of neurotransmitters into synaptic vesicles have not been characterized at the molecular level. The cloning of several plasma membrane neurotransmitter transporters has revealed that these proteins share 37 to 67% sequence identity with one another (*16*). Like SV2, these transporters have 12 membrane-spanning domains. However, amino acid sequence comparisons of SV2 to the γ-aminobutyric acid, glycine, and dopamine transporters revealed no significant identity or homology. It is known that the neurotransmitter transporters of synaptic vesicles are functionally distinct from transmitter uptake transporters on the plasma membrane. Vesicle-localized transporters require a membrane H^+ gradient, whereas plasma membrane transporters are dependent on Na^+ (*17*). The similarity between SV2 and transporter proteins suggests that SV2 may be a vesicle-localized neurotransmitter transporter. As is the case with plasma membrane neurotransmitter transporters, there may be a family of vesicle-localized transporters. Although evidence suggests that all of the cDNA clones isolated in our initial screen are derived from a single gene, further work will determine whether SV2 is the first member of a gene family.

SV2 diverges from the bacterial transporters after the seventh transmembrane domain. No significant homologies between this region of SV2 and proteins in GenBank were found. This divergence suggests that SV2 may have additional functions or that its function has diverged from that of the transporters with which it shares homology. This proposal is supported by the finding that SV2, which is at least 20 kD larger than the other transporters, has a longer, highly charged NH_2-terminal region and contains a more extensive hydrophilic region between transmembrane domains 7 and 8. Synaptic vesicles also contain proteins that transport ions, most notably Cl^-. Both Cl^- pump (*18*) and Cl^- channel (*19*) activities have been described, though the proteins mediating these activities have not been fully characterized. Rather than function as a transmitter transporter, SV2 may therefore function as an ion transporter or channel. Another protein with similarities to a different class of transporters, the cystic fibrosis gene product, displays Cl^- channel activity (*20*), which demonstrates that some ion channels are structurally related to transporters.

Finally, SV2 shares homology with transporters that move substances both into and out of cells. Therefore, the direction of transport by SV2 cannot be assumed on the basis of the homologies reported here. It is possible that SV2 is responsible for the movement of substances out of synaptic vesicles. For example, SV2, in conjunction with a plasma membrane component, may participate in the initial release of transmitter substances, which has been shown to occur via a channel-like pore (*21*). The localization of SV2 to secretory vesicles in a variety of neural and endocrine cells, together with its conservation across species, implicates its importance in vesicle functioning. The finding that SV2 shares significant homology to transporter proteins suggests that it moves ions or molecules across the vesicle membrane.

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23. We thank R. Kelly for the anti-SV2–producing cell line, J. Guastella for rat brain RNA and helpful discussions, and T. Kreiner and M. Bennett for critical reading of the manuscript. Amino acid sequencing was performed by the Stanford Protein and Nucleic Acid Facility. Supported by the National Institute of Mental Health.

8 June 1992; accepted 17 July 1992

Inhibition of Long-Term Potentiation by NMDA-Mediated Nitric Oxide Release

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Activation of *N*-methyl-D-aspartate (NMDA) receptors before tetanic stimulation blocks long-term potentiation (LTP) in the CA1 region of the hippocampus. This NMDA-mediated inhibition of LTP can be reversed by the nitric oxide (NO) inhibitors L-N^G-monomethylarginine or hemoglobin and mimicked by sodium nitroprusside. These results indicate that the timing of NO release relative to high-frequency activation of CA1 synapses may be an important determinant of LTP generation and suggest that NO may play a positive or negative modulatory role in LTP depending on prior events at the tetanized synapse and the ambient concentration of excitatory amino acids.

The mechanisms underlying the induction and maintenance of LTP at CA1 hippocampal synapses are uncertain. Quantal analysis has indicated that enhanced release of glutamate, the presumed excitatory neurotransmitter at these synapses, is a crucial event (*1*). Other studies have demonstrated that postsynaptic changes in transmitter sensitivity are also important (*2*). It is clear that CA1 LTP is critically dependent on the activation of NMDA receptors (*3*) and the influx of Ca^{2+} into postsynaptic neurons (*4, 5*). This has prompted the hypothesis that postsynaptic

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NMDA receptors promote the release of a transsynaptic messenger that mediates the enhanced neurotransmitter release from presynaptic terminals. Nitric oxide is currently a leading candidate for this retrograde messenger because NMDA evokes a Ca^{2+}-dependent release of NO (6) and NO inhibitors block the induction of CA1 LTP (7). Furthermore, high concentrations of sodium nitroprusside (SNP), an agent that spontaneously releases NO (8), augment synaptic responses in the CA1 region of hippocampal slices (9), and perfusion of NO increases the frequency of spontaneous miniature excitatory synaptic currents in cultured hippocampal neurons (10).

Although NMDA receptor activation is a key event underlying CA1 LTP generation, applications of NMDA under physiological conditions produce only a short-lived enhancement of synaptic responses and do not mimic LTP (5, 11). This suggests that NMDA receptor activation alone is insufficient to produce LTP. Alternatively, NMDA may activate processes that interfere with LTP development. In support of the latter hypothesis, studies have demonstrated that both synaptic (12, 13) and pharmacologic (14) activation of NMDA receptors before tetanic stimulation inhibit the development of CA1 LTP. The NMDA-mediated inhibition of LTP is mimicked by glutamate and aspartate at concentrations similar to those present in the extracellular fluid of the central nervous system (CNS) (15) and does not result from a depression of postsynaptic NMDA receptor–mediated responses (14). However, the inhibition of LTP is reversed by low concentrations of the competitive NMDA receptor antagonist, 2-amino-5-phosphonovalerate (APV) (12–14) or by administration of solutions containing low concentrations of Ca^{2+} with high concentrations of Mg^{2+} (14). Because of the apparent dependence on NMDA receptors and Ca^{2+}, we have examined the role of NO in this LTP-blocking action of NMDA by administering NO inhibitors before and during exposure to NMDA.

Experiments were performed in the CA1 region of hippocampal slices prepared from 29- to 36-day-old rats (16). In control slices, tetanic stimulation produced an increase of 49.5 ± 7.0% (mean ± SEM, n = 13) in the slope of extracellularly recorded excitatory postsynaptic potentials (EPSPs) and an increase of 87.5 ± 14.8% (n = 18) in the amplitude of population spikes, as measured at the 50% point on the respective stimulus-response curves 60 min after the tetanus (Figs. 1 and 2). Consistent with our previous observations (14), we found that 1 μM NMDA administered to slices for 5 min before the tetanus inhibited LTP in six of seven slices (−4.5 ± 6.9% change in EPSP slope measured 60 min after the tetanus). Similarly, 1 μM NMDA inhibited LTP when administered for 5 min immediately after the tetanus in all six slices studied (+1.5 ± 6.0% change in EPSP slope). The administration of L-N^G-monomethylarginine (L-NMMA), a competitive NO synthase inhibitor (17), for 5 min before and during perfusion of 1 μM NMDA allowed LTP generation when both were administered before the tetanus. Four of seven slices exhibited LTP in the presence of 30 μM L-NMMA plus 1 μM NMDA (+21.5 ± 9.8% change in EPSP slope), whereas all five slices exhibited LTP in the presence of 100 μM L-NMMA plus 1 μM NMDA (+36.8 ± 7.1% change in EPSP slope). Pretreatment of slices with 30 μM L-NMMA for 10 min also allowed LTP in all slices treated with 1 μM NMDA immediately after the tetanus (+54.7 ± 16.2% change in EPSP slope) (n = 5). When applied alone for 10 min before the tetanus, 100 μM L-NMMA did not inhibit LTP (+30.8 ± 13.3% change in EPSP slope) (n = 5).

If the L-NMMA reversal of NMDA-mediated LTP inhibition is produced by an effect on NO synthase, then the effect should be reversed by L- but not D-arginine (18). Consistent with this hypothesis, application of 1 mM L-arginine with 100 μM L-NMMA and 1 μM NMDA blocked the development of LTP in all slices studied (+1.0 ± 3.8% change in EPSP slope) (n = 6) (Fig. 2). However, 1 mM D-arginine did not reverse the effect of 100 μM L-NMMA (+33.8 ± 11.4% change in EPSP slope) (n = 6). None of these agents administered alone altered baseline synaptic responses, and 1 mM L-arginine alone did not alter LTP generation (+34.5 ± 6.7% change in EPSP slope) (n = 5).

We found that L-N^G-nitroarginine (L-NOArg), which like L-NMMA is a competitive NO synthase inhibitor (19), was less effective than L-NMMA in reversing the NMDA inhibition of LTP. Only two of five slices treated with 1 μM L-NOArg plus 1 μM NMDA before the tetanus exhibited LTP (+56.2 ± 52.4% change in EPSP slope) (n = 5). However, four of six slices exhibited LTP in the presence of 1 μM L-NOArg alone (+36.4 ± 22.4% change in EPSP slope). At higher concentrations (≥10 μM), L-NOArg altered baseline population spikes. Preliminary histologic studies indicate that L-NOArg may be toxic to hippocampal slices at higher concentrations, producing shrinkage of pyramidal neurons (20). In contrast, hemoglobin (0.1 to 1.0 μM), which inhibits NO action by binding the molecule (21), reversed the inhibitory effect of 1 μM NMDA in all slices studied (n = 10). EPSP slopes were increased by 42.5 ± 6.2% (n = 5) 1 hour after the tetanus in slices treated with 0.1 μM hemoglobin for 10 min and 1 μM NMDA for 5 min before the tetanus (Fig. 2).

The effects of the NO inhibitors suggest that pretetanic NMDA receptor activation

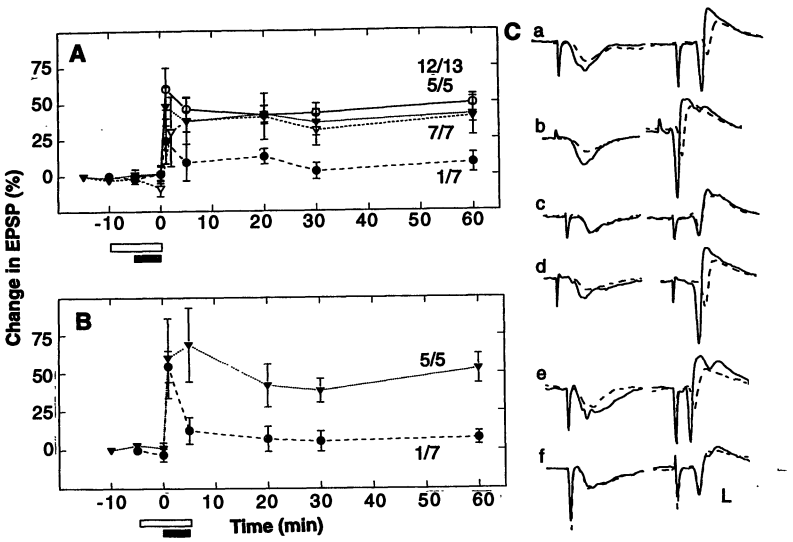

Fig. 1. L-NMMA reverses NMDA-mediated LTP inhibition. (**A**) Time course of change in EPSP slope (mean ± SEM) for a series of control slices (open circles) and slices treated with 1 μM NMDA (filled circles), 1 μM NMDA plus 100 μM L-NMMA (filled triangles), and 100 μM L-NMMA alone (open triangles). The numbers in the graph indicate the proportion of slices exhibiting LTP. In these experiments NMDA was applied for 5 min immediately before the tetanus (solid bar), whereas L-NMMA was applied for 10 min before the tetanus (open bar). The tetanus was delivered at time 0. (**B**) Effect of 1 μM NMDA (solid bar) administered for 5 min after the tetanus (circles) and 30 μM L-NMMA (open bar) administered for 5 min before and during perfusion with 1 μM NMDA (triangles). (**C**) Traces (a to f) depict field EPSPs (left) and population spikes (right) in individual experiments measured 5 min before (dashed traces) and 60 min after (solid traces) the tetanus in slices treated with 1 μM NMDA plus 100 μM L-NMMA (a), 100 μM L-NMMA (b), 1 μM NMDA (c), and 1 μM NMDA plus 0.1 μM hemoglobin (d), before the tetanus. The traces in (e) and (f) show the effect of 1 μM NMDA administered posttetanically with (e) and without (f) 30 μM L-NMMA. Calibration bar, 1 mV, 2 ms.

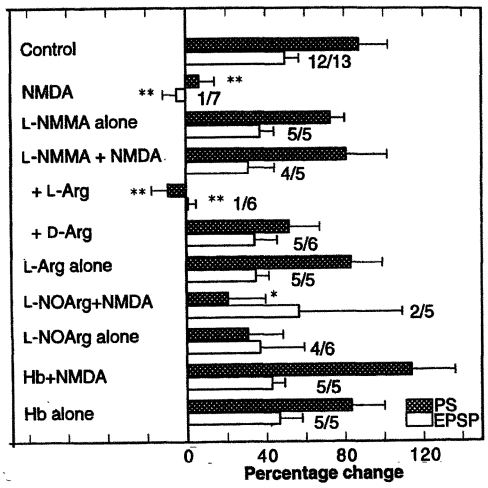

Fig. 2. Nitric oxide inhibitors and NMDA-mediated LTP inhibition. The graph shows changes in population spike (PS) amplitude (hatched bars; mean ± SEM) and EPSP slope (open bars) measured 60 min after the tetanus in various conditions. The stimuli were chosen to evoke a 50% maximal population spike (hatched bars) or EPSP (open bars) during the baseline stimulus-response curve. The numbers in the graph indicate the proportion of slices exhibiting LTP. For these experiments, NMDA was administered for 5 min immediately before the tetanus, whereas other agents were administered for 10 min before the tetanus, as described for Fig. 1. The concentrations used in these experiments were 1 μM NMDA, 100 μM L-NMMA, 1 mM L- and D-arginine, 1 μM L-NOArg, and 0.1 μM hemoglobin (Hb). $^{**}P < 0.01$, $^{*}P < 0.05$ compared to control by Mann-Whitney *U* test.

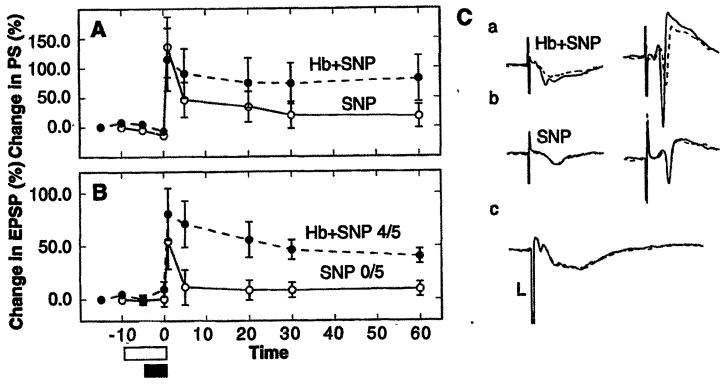

Fig. 3. Time course of change in (**A**) population spike (PS) amplitude and (**B**) EPSP slope for slices treated with 10 μM SNP (open circles) and 10 μM SNP plus 1 μM hemoglobin (Hb) (filled circles). The numbers in the EPSP graph indicate the proportion of slices exhibiting LTP. In these experiments SNP was applied for 5 min before the tetanus (solid bar), and hemoglobin was applied for 10 min (open bar). The tetanus was delivered at time 0. The traces depict EPSPs (left) and PS (right) 5 min before (dashed traces) and 60 min after (solid traces) tetanic stimulation in SNP-treated slices with (a) and without (b) hemoglobin pretreatment. The traces in (**C**) show the effect of 10 μM SNP on the NMDA-component of synaptic responses recorded in the presence of 25 μM 6,7-dinitroquinoxaline-2,3-dione and 0.1 mM Mg^{2+}. Calibration bar, 1 mV, 2 ms.

prevents CA1 LTP by promoting the release of NO. We further examined this issue by treating slices with SNP (*8*). The administration of 10 μM SNP for 5 min before tetanus blocked LTP in all five slices studied (−5.8 ± 6.9% change in EPSP slope) without altering baseline synaptic transmission (Fig. 3). This inhibitory effect of SNP on LTP did not result from blockade of NMDA receptors because 10 μM SNP failed to alter the NMDA component of synaptic potentials in CA1 (+6.4 ± 6.8% change) ($n = 6$). Furthermore, the inhibitory effect of 10 μM SNP on LTP could be reversed by the administration of 1 μM hemoglobin, which allowed LTP development in four of five slices (+47.0 ± 16.5% change in EPSP slope).

The inhibition of LTP is most likely mediated by NO produced locally in the CA1 region. The highly reactive nature of the NO molecule results in a half-life measured in seconds (*22*), which suggests that NO acts over limited distances. However, bath perfusions of NMDA could promote NO release from any of the hippocampal subfields, particularly the dentate gyrus, which contains high levels of NO synthase (*23*). To address this issue, we examined the inhibitory effect of NMDA and the reversal of NMDA's effects by NO inhibitors in slices in which the dentate gyrus and CA3 region were surgically removed. In four of five control CA1 slices, LTP was produced (+31.1 ± 16.9% change in EPSP slope). However, in CA1 slices, 1 μM NMDA inhibited LTP (+8.6 ± 6.3% change in EPSP slope) ($n = 5$) and 100 μM L-NMMA reversed the inhibition (+118.2 ± 32.6% change in EPSP slope) ($n = 3$).

These results indicate that untimely release of NO by activation of NMDA receptors blocks the development of CA1 LTP. Coupled with previous studies demonstrating that NO inhibitors block CA1 LTP (*7*, *10*) and that high concentrations of SNP promote LTP (*9*), it is likely that the timing and degree of NO release relative to high-frequency activation of synapses is critical. In support of a role for NO in LTP generation, we found that a 10-min application of 1 μM hemoglobin before tetanic stimulation blocked LTP in five of eight slices (+14.0 ± 8.0% change in EPSP slope). It proved difficult to study the effect of higher concentrations of hemoglobin because of the instability of the solutions during perfusion. Consistent with the findings of O'Dell *et al.* (*10*), 50 μM L-NOArg also blocked LTP in four of five slices when administered for 30 min before and for 10 min after the tetanus (−5.9 ± 11.2% change in EPSP slope).

Nitric oxide may be an important mediator of the delayed toxic effects of NMDA in cultured neurons (*24*) and in hippocampal slices (*25*). However, these toxic effects require significantly higher concentrations of NMDA than those that block LTP (100 μM compared to 1 μM in hippocampal slices). Thus, the effects of NMDA on LTP do not appear to be mediated by a toxic effect on CA1 neurons. Rather, the effects of low NMDA concentrations may mimic the baseline physiologic state of neurons because extracellular brain fluid contains low micromolar concentrations of glutamate and aspartate (*15*). On the basis of observations by Huang *et al.* (*13*), it appears that pretetanic NMDA receptor activation alters the threshold for LTP generation at CA1 synapses and may help to prevent excessive excitation in the CNS. Our results indicate that these changes are mediated by the release of NO. This suggests that NO may play a positive or negative modulatory role in LTP, depending on when NO is released relative to high-frequency synaptic activation. The inhibitory effects may reflect disruption of NO-mediated biochemical processes necessary for LTP or activation of competing processes (*26*). In future experiments it will be important to determine the nature of the NO-mediated biochemical changes and to examine how NMDA and other factors modulate these processes.

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26. Nitric oxide could also have inhibited LTP by blocking NMDA-gated responses [O. Manzoni *et al.*, *Neuron* **8**, 653 (1992)]. However, at the concentrations studied, NMDA and SNP did not alter NMDA-mediated synaptic responses in CA1 (*12*).
27. We thank J. W. Olney, K. Kato, S. Mennerick, and L. L. Thio for helpful discussions and comments. Supported by Research Scientist Development Award MH00964, grants MH45493 and AG05681, and fellowships from the McDonnell Center for Higher Brain Function and the Bantly Foundation.

20 March 1992; accepted 17 June 1992

Hyperalgesia Mediated by Spinal Glutamate or Substance P Receptor Blocked by Spinal Cyclooxygenase Inhibition

A. B. Malmberg and T. L. Yaksh

Inhibition of cyclooxygenase by nonsteroidal anti-inflammatory drugs (NSAIDs) in the periphery is commonly accepted as the primary mechanism by which these agents produce a selective attenuation of pain (analgesia). NSAIDs are now shown to exert a direct spinal action by blocking the excessive sensitivity to pain (hyperalgesia) induced by the activation of spinal glutamate and substance P receptors. These findings demonstrate that the analgesic effects of NSAIDs can be dissociated from their anti-inflammatory actions. Spinal prostanoids are thus critical for the augmented processing of pain information at the spinal level.

NSAIDs have a potent effect on pain behavior induced by inflammatory conditions but little effect on responses evoked by an acute high-intensity stimulus (*1*, *2*). Observations in the 1960s that tissue injury gives rise to the local formation of prostanoids (*3*), that local application of prostanoids facilitates activity of C fiber primary afferents and evokes pain behavior (*4*), and that the structurally diverse class of agents called NSAIDs powerfully inhibits cyclooxygenase that synthesizes prostanoids (*5*) supported the unifying hypothesis that the analgesic effect of NSAIDs reflects their actions in preventing the sensitization of the peripheral afferent terminal.

Subsequent studies, however, emphasize that NSAIDs can also exert an effect within the central nervous system. Direct administration of NSAIDs into the spinal intrathecal space, like systemic administration, significantly diminishes the pain behavior evoked by the intraperitoneal (*1*, *6*) or subcutaneous (*6*, *7*) injection of irritants without affecting the response to acute pain stimuli. Thus, in the formalin test a small volume of irritant (formalin) is injected into the hind paw of the rat. In humans, an equivalent injection induces a sharp stinging sensation, followed by a longer period of discomfort and hyperalgesia (*8*). In rats, this treatment results in an immediate, transient licking and flinching of the injected paw (0 to 5 min, phase 1), with a prolonged recurrence of similar behavior (10 to >60 min, phase 2). The time course corresponds to a pronounced short increase in neural activity, followed by an extended interval of elevated background activity in the primary afferent C fibers that innervate the injected skin (*9*). In this behavioral model, NSAIDs diminish the phase 2 but not the phase 1 response in a dose-dependent and stereospecific fashion (with the active isomer producing cyclooxygenase inhibition) (*7*). In the studies noted above the NSAIDs are observed to be 100 to 500 times as active after spinal as after systemic administration.

These observations suggest that the effects of NSAIDs on phase 2 irritant-induced hyperalgesia are likely mediated by inhibition of prostaglandin synthesis within the spinal cord. They also emphasize that the anti-inflammatory component of these

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