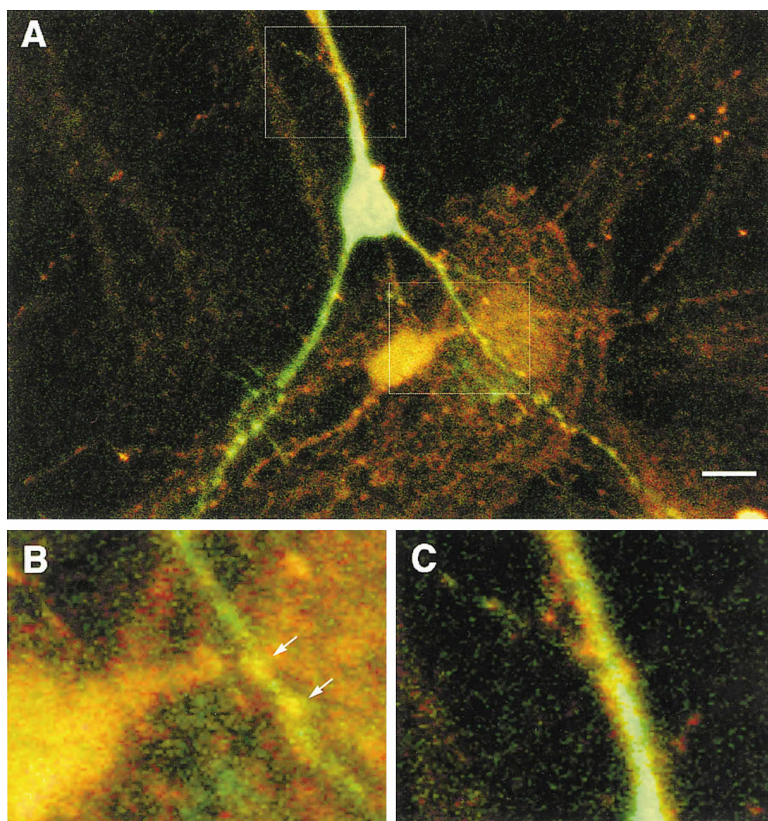


Figure 3. Double Labeling for Fluorescein-Myoglobin (green) and Synaptophysin Immunofluorescence (red) Showing That Myoglobin Diffuses to Synaptic Regions of the Injected Cell

A neuron was loaded with fluorescein-myoglobin for 12 min as shown in Figure 2, immediately fixed in paraformaldehyde, processed for synaptophysin immunofluorescence, and imaged with a confocal microscope.

(A) The cell body and process of the injected neuron (green) and synaptophysin labeling of neighboring neurons (red). Synaptophysin is concentrated in synaptic terminals, but can also be seen in the cell bodies of cultured neurons (Fletcher et al., 1991). Scale bar = 15 μ m.

(B) Higher power view of the lower right-hand box in (A). The two arrows point to yellow spots where myoglobin and synaptophysin are colocalized, which may be presynaptic terminals on the axon of the injected neuron. (C) Higher power view of the upper left-hand box in (A) showing an area where myoglobin and synaptophysin are adjacent, which may be postsynaptic zones on a dendrite of the injected neuron.

These results suggest that NO is involved in potentiation at synapses between individual hippocampal neurons in culture, and that NO synthase is activated in the postsynaptic neuron during induction of potentiation (perhaps by Ca^{2+} influx through NMDA receptor channels). As an additional test of the involvement of NO, we applied exogenous NO to see if it induced long-lasting potentiation of the EPSC. Brief (30–60 s) bath application of NO (10 nM) paired with a weak tetanus (a 50 Hz, 0.5 s train of depolarizations in normal Mg^{2+} and 50 μ M APV, to avoid potentiation by the tetanic stimulation alone) produced a rapid enhancement of the EPSC that lasted for the remainder of the experiment (Figure 6). NO had no consistent effect on the currents in either the pre- or postsynaptic cell during the tetanus, suggesting that it does not act simply by enhancing the effectiveness of the tetanus. Cells that received either vehicle solution paired with weak tetanus or NO alone showed no increase in EPSC amplitude, compared to control cells that received only test stimulation.

These data are similar to previous results in slices (Zhuo et al., 1993) and support the hypothesis that NO is involved in LTP. However they do not discriminate between possible sites of action of NO. We therefore injected MbO_2 (50 μ M) into either the post- or presynaptic neuron through the patch electrode and then applied exogenous NO paired with weak tetanus. Following injection of MbO_2 into the postsynaptic neuron, paired stimulation induced significant potentiation (Figure 6). The EPSC was still potentiated 45 min after NO paired with weak tetanus in four experiments that lasted that long (\bar{X} = 37% increase). By contrast, injection of MbO_2

into the presynaptic neuron blocked the potentiation, whereas injection of $Mbmet$ did not (Figure 6E). A two-way ANOVA revealed that the six training procedures in Figure 6D produced significantly different amounts of potentiation ($F[5,71] = 11.07$, $p < .01$), and there was no significant interaction. Subsequent analysis showed that NO paired with weak tetanus with or without postsynaptic MbO_2 produced significantly greater potentiation than each of the other training procedures ($p < .05$ in each case), which were not significantly different from each other.

These results demonstrate that NO must gain access to the presynaptic neuron to produce potentiation. As an additional test of the site of action of NO, we used a membrane-impermeant NO donor, CNO-4, that releases NO only upon photolysis with UV light (Makings and Tsien, 1994; Lev-Ram et al., 1995). This approach also permits better temporal and spatial control of the release of NO. Consistent with our results when NO was applied in the bath, intracellular injection of CNO-4 (20 μ M) into the postsynaptic neuron, followed by photolytic release of NO (~ 10 nM) starting at the same time as weak tetanic stimulation (paired training), caused a rapid and long-lasting enhancement of the EPSC (Figure 7). The EPSC was still potentiated 45 min after paired training in one experiment that lasted that long (37% increase). There was no potentiation if either the CNO-4, UV light flash, or weak tetanus was omitted. There was also no potentiation if photolytic release of NO started immediately after the end of the weak tetanus (unpaired training), demonstrating that the effects of NO and the weak tetanus are synergistic and not simply additive during paired

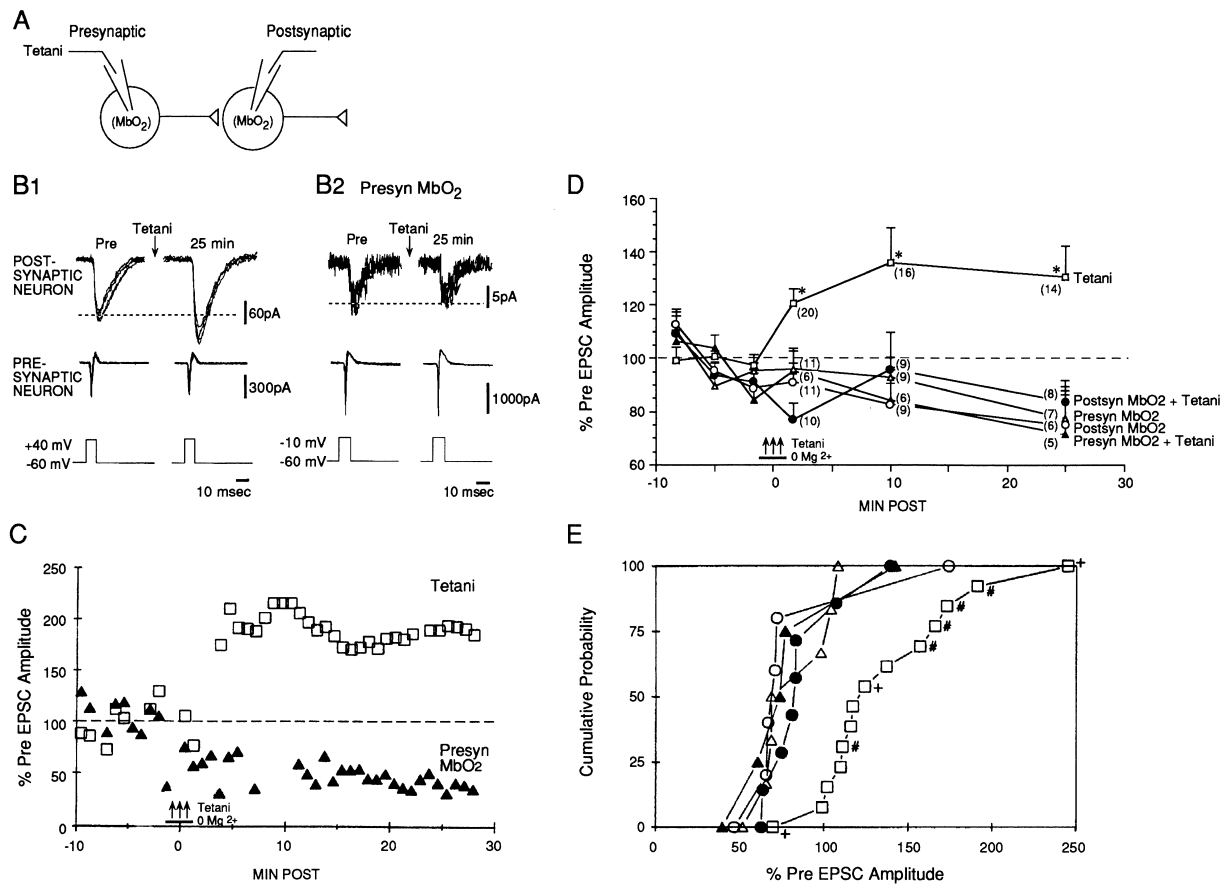


Figure 4. Tetanus Induced LTP Can Be Blocked by Injection of Oxmyoglobin (MbO₂) into the Pre- or Postsynaptic Neuron

(A) Experimental arrangement.

(B₁) Example of potentiation by tetanic stimulation in an interleaved experiment without MbO₂.

(B₂) Example of block of potentiation by injection of MbO₂ into the presynaptic neuron.

(C) Trial-by-trial results from two experiments like the ones shown in (B).

(D) Average potentiation by tetanic stimulation during brief perfusion with 0 Mg²⁺ saline (open squares). In some of these experiments, Mbmet was injected into either the presynaptic (n = 6) or the postsynaptic (n = 3) neuron. Results with and without Mbmet were not significantly different and have been pooled. By contrast, the potentiation was blocked by injection of MbO₂ into either the presynaptic neuron (closed triangles) or the postsynaptic neuron (closed circles). Intracellular injection of MbO₂ alone into either the presynaptic cell (open triangles) or the postsynaptic cell (open circles) had no effect on the EPSC amplitude. Average pre values were 69 pA, 174 pA, 60 pA, 178 pA, and 224 pA, not significantly different by a one-way ANOVA.

(E) Cumulative probability distributions of the potentiation 25 min after training in the five groups shown in (D). # indicates experiments in which Mbmet was injected into the presynaptic neuron, and + indicates experiments in which it was injected into the postsynaptic neuron.

training. A two-way ANOVA revealed that the five training procedures in Figure 7D produced significantly different amounts of potentiation ($F[4,38] = 6.61, p < .01$), and there was no significant interaction. Subsequent analysis showed that photolysis of caged NO paired with weak tetanus produced significantly greater potentiation than each of the other training procedures ($p < .01$ in each case), which were not significantly different from each other. These results show that potentiation by NO paired with presynaptic activity has strict temporal specificity, comparable to that of potentiation by postsynaptic depolarization paired with presynaptic activity (Kelso et al., 1986).

Release of NO in the postsynaptic cell corresponds to the presumed physiological situation. Enhancement by postsynaptic release of NO was blocked by the addition of MbO₂ (10 μM) to the bath (Figure 8), consistent

with the hypothesis that NO produced in the postsynaptic neuron must cross the extracellular space to produce potentiation. By contrast, intracellular injection of CNO-4 into the presynaptic neuron, followed by photolytic release of NO at the same time as weak tetanic stimulation, caused a rapid and long-lasting increase of the EPSC even with MbO₂ in the bath. There was no potentiation if either the UV light flash or the weak tetanus was omitted. A two-way ANOVA revealed that the four training procedures in Figure 8D produced significantly different amounts of potentiation ($F[3,30] = 8.12, p < .01$), and there was no significant interaction. Subsequent analysis showed that photolysis of caged NO in the presynaptic neuron paired with weak tetanus produced significantly greater potentiation than each of the other training procedures ($p < .01$ in each case), which were not significantly different from each other. These

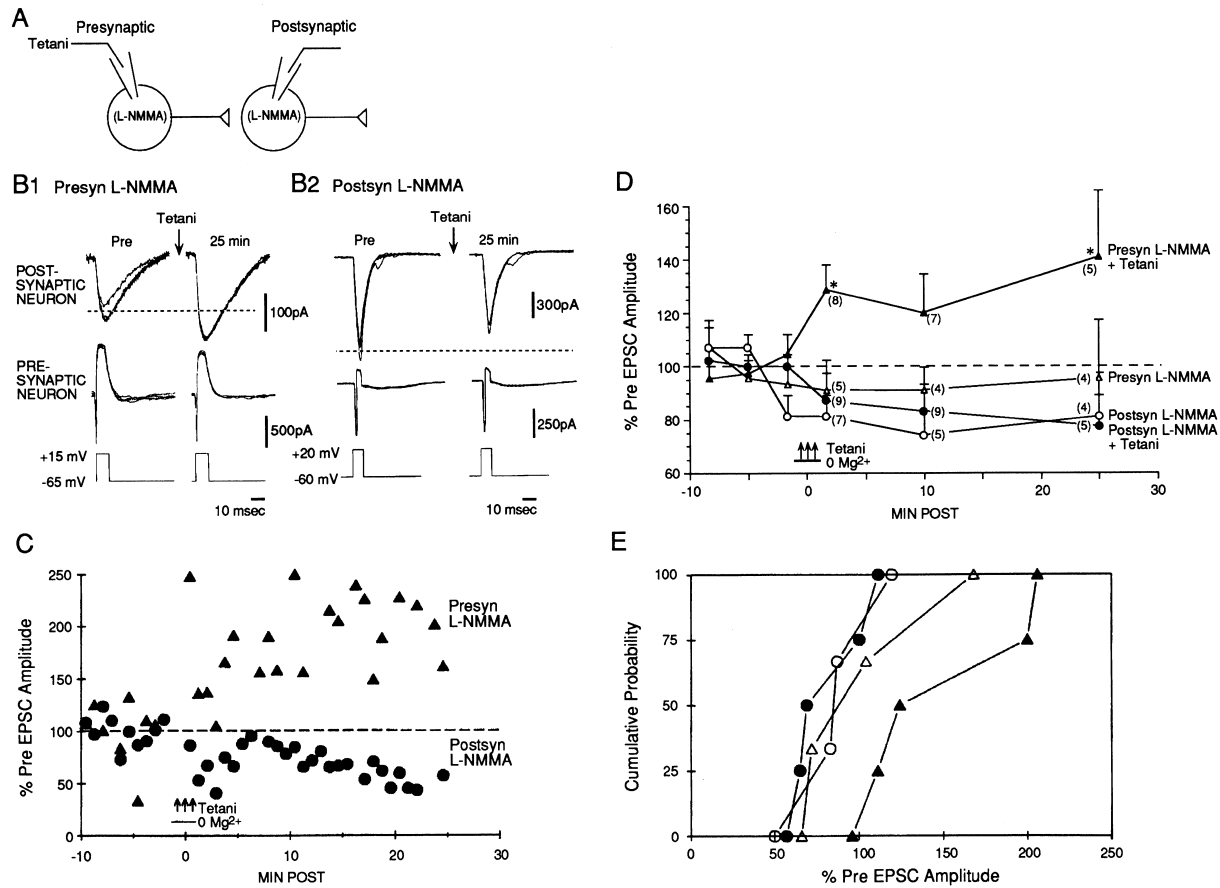


Figure 5. Tetanus-Induced LTP Can Be Blocked by Injection of the NO-Synthase Inhibitor, N^G-monomethyl-L-arginine (L-NMMA) into the Postsynaptic but Not the Presynaptic Neuron

(A) Experimental arrangement.

(B₁) Example of potentiation by tetanic stimulation following injection of L-NMMA into the presynaptic neuron.

(B₂) Example of block of potentiation by injection of L-NMMA into the postsynaptic neuron.

(C) Trial-by-trial results from two experiments like the ones shown in (B).

(D) Average potentiation by tetanic stimulation following injection of L-NMMA into the presynaptic neuron (closed triangles), and block of potentiation following injection of L-NMMA into the postsynaptic neuron (closed circles). Injection of the NO-synthase inhibitor alone into the postsynaptic (open circles) or the presynaptic (open triangles) cell had no effect on EPSC amplitude. Average Pre values were 151 pA, 741 pA, 135 pA, and 537 pA, not significantly different by a one-way ANOVA.

(E) Cumulative probability distributions of the potentiation 25 min after training in the four groups shown in (D).

results demonstrate that NO need be present only in the presynaptic neuron to produce activity-dependent long-lasting potentiation.

Discussion

Our data are consistent with previous results from experiments on hippocampal slices, where LTP can be blocked by oxyhemoglobin in the bath or NO synthase inhibitors in either the bath or the postsynaptic neuron (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992), and bath application of NO donors or NO can produce activity-dependent long-lasting potentiation (Bohme et al., 1991; Zhuo et al., 1993). The data are also consistent with previous observations on cultured hippocampal neurons, where NO produces a long-lasting increase in the frequency of spontaneous miniature EPSCs (mEPSCs), suggesting

a presynaptic effect (O'Dell et al., 1991). However, those previous results did not unequivocally identify the source of NO or its site of action. For example, NO might act indirectly on the presynaptic neuron via another cell, or an increase in mEPSC frequency might be due to a postsynaptic effect (Isaac et al., 1995; Liao et al., 1995). We have taken advantage of the cell culture system, where it is possible to inject substances into either the pre- or postsynaptic neuron, to demonstrate more directly that (i) NO is synthesized in the postsynaptic and not the presynaptic neuron, (ii) NO must travel through the extracellular space to produce potentiation, and (iii) NO acts directly in the presynaptic and not the postsynaptic neuron.

These results are all consistent with the hypothesis that NO acts as a retrograde messenger during the induction of LTP. However, some alternative explanations of the data should be considered. First, our results with

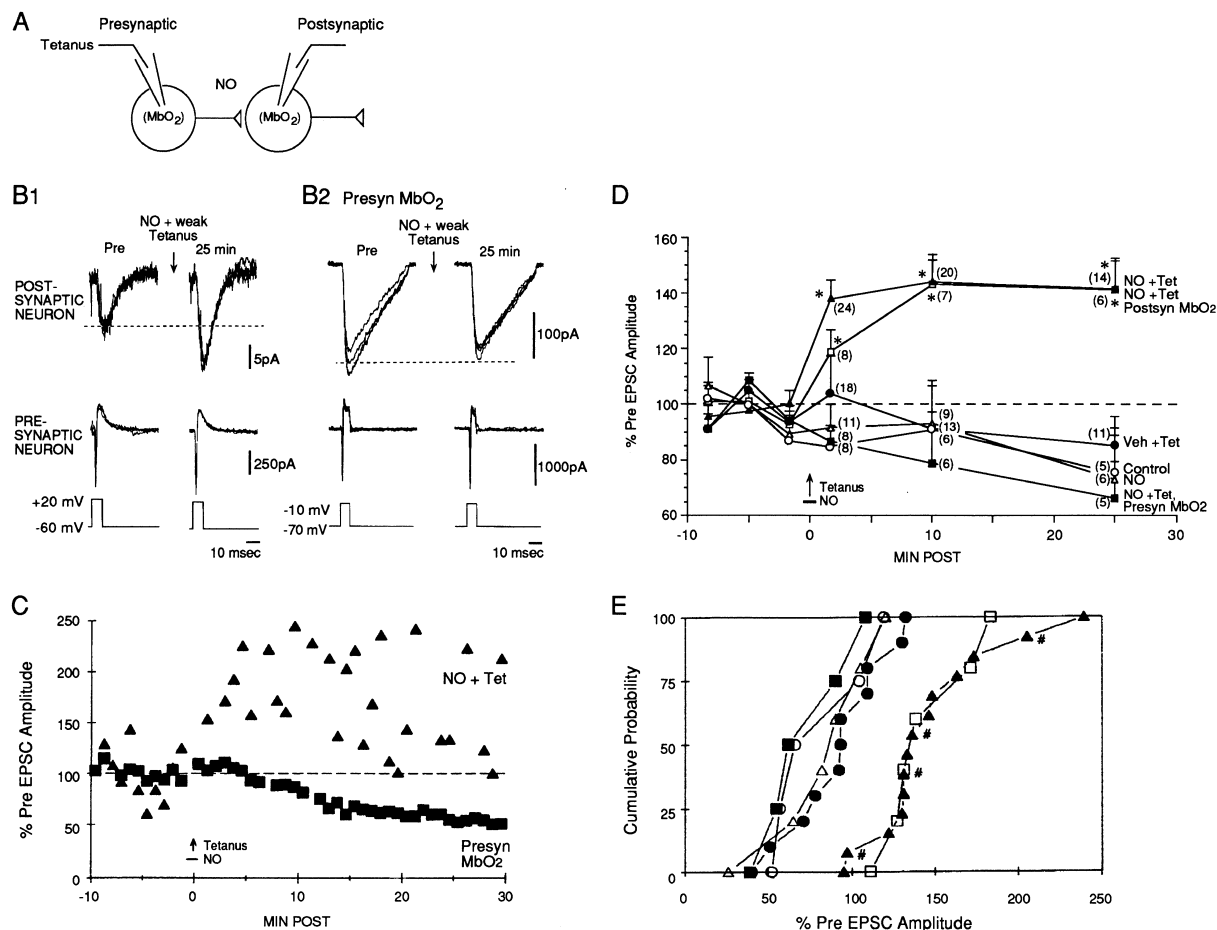


Figure 6. NO Causes Activity-Dependent Long-Lasting Potentiation That Is Blocked by Intracellular Injection of Oxymyoglobin into the Presynaptic, but Not the Postsynaptic, Neuron

(A) Experimental arrangement.

(B.) Example of potentiation by brief (30–60 s) bath application of NO at the same time as weak tetanic stimulation of the presynaptic neuron (a 50 Hz, 0.5 s train of 10 ms positive-voltage steps eliciting inward currents in the presynaptic neuron) (“paired” training) in the presence of 2 mM Mg^{2+} and 50 μ M APV.

(B.) Example of block of potentiation by injection of MbO₂ into the presynaptic neuron.

(C) Trial-by-trial results from two experiments like the ones shown in (B).

(D) Average potentiation by NO paired with weak tetanus (closed triangles). In some of these experiments, Mbmet was injected into the presynaptic neuron (n = 8). Results with and without Mbmet were not significantly different and have been pooled. Vehicle paired with weak tetanus (closed circles) or NO alone (open triangles) had no effect on EPSC amplitude compared to test alone control (open circles). Potentiation by NO paired with weak tetanus was blocked by intracellular injection of MbO₂ into the presynaptic neuron (closed squares) but not the postsynaptic neuron (open squares). Average Pre values were 42 pA, 69 pA, 29 pA, 25 pA, 240 pA, and 245 pA.

(E) Cumulative probability distributions of the potentiation 25 min after training in the six groups shown in (D). # indicates experiments in which Mbmet was injected into the presynaptic neuron.

injected substances could be due in part to differences in diffusion distance from the cell body of the pre- or postsynaptic neuron to the synapses. This seems unlikely because L-NMMA was more effective in the postsynaptic neuron (Figure 5), whereas MbO₂ and CNO were more effective in the presynaptic neuron (Figures 6 and 8). Second, because MbO₂ acts as a sink for NO, its effects might not be spatially limited to the cellular compartment containing the MbO₂. If so, our results may be due to some other effect of MbO₂ such as anoxia or selective binding of different NO species. Our results by no means exclude the possibility that potentiation involves different NO species in addition to NO itself. However, Makings and Tsien (1994) have shown that

NO binding by HbO₂ (which acts similarly to MbO₂) is effectively restricted to the cellular compartment containing the HbO₂. Such compartmentalized binding can account for all of our results including failures of MbO₂ to block potentiation (Figures 6 and 8) as well as successes (Figures 1, 4, 6, and 8). Finally, NO might act as a modulator of other processes that are involved in LTP production. This hypothesis may not be distinguishable from the activity-dependent retrograde messenger hypothesis if NO is produced in the postsynaptic neuron and acts in the presynaptic neuron, as our data suggest (Hawkins et al., 1993).

Although our results support the hypothesis that NO acts as a retrograde messenger during LTP in culture,

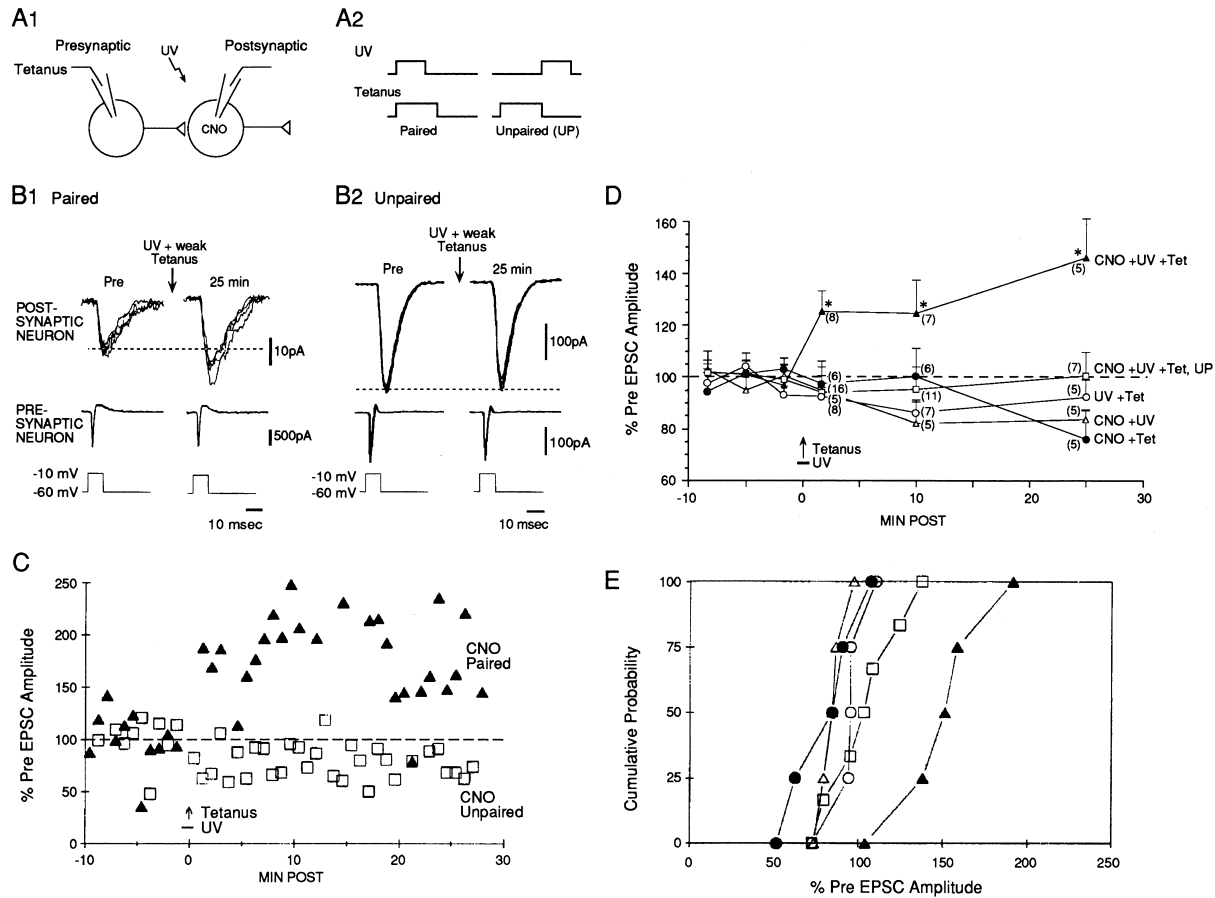


Figure 7. Photolysis of Intracellular Caged NO Causes Activity-Dependent Long-Lasting Potentiation

(A₁) Experimental arrangement. Caged NO was injected into the postsynaptic neuron.
 (A₂) Experimental protocols for photolysis of caged NO either “paired” or “unpaired” with weak tetanic stimulation.
 (B₁) Example of potentiation by photolysis of caged NO with a 350 ms UV light flash starting at the same time as weak tetanic stimulation (paired training) in the presence of 2 mM Mg²⁺ and 50 μM APV.
 (B₂) Example of lack of potentiation by photolysis of caged NO starting immediately after the end of weak tetanic stimulation (unpaired training).
 (C) Trial-by-trial results from two experiments like the ones shown in (B).
 (D) Average potentiation by paired training (closed triangles). There was no potentiation if the caged NO (open circles), UV light flash (closed circles), or weak tetanus (open triangles) was omitted. In three of the experiments in which the weak tetanus was omitted, there was 10 μM MbO₂ in the bath. Results with and without MbO₂ were similar and have been pooled. There was also no potentiation if photolysis of caged NO started immediately after the end of the weak tetanus (unpaired, UP) (open squares). In half of the unpaired experiments, caged NO was injected into the presynaptic neuron. Results with caged NO in either the pre- or postsynaptic neuron were not significantly different and have been pooled. Average Pre values were 35 pA, 95 pA, 19 pA, 117 pA, and 63 pA, not significantly different by a one-way ANOVA.
 (E) Cumulative probability distributions of the potentiation 25 min after training in the five groups shown in (D).

previous results with bath-applied NO synthase inhibitors and NO donors in slices have been more variable (Bliss and Collingridge, 1993; Hawkins, 1996). In particular, release of caged NO in slices has thus far failed to produce potentiation (Boulton et al., 1994; Murphy et al., 1994). Further experiments will be necessary to determine whether these different results are due to use of a different caged NO compound, a different dose of NO, different temporal and spatial localization of the NO, or differences between neurons in culture and slices. We have previously noted that NO tends to be toxic at doses greater than 10 nM in culture, suggesting that dose may be an important parameter (O’Dell et al., 1991). Similarly, experiments on LTP in mice with targeted mutations of different isoforms of NO synthase indicate that LTP in slices has both NO-dependent and NO-independent

components (Son et al., 1996), whereas our experiments indicate that LTP in culture appears to be completely NO dependent. Again, these different results are presumably due to different experimental conditions, probably including the use of less mature neurons in the culture system. Finally, our results do not allow us to assign a physiological function to any specific isoform of NO synthase, but the results of the accompanying paper (Son et al., 1996) suggest that both the neuronal and endothelial isoforms are involved.

NO has several molecular mechanisms of action, including stimulating production of cGMP (East and Garthwaite, 1991; Schuman et al., 1994; Meffert et al., 1996), and presynaptic injection of cGMP produces activity-dependent long-term potentiation in cultured hippocampal neurons (Arancio et al., 1995). Together, these

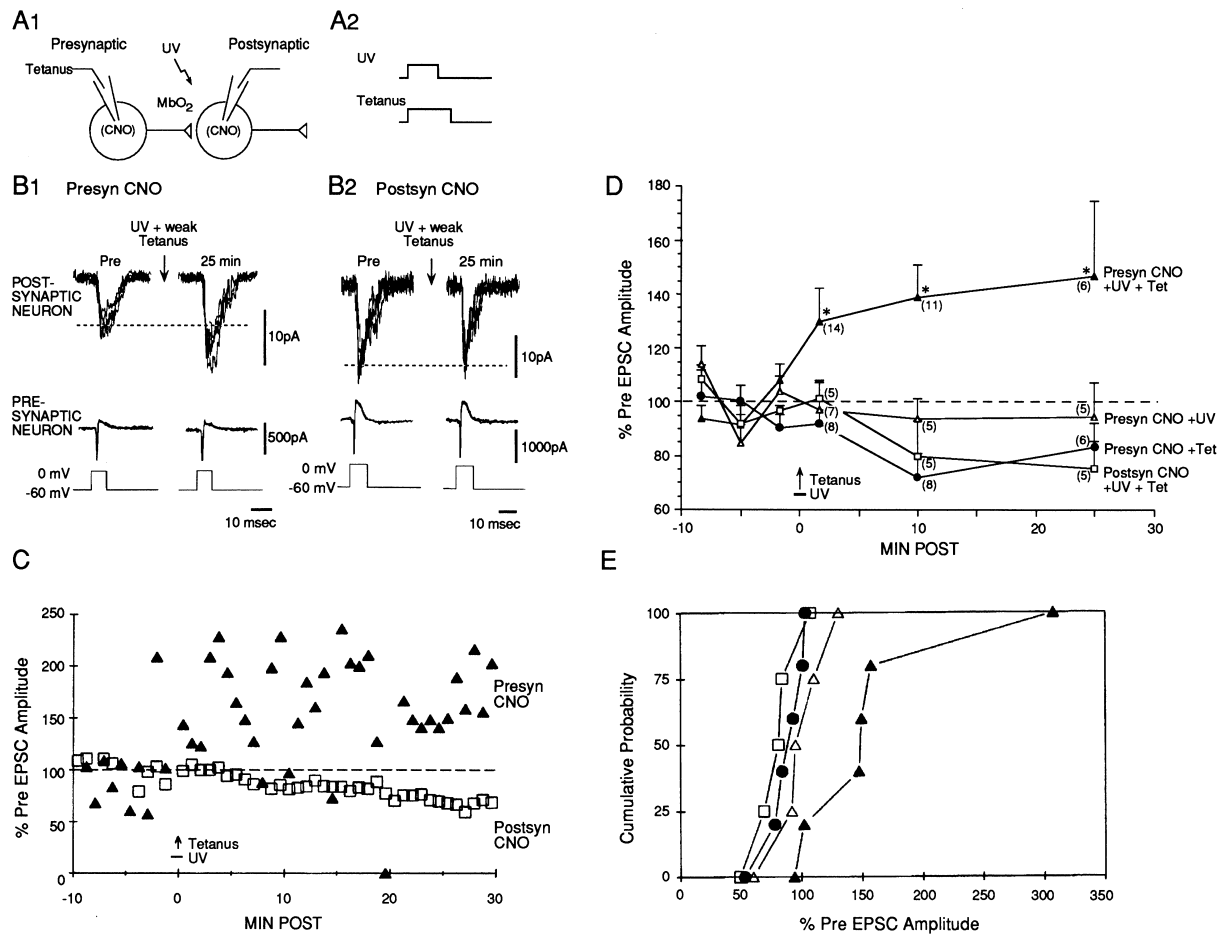


Figure 8. Potentiation by Photolysis of Caged NO in the Postsynaptic, but Not the Presynaptic, Neuron Is Blocked by Extracellular MbO₂ (A₁) Experimental arrangement. (A₂) Experimental protocol. (B₁) Example of potentiation by photolysis of caged NO in the presynaptic neuron with MbO₂ in the bath. (B₂) Example of lack of potentiation by photolysis of caged NO in the postsynaptic neuron with MbO₂ in the bath. (C) Trial-by-trial results from two experiments like the ones shown in (B). (D) Average potentiation by photolysis of caged NO in the presynaptic neuron paired with weak tetanus in the presence of 2 mM Mg²⁺ and 50 μM APV (filled triangles). There was no potentiation if the UV light flash (filled circles) or weak tetanus (open triangles) was omitted. There was also no potentiation by photolysis of caged NO in the postsynaptic neuron paired with weak tetanus (open squares). Average Pre values were 41 pA, 60 pA, 45 pA, and 78 pA, not significantly different by a one-way ANOVA. (E) Cumulative probability distributions of the potentiation 25 min after training in the four groups shown in (D).

data support the following hypothesis: tetanic stimulation causes Ca²⁺ influx through postsynaptic NMDA receptors, thereby activating NO synthase to form NO in the postsynaptic neuron. NO then diffuses across the extracellular space to the presynaptic terminal, where it activates molecules including soluble guanylyl cyclase and cGMP-dependent protein kinase to produce activity-dependent long-lasting enhancement of transmitter release (Zhuo et al., 1994; Arancio et al., 1995). Thus, NO is thought to provide a bridge between events required for induction of LTP in the postsynaptic cell and expression of potentiation in the presynaptic cell. These results therefore suggest that signaling can occur in a retrograde, as well as in an anterograde, direction during LTP at hippocampal synapses and support the idea that retrograde signaling may play an important role during synaptic plasticity and development more generally (Murphy and Davis, 1994).

Experimental Procedures

Electrophysiology

Dissociated cell cultures of hippocampal neurons from 1- to 2-day-old Sprague-Dawley rats were prepared as previously described (O'Dell et al., 1991). The bath and electrode solutions were also as previously described (Arancio et al., 1995), except that the bath solution was not oxygenated in these experiments. The pre- and postsynaptic cells were maintained under ruptured patch whole-cell voltage clamp throughout the experiments (Arancio et al., 1995), and the input resistances of both cells were checked for constancy. A 10 ms positive voltage step that was sufficient to elicit a large inward current in the presynaptic neuron produced an EPSC in the postsynaptic neuron once every 50 s. EPSC amplitudes were measured automatically by computer between the peak and the mean of the baseline just before the start of the EPSC. The EPSC data were log transformed to make them more normally distributed for analysis with parametric statistics. MbO₂ was prepared as previously described (Lev-Ram et al., 1995). NO was prepared by bubbling the gas until saturation in helium-saturated distilled water, which was then diluted in bath solution to reach a final concentration

of 10 nM immediately before application (O'Dell et al., 1991). All chemicals were from Sigma (St. Louis, MO) unless otherwise indicated.

Diffusion of Fluorescein-Labeled Myoglobin

Fluorescein-labeled myoglobin was prepared by mixing horse skeletal muscle myoglobin (Calbiochem, La Jolla, CA) with fluorescein isothiocyanate (FITC, Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The unconjugated FITC was removed by size exclusion chromatography using Sephadex G-25 (Pharmacia, Piscataway, NJ). Cells were loaded with FITC-myoglobin through the patch pipette in ruptured whole-cell patch configuration, then imaged digitally with videoprobe hardware and software (ETM Systems, Irvine, CA) and an intensified CCD (Hamamatsu, Japan) attached to an epifluorescence microscope (Nikon TMD, Japan). Constant gain and offset settings for the camera and intensifier were used throughout the experiment.

Double Labeling with Fluorescein-Myoglobin and Synaptophysin Immunofluorescence

Cells were loaded with FITC-myoglobin through the patch pipette as described above for 12 min, immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) at room temperature for 15 min, washed with PBS, and preincubated with blocking solution containing 10% heat inactivated goat serum (HIGS) and 0.1% Triton X-100 in PBS for 30 min. Cells were then incubated in rabbit anti-human synaptophysin (DAKO, Carpinteria, CA) diluted 1:100 in PBS containing 1% HIGS and 0.1% Triton X-100 for 48 hr. After 3 washes with PBS, cells were incubated in Texas red-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1:500 for 30 min. Cells were then washed 3 times with PBS, mounted on glass slides with a ProLong antifade kit (Molecular Probes), and examined with a MRC-1000 Laser confocal microscope (Bio-Rad) coupled to a Zeiss Axiovert 100 inverted microscope equipped with a Zeiss 63 × 1.3 NA objective. The cells were excited using the 488 nm and the 568 nm lines of a krypton-argon laser to image FITC and Texas red, respectively. Four Z-series optical sections (0.72 μm steps) were averaged by the Kalman method for each picture. Similar results were obtained with single optical sections.

Caged NO

Twenty micromolar CNO-4 dipotassium salt (Makings and Tsien, 1994) was injected into either the pre- or postsynaptic neuron by inclusion in the intracellular solution of the patch pipette. The UV light was produced by a 100 W DC mercury arc lamp with an electro-mechanical shutter (Vincent Associates, Rochester, NY). UV wavelengths were filtered through a glass filter with peak transmission at 360 nm ± 20 nm. Light was conveyed to the epifluorescence port of an inverted microscope with an air gap of several inches to avoid transmission of shutter vibration. The microscope was equipped with an EF25X objective, NA 0.50. Calibration with a silicon photodiode photometer (IL 1700, International Light, Newburyport, MA) showed that light intensity was 3.2 mW/cm² at the plane of focus, where the cell culture normally sits. Calculations from the extinction coefficient and quantum yield of CNO-4 (Makings and Tsien, 1994) indicate that the UV light should have released approximately 10 nM NO per 350 ms exposure through the shutter. This estimate agrees fairly well with measurements of NO release under the experimental conditions by the determination of nitrites based on a diazotization reaction (Vogel, 1989).

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References

- Arancio, O., Kandel, E.R., and Hawkins, R.D. (1995). Activity-dependent long-term enhancement of transmitter release by presynaptic 3', 5'-cyclic cGMP in cultured hippocampal neurons. *Nature* **376**, 74–80.
- Bekkers, J.M., and Stevens, C.F. (1990). Presynaptic mechanism for long-term potentiation in the hippocampus. *Nature* **346**, 724–729.
- Bliss, T.V.P., and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39.
- Böhme, G.A., Bon, C., Stutzmann, J.-M., Doble, A., and Blanchard, J.-C. (1991). Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.* **199**, 379–381.
- Boulton, C.L., Irving, A.J., Southam, E., Potier, B., Garthwaite, J., and Collingridge, G.L. (1994). The nitric oxide-cyclic GMP pathway and synaptic depression in rat hippocampal slices. *Eur. J. Pharmacol.* **6**, 1528–1535.
- East, S.J., and Garthwaite, J. (1990). Nanomolar N^g-nitroarginine inhibits NMDA-induced cyclic GMP formation in rat cerebellum. *Eur. J. Pharmacol.* **184**, 311–313.
- East, S.J., and Garthwaite, J. (1991). NMDA receptor activation in rat hippocampus induces cGMP formation through the L-arginine-nitric oxide pathway. *Neurosci. Lett.* **123**, 17–19.
- Fletcher, T.L., Cameron, P., DeCamilli, P., and Banker, G. (1991). The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J. Neurosci.* **11**, 1617–1626.
- Gorbanov, N.V., Osipov, A.N., Day, B.W., Zoyas-Rivera, B., Kagan, V.E., and Elsayed, N.M. (1995). Reduction of ferrylmyoglobin and ferrylhemoglobin by nitric oxide: a protective mechanism against ferryl hemoprotein-induced oxidations. *Biochemistry* **34**, 6689–6699.
- Haley, J.E., Wilcox, G.L., and Chapman, P.F. (1992). The role of nitric oxide in hippocampal long-term potentiation. *Neuron* **8**, 211–216.
- Hawkins, R.D. (1996). NO Honey, I don't remember. *Neuron* **16**, 465–467.
- Hawkins, R.D., Kandel, E.R., and Siegelbaum, S.A. (1993). Learning to modulate transmitter release: themes and variations in synaptic plasticity. *Annu. Rev. Neurosci.* **16**, 625–665.
- Isaac, J.T., Nicoll, R.A., and Malenka, R.C. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* **15**, 427–434.
- Kelso, S.R., Ganong, A.H., and Brown, T.H. (1986). Hebbian synapses in hippocampus. *Proc. Natl. Acad. Sci. USA* **83**, 5326–5330.
- Lev-Ram, V., Makings, L.R., Keitz, P.F., Kao, J.P.Y., and Tsien, R.Y. (1995). Long-term depression in cerebellar Purkinje neurons results from coincidence of nitric oxide and depolarization-induced Ca²⁺ transients. *Neuron* **15**, 407–415.
- Liao, D., Hessler, N.A., and Malinow, R. (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slices. *Nature* **375**, 400–404.
- Makings, L.R., and Tsien, R.Y. (1994). Caged nitric oxide. Stable organic molecules from which nitric oxide can be photoreleased. *J. Biol. Chem.* **269**, 6282–6285.
- Malgaroli, A., and Tsien, R.W. (1992). Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons. *Nature* **357**, 134–139.
- Meffert, M.K., Calakos, N.C., Scheller, R.H., and Schulman, H. (1996). Nitric oxide modulates synaptic vesicle docking/fusion reactions. *Neuron* **16**, 1229–1236.
- Murphey, R.K., and Davis, G.W. (1994). Retrograde signaling at the synapse. *J. Neurobiol.* **25**, 595–598.
- Murphy, K.P.S.J., Williams, J.H., Bettache, N., and Bliss, T.V.P. (1994). Photolytic release of nitric oxide modulates NMDA receptor-mediated transmission but does not induce long-term potentiation at hippocampal synapses. *Neuropharmacology* **33**, 1375–1385.
- O'Dell, T.J., Hawkins, R.D., Kandel, E.R., and Arancio, O. (1991).

Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. USA* **88**, 11285–11289.

Popov, S., and Poo, M.-M. (1992). Diffusional transport of macromolecules in developing nerve processes. *J. Neurosci.* **12**, 77–85.

Schuman, E.M., and Madison, D.V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* **254**, 1503–1506.

Schuman, E.M., Meffert, M.K., Schulman, H., and Madison, D.V. (1994). An ADP-ribosyl transferase as a potential target for nitric oxide action in hippocampal long-term potentiation. *Proc. Natl. Acad. Sci. USA* **91**, 11958–11962.

Son, H., Hawkins, R.D., Martin, K., Kiebler, M., Huang, P.L., Fishman, M.C., and Kandel, E.R. (1996). Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell*, this issue.

Stevens, C.F., and Wang, Y.Y. (1993). Reversal of long-term potentiation by inhibitors of heme oxygenase. *Nature* **364**, 147–149.

Vogel, A.I. (1989). *Textbook of Quantitative Chemical Analysis* (Harlow, Essex, UK: Longman Scientific and Technical).

Zhuo, M., Small, S.A., Kandel, E.R., and Hawkins, R.D. (1993). Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in the hippocampus. *Science* **260**, 1946–1950.

Zhuo, M., Hu, Y., Schultz, C., Kandel, E.R., and Hawkins, R.D. (1994). Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation. *Nature* **368**, 635–639.