NMDA receptor activation enhances inhibitory GABAergic transmission onto hippocampal pyramidal neurons via presynaptic and postsynaptic mechanisms

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ABSTRACT

NMDA receptors (NMDARs) are implicated in synaptic plasticity and modulation of glutamatergic excitatory transmission. Effect of NMDAR activation on inhibitory GABAergic transmission remains largely unknown. Here we report that a brief application of NMDA could induce two distinct actions in CA1 pyramidal neurons in mouse hippocampal slices: (1) an inward current attributed to activation of postsynaptic NMDARs and (2) fast phasic synaptic currents, namely spontaneous IPSCs (sIPSCs), mediated by GABA\textsubscript{A} receptors in pyramidal neurons. The mean amplitude of sIPSCs was also increased by NMDA. This profound increase in the sIPSC frequency and amplitude was markedly suppressed by the sodium channel blocker tetrodotoxin, whereas the frequency and mean amplitude of miniature IPSCs were not significantly affected by NMDA, suggesting that NMDA elicits repetitive firing in GABAergic interneurons, thereby leading to GABA release from multiple synaptic sites of single GABAergic axons. We found that the NMDAR open-channel blocker MK-801 injected into recorded pyramidal neurons suppressed the NMDA-induced increase of sIPSCs, which raises the possibility that the firing of interneurons may not be the sole factor and certain retrograde messengers may also be involved in the NMDA-mediated enhancement of GABAergic transmission. Our results from pharmacological tests suggest that the nitric oxide signaling pathway is mobilized by NMDAR activation in CA1 pyramidal neurons, which in turn retrogradely facilitates GABA release from the presynaptic terminals. Thus, NMDARs at glutamatergic synapses on both CA1 pyramidal neurons and interneurons appear to exert feedback and feedforward inhibition for determining the spike timing of the hippocampal microcircuit.
Inhibitory GABAergic interneurons critically modify the outcome of neuronal network. For example, they regulate the dendritic electrogenesis and spike generation of hippocampal pyramidal neurons (Miles et al. 1996; Pouille and Scanziani 2001) as well as the maintenance of rhythmic oscillations associated with specific behavioral states (Cobb et al. 1995; Klausberger and Somogyi 2008). Moreover, the interneuron activity also controls not only the development of cortical circuitry (Allendoerfer and Shatz 1994) but also the long-term synaptic plasticity of excitatory synapses (McBain and Fisahn 2001; Wigstrom and Gustafsson 1985). It would be therefore crucial and informative to understand the mechanisms underlying the regulation of GABA release from interneurons. To date, a variety of neurotransmitters including dopamine (Zhou and Hablitz 1999b), serotonin (Zhou and Hablitz 1999a) and glutamate (Satake et al. 2000) as well as GABA itself (Jarolimek and Misgeld 1997) have been reported to modulate the GABA release from the presynaptic terminals. In particular, the role of the major excitatory transmitter glutamate is of physiological importance in modulation of GABAergic interneurons, because it directly controls the excitatory and inhibitory neuronal circuits. In the hippocampus, ambient glutamate released by basal activity increases the efficacy of GABAergic synapses by activating presynaptic kainite receptors (KARs) (Jiang et al. 2001). In contrast, activation of postsynaptic KARs by a high concentration of KR agonists has been shown to markedly increase the firing of interneurons, leading to massive GABA release and hence a use-dependent depression of stimulation-evoked GABAergic synaptic response (Frerking et al. 1998). The roles of metabotropic glutamate receptor (mGluR) in modulation of GABA release have also been well documented: bath application of a specific group II mGluR agonist selectively inhibits inhibitory GABAergic transmission that
originates from interneurons in the stratum radiatum but not in the stratum oriens (Poncer et al. 2000), and synaptic activation of group III mGluRs could disinhibit GABAergic interneurons and thereby dampen the hippocampal excitability (Belen and Kostyuk 2002; Semyanov and Kullmann 2000).

Compared to KAR- and mGluR-mediated modulation of GABAergic transmission, short-term plasticity of GABAergic inhibitory transmission in the hippocampus following transient activation of NMDA receptors (NMDARs) have remained largely unknown except previous studies on cultured and acutely dissociated hippocampal neurons (Araque et al. 1998; Chen and Wong 1995; Marsden et al. 2007). Thus, this study using acute hippocampal slices aimed to examine how pyramidal neurons and interneurons respond to brief application of NMDA. We found that NMDA induces a profound increase in GABA\textsubscript{A} receptor-mediated spontaneous inhibitory postsynaptic current (sIPSC) superimposed on inward current responses in pyramidal neuron. The NMDA-induced sIPSCs were abolished by tetrodotoxin (TTX), suggesting that NMDA causes robust firing of GABAergic interneurons, leading to the increase in sIPSCs in pyramidal neurons. Both N- and P/Q-type voltage-gated calcium channels appear to mediate NMDAR-mediated GABA release from hippocampal interneurons because ω-conotoxin GVIA and ω-agatoxin TK partially suppressed the NMDA-induced sIPSC increase. Interestingly, the action of NMDA to increase sIPSCs was markedly attenuated both by injecting the NMDAR antagonist into postsynaptic pyramidal neurons and treating slices by the NO scavenger PTIO. Our data suggest that postsynaptic activation of NMDARs in hippocampal pyramidal neurons triggers nitric oxide synthesis and causes release of a retrograde messenger, possibly nitric oxide, thereby resulting in short-term facilitation of GABA release from interneurons.
METHODS

Slice preparation

All experiments were carried out in accordance with Tokushima Bunri University guidelines for animal experiments and approved by the Institutional Animal Care and Use Committee. C57BL6/J mice with the age of postnatal 11-16 days were deeply anesthetized by inhalation of halothane and immediately decapitated. The brain was rapidly removed and placed into ice-cold “cutting solutions” containing (in mM) 110 choline chloride, 2.5 KCl, 2.0 NaH$_2$PO$_4$, 25 NaHCO$_3$, 0.5 CaCl$_2$, 7 MgCl$_2$, 20 Glucose, 1.3 sodium ascorbate, 0.6 sodium pyruvate (Toni et al. 2008). Transverse slices of the right hemisphere were cut at 300 µm thickness using a vibrating microtome (VT1000S, Leica, Nussloch, Germany) and stored in a humidified and oxygenated incubating chamber with an interface of artificial cerebrospinal fluid (ACSF) that contained (in mM): NaCl 138.6, KCl 3.4, CaCl$_2$, 2.5, MgCl$_2$ 1.0, NaHCO$_3$ 21.0, NaH$_2$PO$_4$ 0.6 and glucose 10.0 (bubbled with 95% O$_2$ and 5% CO$_2$ and kept at pH 7.4) for at least 1 hr at room temperature. After incubation, the slice was transferred to a recording chamber on the stage of microscope (Axioskop-FS, Carl Zeiss, Jena, Germany) and continuously perfused with the oxygenated ACSF at a flow rate of 1.0-1.5 ml/min, and all the experiments were performed at room temperature unless otherwise stated.

Electrophysiology

Whole-cell voltage-clamp recordings were made from CA1 hippocampal pyramidal neurons under infrared-differential interface contrast (IR-DIC) video microscopy at room temperature using a Multiclamp 700B amplifier (Axon Instruments, Foster City, CA). Patch electrodes had a resistance of 3–4 MΩ when filled with an intracellular solution containing (in mM): CsCl 140, HEPES 10, QX-314 2, Mg-ATP 3, EGTA 0.1, Na$_3$GTP 0.4 (pH 7.4 adjusted with CsOH). When tested the effects of intracellularly applied NMDA channel blocker, 1 mM MK-801 was included in the pipette (Harney et al. 2008). The accessing resistance monitored by a constant membrane test pulse throughout experiments was in the range from 25–35 MΩ and was not compensated. Data were discarded if this value changed by more
than 20%. CA1 pyramidal neurons were held at –60 mV to record membrane currents and synaptic currents. Signals were digitized by the pClamp9 program through an A/D converter, Digidata 1322A (Axon Instruments). NMDA (100 µM) prepared in Mg$^{2+}$-free ACSF was puff-applied through the micropipette placed in the vicinity of recorded pyramidal neurons. To investigate the effects of NMDA on interneurons, hippocampal slices were prepared from VGAT (vesicular GABA transporter)-Venus transgenic mice (Wang et al. 2009) whose GABAergic interneurons could be clearly identified under fluorescence microscope, and recordings were made from interneurons under current-clamp mode without current injection. Because puff application of NMDA induced highly frequent firings in interneurons, signals were analyzed by the Clampfit program (Axon Instruments) to measure the peak amplitude and decay time of postsynaptic NMDA response. Basal and NMDA-induced synaptic events were analyzed by the Minianalysis program (Synaptosoft, Fort Lee, NJ). The threshold level was set as five times of the baseline noise (root-mean-square, RMS), and synaptic events detected automatically were visually checked to minimize errors. The paired two-tailed Student’s t-test was used for comparisons between two groups. Data are expressed as means ± SEM in all cases.

**Drug application**

D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV), N$^G$-Nitro-L-arginine methyl ester hydrochloride (L-NAME hydrochloride), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), N-Methyl-D-aspartic acid (NMDA) and SR95331 hydrobromide were obtained from Tocris Bioscience (Bristol, UK); Tetrodotoxin from Wako (Osaka, Japan); (+)-MK-801 hydrogen maleate, and PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) from Sigma (St. Louis, USA); ω-Agatoxin TK and ω-Conotoxin GVIA from Peptide Institute (Osaka, Japan). All drugs were prepared in stock solutions and diluted into a working concentration immediately before each experiment. BSA (0.25–0.5 mg/ml) was co-applied when tested the effects of calcium channel blockers to prevent the absorption to perfusion line and non-specific binding (Poncer et al. 1997).

**RESULTS**
NMDA increases GABA<sub>A</sub>-receptor mediated IPSCs in CA1 pyramidal neurons

When recorded from hippocampal CA1 pyramidal neurons, application of NMDA (100 µM) from the micropipette by a brief pressure pulse to the vicinity of the recorded site elicited not only an inward current but also fast phasic synaptic responses superimposed on the current response, which lasted several seconds (Fig. 1). There were two distinct types of NMDA-induced responses, which we termed type I and II. The type I response was observed in a majority of recordings (94 out of 100 cells): in this group, the amplitude of NMDA-induced phasic responses was small (Fig. 2C, mean amplitude change, 227 ± 21%, n = 13), and the increase in the frequency was marked (524 ± 48%, n = 13). In contrast, in a minority of neurons observed (6 out of 100 neurons), NMDA elicited a type II response: the mean amplitude of phasic responses, i.e., sIPSCs, was tremendously increased by NMDA (Fig. 2C, 1091 ± 74%, n = 6, p<0.01 as compared to the type I response), while the frequency increase was in the same extent to the type I response (402 ± 58%, n = 6, p>0.05 between type II and I responses).

When the bath temperature was increased to 35 °C, NMDA elicited an intense enhancement of phasic responses (Fig. 1C): mean increases in the amplitude and frequency of sIPSCs were 283 ± 112 and 706 ± 127%, respectively (n = 6). There was no significant difference in the extents of NMDA-induced responses between the two different temperatures (p>0.05, for both frequency and amplitude changes).

Because both excitatory postsynaptic currents (EPSCs) and IPSCs were in an inward direction under our recording conditions, we then examined whether NMDA increases either EPSCs or IPSCs. As illustrated in Fig. 2, A and B, addition of the GABA<sub>A</sub> receptor antagonist SR95531 (30 µM) completely abolished NMDA-induced increases in phasic synaptic activities in both type I and II responses, indicating that
NMDAR activation increases the frequency of \( \text{GABA}_\text{A} \) receptor-mediated IPSCs. Moreover, basal spontaneous synaptic events were also markedly inhibited by SR95531 (Fig. 2D): the frequency of spontaneous events was 2.02 ± 0.28 Hz in control solution (n = 9) and 0.11 ± 0.02 in the presence of SR95531 (\( p < 0.0001 \)). Spontaneous activities recorded in CA1 pyramidal neurons thus appeared to be mostly GABAergic in nature.

On the other hand, the AMPA/KA receptor antagonist NBQX exhibited variable effects on the NMDA-induced phasic inward current responses. In most cells examined (12 out of 15 cells), NBQX exhibited no significant effect on the frequency and the amplitude of NMDA-induced phasic synaptic currents: the frequency changes were 593 ± 143% in control ACSF and 513 ± 168% in NBQX (10 \( \mu \text{M} \)) containing ACSF (n = 12, \( p > 0.05 \)); the amplitude changes were 434 ± 98% in control ACSF and 391 ± 117% in NBQX containing ACSF (n = 12, \( p > 0.05 \)). In 3 out of 15 cells, NBQX markedly suppressed the NMDA-induced phasic synaptic currents without affecting the tonic inward current (data not shown). The observations suggest that AMPA/KA receptors do not play a substantial role in the NMDA-induced increase in the GABAergic synaptic activity in most CA1 pyramidal neurons and that a minority of pyramidal neurons might make synaptic contacts with GABAergic interneurons via axon collaterals to induce AMPAR-mediated feedback GABAergic IPSCs as demonstrated in the neocortical pyramidal neurons-interneuron interaction (Ren et al. 2007).

Application of either specific NMDAR antagonists, d-APV or \( \text{Mg}^{2+} \)-rich ACSF, markedly suppressed both the inward current responses and the increase in spontaneous GABAergic IPSCs produced by puff-applied NMDA (Fig. 3, A and B). Summary data depicted in Fig. 3C show that 50 \( \mu \text{M} \) d-APV completely blocked the NMDA-induced increases in the frequency as well as the amplitude of sIPSCs: for the
frequency change, 553 ± 111% in control and 97 ± 8.2% in the presence of D-APV (n = 5, p<0.05); for amplitude change, 220 ± 19% in control and 123 ± 6.9% in the presence of D-APV (n = 5, p<0.01). Switching from normal ACSF that contained 1 mM Mg$^{2+}$ to high Mg$^{2+}$ (8 mM)-containing ACSF also significantly reduced the NMDA-induced responses: for the frequency change, 452 ± 70% in control and 97 ± 10% in high Mg$^{2+}$ ACSF (n = 9, p<0.01); for the amplitude change, 203 ± 36% in control and 99 ± 3.5% in high Mg$^{2+}$ ACSF (n = 9, p<0.05).

We further asked whether glutamate itself could increase GABAergic IPSCs in pyramidal neurons. To test this issue, glutamate was iontophoretically applied from the glass micropipette to the vicinity of the recorded cells. In fact, glutamate also induced IPSCs in a APV-sensitive manner (Fig. S1A and B). As compared to the complete blockade of NMDA-induced sIPSCs by APV, the glutamate-induced inward current and sIPSCs were only partially suppressed by APV (Fig. S1C), suggesting that glutamate activates both NMDA- and AMPA-type glutamate receptors to induced GABAergic IPSCs as well as an inward current response in pyramidal neuron.

1The online version of this article contains supplemental data.

Characterization of NMDAR activation responsible for the increase in sIPSCs

Repeated activation of NMDARs has been known to undergo a progressive and use-dependent decline in either a calcium-dependent or a calcium-independent mechanism (Rosenmund and Westbrook 1993; Vissel et al. 2001; Wang et al. 1996). It is therefore needed to optimize the way of NMDA applications for reproducible responses. For this purpose, we examined the effects of changing the duration of puffs applied to the NMDA-filled pipette (0.5 to 20 sec). Both the amplitude and the decay time of NMDA-induced inward currents in pyramidal neurons increased in a puff-
duration dependent manner, but the latter was more linearly related to the puff duration (Fig. 4A). The peak amplitude of NMDA-induced currents showed a biphasic increase: it gradually increased in the amplitude with puffs less than 10 sec, then declined with 10 sec-puff and increased again with puffs more than 10 sec, which suggests that there could be two components of NMDA-induced responses, one being postsynaptic direct action and the other a trans-synaptic action (data not shown). Figure 4C illustrates the effects of changing the puff duration on the amplitude and the frequency of NMDA-induced sIPSC increases. There was little difference in the amplitude of sIPSCs when the NMDA puff durations were changed, and the amplitude increase stayed around 200% of the control with individual puff durations (n = 5). In contrast, the frequency change of sIPSCs induced by NMDA puffs reached a maximal increase of 519 ± 114% at the duration of 3 sec with a gradual decline following further increase in the duration. Thus, it seemed to be appropriate to use the puff duration of 3 to 5 sec. Indeed, the NMDA-induced increases in the amplitude and frequency of sIPSCs remained almost constant when NMDA applications were repeated with an interval of 180 sec throughout the period of experiments (Fig. 4, B and D). In the following experiments, we therefore decided to apply NMDA under these conditions to perform further pharmacological experiments for exploring the mechanisms underlying the NMDA-induced enhancement of GABAergic sIPSCs.

NMDAR activation induces interneuron firing to enhance TTX-sensitive IPSCs in CA1 pyramidal neurons

The NMDA-induced increases in the frequency and amplitude of sIPSCs in CA1 pyramidal neurons could be attributed to an increase in the firing of presynaptic GABAergic interneurons or partly to an increase in the sensitivity of postsynaptic GABA_A receptors in pyramidal neurons. To distinguish the two different mechanisms,
we examined the effects of a sodium channel blocker, TTX, on the NMDA-induced
responses. In the presence of TTX (0.5 μM), puff application of NMDA gave rise to
the NMDAR-mediated inward current, whereas TTX almost completely abolished the
NMDA-induced increase in synaptic activities (Fig. 5, A and B): the frequency
changes were 394 ± 59% in the control ACSF and 83 ± 6.2% in the presence of
TTX (n = 6, p<0.01); and the amplitude changes were 223 ± 34% in the control
medium; 118 ± 16 % in the TTX-medium (n = 6, p<0.05). The result is consistent
with the possibility that the firing of presynaptic GABAergic interneurons increases
during NMDA application.

To obtain further support for this notion, we next attempted to record from
GABAergic interneurons in CA1 subfield and examined the effects of NMDA on
them. For this purpose, we recorded from fluorescent GABAergic interneurons in
hippocampal slices cut from VGAT-Venus transgenic mice (Wang et al. 2009). Puff
application of NMDA induced an intense discharge of action potentials during the
rising phase of depolarization under current clamp mode in 1 out of 9 Venus-positive
interneurons (Fig. 5D). In interneurons with spontaneous firing, NMDA initially
increased the rate of action potential generation and ceased it when the depolarizing
action reached a plateau (Fig. 5C). Taken together, it is most likely that brief
application of NMDA not only induces an inward current in CA1 pyramidal neurons
but also activates NMDARs located at soma/dendrites of interneurons to increase
their firings, thereby trans-synaptically enhancing GABAergic sIPSCs in pyramidal
neurons. The fact that there were at least two types of interneurons in terms of the
spontaneous firing and the NMDA sensitivity (see Fig. 5, C and D) could explain the
observations that NMDA induced two distinct (type I and II) changes in sIPSCs in
pyramidal neurons: the NMDA-induced type I response (namely relatively small
sIPSCs evoked in pyramidal neurons) could be produced by interneurons shown in
Fig. 5C, and interneurons with the firing property shown in Fig. 5D could be responsible for the type II response with large sIPSCs in pyramidal neurons.

NMDAR activation mobilizes retrograde nitric oxide-mediated signaling from pyramidal neurons to interneurons

The data presented above clearly demonstrate that NMDARs in presynaptic GABAergic interneurons play a major role in the NMDA-induced enhancement of sIPSCs in CA1 pyramidal neurons. In the following experiments, we further examined whether postsynaptic NMDARs in pyramidal neurons could be involved in the NMDA-induced IPSC acceleration. To test this possibility, we attempted to selectively block the postsynaptic NMDAR activation by loading pyramidal neurons with an irreversible NMDAR blocker, MK-801 (1 mM), through the recording patch-pipette under the whole-cell configuration (Lien et al. 2006; Rodriguez-Moreno and Paulsen 2008). To minimize the leakage of MK-801 from the recording pipette, we adopted two ways to introduce MK-801 into pyramidal neurons. First, we took care of reducing the amount and time of positive pressure adding to the patch-pipette, so that pressure was applied immediately before approaching onto the cell surface. To our surprise, the inclusion of MK-801 in the pipette not only gradually reduced the amplitude of NMDA-induced inward currents as we expected (Fig 6B), but also markedly inhibited the NMDA-induced increases in the frequency and the amplitude of sIPSCs in 5 out of 8 pyramidal neurons (Fig. 6, A and C). Second, we used a two-step filling of MK-801: the tip of the recording pipette was first filled with the control internal solution, and then 1 mM MK801-containing internal solution was superimposed on it. Under these conditions, the NMDA-induced current decreased to 0.20 ± 0.05 of control at 24 min after entering the whole-cell mode, while the NMDA-induced changes in sIPSC were also largely inhibited: the frequency and
amplitude increases were 0.43 ± 0.12 and 0.46 ± 0.06 of the controls, respectively, (n = 6). In the control experiments where MK-801 was not included in the patch-pipette, repeated applications of NMDA produced reproducible increases in sIPSCs without any significant sign of rundown (see Fig. 4D). Thus, the findings from the experiments of intracellular applications of MK-801 strongly suggest that activation of postsynaptic NMDARs also plays a role in the NMDA-induced enhancement of sIPSCs.

One plausible consequence would be that postsynaptic NMDAR activation could recruit a retrograde messenger that diffuses from pyramidal neurons to the presynaptic terminal of GABAergic interneurons to facilitate GABA release. Several lines of evidence support nitric oxide (NO) as a candidate for retrograde messenger: (1) NO increases the GABA release at several central nervous system (CNS) synapses (Li et al. 2002; Yang and Cox 2007) and induces long term potentiation of inhibitory GABAergic synapses in the ventral tegmental area (Nugent et al. 2009; Nugent et al. 2007); and (2) Ca\(^{2+}\)/calmodulin-dependent neuronal nitric oxide synthase (nNOS) occurs in hippocampal pyramidal neurons where NO could be produced following NMDAR activation (Ledo et al. 2005; Prast and Philippu 2001). We, therefore, tested the effect of a commonly used NO scavenger, PTIO, on the NMDA-induced GABAergic synaptic responses. As can be seen from the experiments shown in Fig. 7, the addition of 100 µM PTIO reversibly inhibited the NMDA-induced increase in the sIPSC frequency (8 out of 14 pyramidal neurons tested), while the increase in the sIPSC amplitude remained unaltered (Fig. 7, A and C): NMDA increased the frequency of sIPSCs to 1196 ± 268% in control ACSF and 711 ± 140% in the presence of PTIO, respectively (n = 8, p<0.05).

We then examined the effects of L-NAME, a none-specific NO synthase inhibitor, to further test the possible involvement of NO signaling in the NMDAR-
mediated response. As shown in Fig 7D, the pretreatment of slices with 300 µM L-NAME for at least 2 hr largely suppressed the NMDA-induced increase of sIPSCs in 7 of 11 cells: the frequency and amplitude increases were 349 ± 45 and 145 ± 26%, respectively, and L-NAME significantly inhibited the NMDA-induced responses as compared to the control responses (p<0.05, n = 7). Together, these data supported the notion that NMDA enhances GABAergic inhibitory transmission through the activation of both presynaptic NMDARs in GABAergic interneurons and postsynaptic NMDARs in pyramidal neurons, the latter leading to mobilization of the retrograde messenger NO signaling pathway to enhance GABA release from interneuron terminals. The NMDA-induced inward current was also truncated by PTIO, which might suggest that NO could act on pyramidal neurons in an autocrine manner to enhance the NMDAR activity as well.

Differential involvements of presynaptic calcium channel subtypes in NMDAR-mediated increase in GABAergic transmission

Previous studies have shown that GABA release from hippocampal interneurons is exclusively mediated by either N-type or P/Q-type voltage gated calcium channels (VGCCs) depending on the cell type of interneurons (Hefft and Jonas 2005; Poncer et al. 1997). We therefore asked whether any specific subtype of presynaptic VGCCs is involved in the NMDA-induced enhancement of GABAergic transmission from interneurons to pyramidal neurons. Application of a selective N-type VGCC blocker, ω-conotoxin GVIA (ω-CTx-GVIA, 1 µM), caused little effect on the increases in the frequency and amplitude of sIPSCs following NMDAR activation (Fig. 8, A and D): the NMDA-induced increases in the sIPSC frequency and amplitude were 654 ± 95 and 383 ± 79% in control ACSF, and 574 ± 76 and 339 ± 69% in ω-CTx-GVIA-containing ACSF, respectively (n = 7, p >0.05).
In contrast, a selective P/Q-type VGCC blocker, ω-Agatoxin TK (ω-Aga-TK, 0.25 µM), almost completely suppressed the NMDA-induced increases of the sIPSC frequency in 5 out of 8 pyramidal neurons examined (Fig. 8, B and D): 527 ± 84% in control ACSF and 140 ± 37% in ω-Aga-TK-containing ACSF (n = 5, p<0.01). The effect of ω-Aga-TK was less effective on the sIPSC amplitude: 295 ± 31% in control ACSF and 189 ± 37% in the toxin-containing ACSF, n = 5, p>0.05). In the other 3 pyramidal neurons, ω-Aga-TK strongly inhibited a small component of sIPSCs induced during the rising phase of NMDA-induced inward current with little effect on a much larger component of sIPSCs observed during the late phase of NMDAR-mediated current (Fig. 8C, middle trace).

Interestingly, further addition of 1 µM ω-CTx-GVIA completely abolished the NMDA-induced larger sIPSCs (Fig. 8C, bottom trace; Fig. 8E). It is therefore likely that CA1 pyramidal neurons are connected by at least two different populations of interneurons selectively expressing either P/Q- or N-type VGCCs at their nerve terminals and that the P/Q-type VGCC expressing type is dominantly involved in the NMDAR-mediated enhancement GABAergic transmission between hippocampal CA1 interneurons and pyramidal neurons. Furthermore, the N-type VGCC in certain interneurons appeared to be very efficient in synchronous release of GABA from their terminals to produce much larger IPSCs in pyramidal neurons. In this context, it seems that interneurons with P/Q-type VGCC could be involved in the NMDA-induced type I response where sIPSCs with relatively small amplitude increased following NMDAR activation, whereas N-type VGCC-expressing interneurons may contribute to type II response with NMDA-induced sIPSCs with large amplitude.

**DISCUSSION**

The main finding of this study is that focal application of NMDA could elicit
marked enhancement of GABA$_A$ receptor-mediated inhibitory transmission between hippocampal CA1 interneurons and pyramidal neurons with two distinct presynaptic and postsynaptic mechanisms. First, activation of NMDARs in presynaptic interneurons appears to contribute to the NMDA-induced enhancement of GABAergic transmission. Second, NMDA is likely to act on postsynaptic NMDARs in pyramidal neurons, linking to liberation of retrograde messengers, possibly NO, thereby enhancing GABA release from presynaptic terminals of interneurons.

A previous study has reported that incubation of a highly purified nerve terminal preparation with NMDA caused an increase in the extracellular GABA concentration, and proposed the existence of presynaptic interneuronal NMDARs responsible for modulation of GABA release from the cytoplasmic pool, presumably through the action of reversing Na$^+$-dependent glutamate transporters (Breukel et al. 1998). Recent histological studies, however, have shown that NMDAR labeling in the hippocampus mostly derived from postsynaptic receptors, but not from presynaptic sites (Nyiri et al. 2003; Takumi et al. 1999). In line with these immunohistochemical data, we find that NMDAR activation elicited the increases in the frequency and amplitude of sIPSCs in a TTX-sensitive manner, but did not alter mIPSCs, arguing against the possible role of presynaptic NMDARs in modulation of spontaneous GABA release from interneuron terminals.

Although the role of NMDARs in long-term synaptic plasticity has been well delineated (Barria and Malinow 2005; Nicoll and Malenka 1999), relatively little was known about how NMDARs could be involved in short-term modulation of neurotransmission at CNS synapses. The results of present study reveal that NMDARs in both hippocampal interneurons and pyramidal neurons play a pivotal role in the information flow through glutamatergic transmission within the hippocampal neuronal circuits. Following activation of AMPA-type receptors
(AMPARs) by the excitatory transmitter released by afferent inputs at excitatory synapses on CA1 pyramidal neurons, there could be a time window in which activation of presynaptic and postsynaptic NMDARs provides a profound inhibitory influence for the processing of afferent signals to hippocampal pyramidal neurons. The fact that diffusible retrograde messengers like NO could be recruited by activation of postsynaptic NMDARs in pyramidal neurons suggests that signal flows through hippocampal CA1 pyramidal neurons may require a period of feedback inhibition provided by a neuronal circuit connecting pyramidal neurons to certain interneurons and back to pyramidal neurons to prevent overexcitation following transient point-to-point transmission through activation of fast-acting AMPARs in postsynaptic pyramidal neurons.

Recently, much attention has been focused on endocannabinoid-mediated retrograde regulation of GABAergic inhibition (Heifets and Castillo 2009). In our experiments, we tested the effects of CBR1 blocker, AM251, on the NMDA-induced sIPSC increase and found among 8 cells that only one cell showed a marginal increase of NMDA-induced IPSCs in the presence of AM251, while other 7 cells showed no significant changes in the NMDA response before and after the CBR1 blocker treatment (data not shown). It seems therefore that hippocampal CA1 GABAergic inhibitory synapses are under reciprocal control by the retrograde regulatory mechanisms mediated by NMDA receptor activation and endocannabinoid liberation.

The results of this study may also support the heterogeneity of hippocampal interneurons (Klausberger and Somogyi 2008; Somogyi and Klausberger 2005). Puff application of NMDA indeed elicited in pyramidal neurons two distinct responses that we referred to as type I and II responses. In the type I response, NMDA recruited GABAergic inputs that elicited IPSCs with relatively small quantal contents, whereas sIPSCs evoked by NMDA in the type II response surprisingly had much larger
amplitude. These observations suggest that the properties of GABAergic synapses
made by interneurons onto pyramidal neurons might be largely different dependent on
the types of interneurons with the distinct properties of GABA release machinery.
This possibility is further supported by the finding that distinct VGCC blockers
differentially affected the NMDA-induced enhancement of GABA release from
interneurons onto pyramidal neurons. The P/Q-type VGCC blocker \( \omega \)-Aga-TK
selectively suppressed the NMDA-induced increase in sIPSCs with small amplitude,
whereas sIPSCs with large amplitude produced in response to NMDA application was
specifically sensitive to the N-type VGCC blocker \( \omega \)-CTx-GVIA. Thus it appears that
one subclass of interneurons that express N-type VGCCs at their terminals provide a
powerful inhibition via synchronous GABA release with large quantal size,
Heterogenous GABAergic interneurons are likely to express distinct calcium channel
subtypes and to be endowed with different release machinery at their presynaptic
terminals, thereby differentially contributing to modulation of the excitability in
hippocampal CA1 pyramidal neurons. It appears therefore that hippocampal
GABAergic interneurons are heterogeneous in terms of not only spiking properties
(Heft and Jonas 2005) but also the release machinery characteristics.

Intracellular injection of the NMDAR open channel blocker MK-801 has been
successfully employed in a number of studies to discriminate the roles of presynaptic
and postsynaptic NMDARs in long-term synaptic plasticity (Humeau et al. 2003; Lien
et al. 2006; Mameli et al. 2005) as well as the processes mediated by synaptic and
extrasynaptic NMDARs (Harris and Pettit 2007). In this study, MK-801 (1 mM, a
commonly used concentration for blockade of postsynaptic NMDARs) included in the
patch-pipette, however, appeared to cause a blocking action through the leakage from
the recording pipette before the establishment of the whole-cell mode. We therefore
adopted a modified protocol in which the amount and the duration of positive pressure
applied to the patch-pipette were reduced before forming GΩ seal. With this method, intracellularly applied MK-801 blocked both postsynaptic NMDA response and NMDA-induced increase of sIPSCs simultaneously. The observation was confirmed by experiments using a two-step filling of MK-801. The results of the two experiments, therefore, point to the possible involvement of retrograde messengers in the NMDAR-mediated enhancement of GABAergic transmission from interneurons to CA1 pyramidal neurons. This possibility is consistent with previous findings that the NO synthesizing enzyme nNOS is densely expressed in hippocampal pyramidal neurons and tightly linked to NMDARs at glutamatergic synapses (Burette et al. 2002; Wendland et al. 1994). The role of NO has been well documented in modulation of neurotransmitter release at excitatory synapses. For example, repetitive stimulation of glutamatergic afferents has been shown to induce calcium influx through NMDAR channels and activate nNOS coupled to NO generation in postsynaptic neurons, thereby retrogradely regulating the release of glutamate to induce long term potentiation (Garthwaite 2008; Haley et al. 1992). The NO-mediated pathway is also implicated in modulation of GABA release from interneurons onto magnocellular neurons in the paraventricular nucleus (Bains and Ferguson 1997; Garthwaite 2008). Furthermore, it has recently been reported that NO could be involved in depolarization-induced suppression of inhibition (DSI) following activation of muscarinic receptors in hippocampal pyramidal neurons where NO has been suggested to exert an inhibitory effect on GABA release (Makara et al. 2007). In contrast to their report, the results from this study and other studies on other brain regions including the thalamus, ventral tegmental area and paraventricular nucleus (Li et al. 2004; Li et al. 2002; Nugent et al. 2009; Yang and Cox 2007) show the enhancement of GABAergic transmission by NO-mediated signaling pathway. Thus, NO appears to be involved in diverse modes of modulation of neurotransmission at a
number of CNS excitatory and inhibitory synapses.

Overall, our findings support the notion that NMDARs in both presynaptic interneurons and postsynaptic pyramidal neurons play a pivotal role in the regulation of GABAergic inhibitory transmission in the CA1 subfield. The activation of postsynaptic NMDARs appears to elicit the liberation of retrograde messenger like NO, and profoundly promote GABA release from interneurons, thereby resulting in feedback inhibition to determine a precise timing of action potentials in hippocampal projecting neurons. By this way, NMDARs likely enhance the computational capacity of the hippocampal microcircuit.

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**Legends to Figures**

Figure 1. NMDA-induced phasic inward currents superimposed on a tonic inward current in a CA1 pyramidal neuron. A: a representative trace from a pyramidal neuron displayed on a slow time base. NMDA (100 μM) was puff-applied adjacent to the recorded neuron during the period indicated by a horizontal line. 

B: expanded traces obtained in the record shown in A: 1, before NMDA application; 2, during the NMDA-induced tonic inward current response; and 3, after returning to the baseline level. Note that NMDA induced not only a tonic inward current but also a burst of phasic inward synaptic currents. 

C: NMDA-induced responses in a pyramidal neuron were recorded at 35 °C.

Figure 2. Two types of responses induced by puff applications of NMDA in CA1 pyramidal neurons in mouse hippocampal slices. A and B: representative traces of NMDA-induced type I (A) and type II (B) responses in control ACSF (*top*) and in SR95531 (30 μM)-containing ACSF (*bottom*). During the period indicated by a horizontal bar, NMDA (100 μM) was applied by a brief puff given to the micropipette placed near the recorded pyramidal neurons. NMDA induced a tonic inward current and phasic inward synaptic currents with small amplitudes in type I response (A) and large amplitudes in type II responses (B). The GABA<sub>A</sub> receptor blocker SR95531 completely abolished phasic synaptic currents without significantly affecting the tonic inward currents. C: summary graph for changes in the frequency (open column) and amplitude (closed column) of sIPSC in NMDA-induced type I and II responses. There was no significant difference in the extents of sIPSC frequency changes between the Type I and II responses (n = 6, p>0.05), whereas the amplitude increase was much larger in the Type II response than in the type I response (n = 6, p<0.01). D: blockade
of basal sIPSCs by bath application of SR95531 (30 μM; n = 9, p<0.0001).

Figure 3. Effects of the NMDAR antagonist D-APV and high Mg$^{2+}$ on NMDA-induced responses in CA1 pyramidal neurons. A and B: NMDA-induced responses were recorded from pyramidal neurons in control ACSF (top traces), D-APV (50 μM)-containing (bottom trace in A) and 8 mM Mg$^{2+}$-containing ACSF (bottom trace in B). NMDA (100 μM) was puff-applied adjacent to the recorded neuron during the period indicated by a horizontal line. The NMDA-induced responses were almost completely abolished in the presence of either D-APV or high Mg$^{2+}$.

C: summary graph for the effects of D-APV and Mg$^{2+}$ on NMDA-induced changes in the sIPSC frequency (open column) and amplitude (closed column). D-APV significantly suppressed the NMDA-induced increases in the sIPSC frequency and amplitude (n = 5, p<0.05 and p<0.01, respectively). Similarly, both the sIPSC frequency and amplitude change were significantly suppressed by high Mg$^{2+}$ (n = 9, p<0.01 and p<0.05, respectively).

Figure 4. Characterization of NMDA-induced responses in CA1 pyramidal neurons. A: effects of changing the puff duration on NMDA-induced responses. Upper and bottom pairs of traces; monitoring puff durations (0.5s, 1s, 3s, 5s in top trace of the upper pair; and 10s, 15s, 20s in top trace of the lower pair), and superimposed NMDA-induced current responses (bottom trace of each pair) produced in response to individual puffs in a single pyramidal neuron. B: the NMDA-induced responses in response to the first (1st, top trace) and 10th NMDA puff (10th, bottom). Both the first and 10th NMDA puffs (during the period indicated by horizontal bars) produced almost reproducible current responses. C: effects of changing the NMDA puff duration on the NMDA-induced changes in the sIPSC frequency (open circles) and
amplitude (closed circles). $D$: time-course change in the NMDA-induced increases in the sIPSC frequency (open circles) and amplitude (closed circles). Each value of increase in the sIPSC frequency and amplitude induced by successive NMDA puffs was expressed as a ratio of that induced by the first puff.

Figure 5. The effects of TTX on NMDA-induced responses in a pyramidal neuron, and the effects of NMDA on interneurons in the CA1 region. $A$: representative traces of NMDA-induced responses in a pyramidal neuron before (top) and after treatment with TTX (0.5 μM, bottom). $B$: Summary graph for the effects of TTX on the NMDA-induced changes in the sIPSC frequency (open column) and amplitude (closed column). TTX markedly suppressed both the increases in the sIPSC frequency ($n = 6$, $p<0.01$) and amplitude ($n = 6$, $p<0.05$). $C$ and $D$: effects of NMDA on two different interneurons. NMDA, applied by puff during the period indicated by a horizontal bar, first increased spontaneous firing of an interneuron together with a large depolarization and subsequently made it quiescent, possibly because of depolarizing blockade of sodium channels (C) and elicited action potentials superimposed on a depolarizing action in another silent interneuron (D).

Figure 6. Effects of the NMDAR open channel blocker MK-801 on the NMDA-induced responses in CA1 pyramidal neurons. $A$: representative traces of current responses produced by the first (left) and eighth NMDA puffs (right) in the same pyramidal neuron into which MK-801 (1 mM) was injected through the recording patch pipette. $B$: time-course change in the amplitude of inward current responses in pyramidal neurons produced in response to repeated puff applications of NMDA with an interval of 3 min. In these neurons, the recordings were made with the patch pipette filled with a MK-801-containing internal solution, and the amplitude of
NMDA-induced currents was expressed as a ratio of that produced by the first NMDA puff. C: time-course changes in the NMDA-induced increases in the sIPSC frequency (open circles) and amplitude (closed circles). These experiments were performed in the same manner as described in B.

Figure 7. Effects of the NO scavenger PTIO and the NOS inhibitor L-NAME on the NMDA-induced increases of sIPSCs in CA1 pyramidal neurons. A: time-course changes in the NMDA-induced increases in the sIPSC frequency (open circles) and amplitude (closed circles). The extent of NMDA-induced changes in sIPSC frequency and amplitude was expressed as a ratio of that produced by the first NMDA puff. PTIO (100 μM) was applied by perfusion during the period indicated by a horizontal line. B: sample traces of NMDA-induced responses before (1), during PTIO application (2), and after washing out the drug (3). Each trace was obtained at the time point labeled by 1 to 3 in the graph (A). C: summary graph for the effects of PTIO on the NMDA-induced increases in sIPSC frequency (open column) and amplitude (closed). The value after PTIO treatment was expressed a ratio of the NMDA-induced increase in control ACSF. D: NMDA-induced responses recorded from pyramidal neurons of a control slice (left) and a slice pretreated with 300 μM L-NAME (right).

Figure 8. Differential effects of the VGCC blockers on the NMDA-induced increases in sIPSCs in CA1 pyramidal neurons. A: the NMDA-induced type I response in control ACSF (top) was not significantly affected by treatment with the N-type VGCC blocker ω-CTx-GVIA (1 μM, bottom). B: the NMDA-induced type I response in control ACSF (top) was almost completely abolished by treatment with the P/Q-type VGCC blocker ω-Aga-TK (0.25 μM, bottom). C: NMDA puff induced a mixed type I
and II response in control ACSF (top) of which the initial phase of small sIPSC increase (type I response) was selectively suppressed by ω-Aga-TK (0.25 μM, middle), and the later phase of large IPSC increases (type II response) was totally abolished by ω-CTx-GVIA (1 μM, bottom). D: summary graph for the effects of ω-CTx-GVIA (1 μM, open column) and ω-Aga-TK (0.25 μM, closed column) on the NMDA-induced increases in the frequency of small IPSCs (type I response). The type I response was selectively suppressed by ω-Aga-TK (n = 5, p<0.01), but not by ω-CTx-GVIA (n = 5, p>0.05). E: summary data for the NMDA-induced changes in sIPSC frequency in control ACSF (NMDA) and in the presence of ω-Aga-TK (0.25 μM) or ω-Aga-TK (0.25 μM) plus ω-CTx-GVIA (1 μM). The data in E were derived from the experiment shown in C.
Figure 1 (Xue et al.)

A

1. NMDA

2. RT

3. 10 s

500 pA

B

1

2

3

10 s

200 pA

200 ms

C

NMDA

35 °C

10 s

200 pA
Figure 2 (Xue et al.)

A. NMDA

B. NMDA

C. Graph showing sIPSC frequency and amplitude for different treatments.

D. Graph showing mean frequency for baseline and SR95531 conditions.

**Baseline**

NMDA

+ SR95531

500 pA

5 s
Figure 3 (Xue et al.)
Figure 4 (Xue et al.)

A

B

C

D

Nominal sIPSC

Puff duration

Time (min)

sIPSC (%)

Frequency

Amplitude

Normalized sIPSC

Normalized sIPSC

0.5 s 3 s 10 s 20 s

0

200 pA

10 s

200 pA

10 s

500 pA

5 s

0

600

400

200

0

0

6

12

18

24

Frequency

Amplitude

0.8

0.4

1.2

Normalized sIPSC (%)

Normalized sIPSC (%)

NMDA 1st puff

NMDA 10th puff
Figure 5 (Xue et al.)
Figure 6 (Xue et al.)

A

NMDA (1st puff)  MK-801

NMDA (8th puff)  MK-801

0 6 12 18 24

0 0.4 0.8 1.2

Time (min)

Normalized peak amplitude of NMDA response

B

Normalized peak amplitude of NMDA response

Time (min)

0 6 12 18 24

Normalized sIPSC Frequency Amplitude

C

Normalized sIPSC

Time (min)

0 6 12 18 24

5 s

500 pA

NMDA (1st puff)  MK-801

NMDA (8th puff)  MK-801

C

5 s

500 pA
Figure 7 (Xue et al.)

A. Graph showing normalized sIPSC frequency and amplitude over time with PTIO treatment.

B. Three panels illustrating the effects of NMDA and PTIO on sIPSCs.

C. Bar graph comparing control, PTIO, control, and PTIO conditions for sIPSC frequency and amplitude.

D. Comparison of NMDA- and L-NAME-induced sIPSCs.