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Nitric Oxide Synthase Inhibitor Decreases NMDA-Induced Elevations of Extracellular Glutamate and Intracellular Ca^{2+} Levels Via a cGMP-Independent Mechanism in Cerebellar Granule Neurons

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These studies were designed to examine the differential effect of nitric oxide (NO) and cGMP on glutamate neurotransmission. In primary cultures of rat cerebellar granule cells, the glutamate receptor agonist N-methyl-D-aspartate (NMDA) stimulates the elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), the release of glutamate, the synthesis of NO and an increase of cGMP. Although NO has been shown to stimulate guanylyl cyclase, it is unclear yet whether NO alters the NMDA-induced glutamate release and $[Ca^{2+}]_i$ elevation. We showed that the NO synthase inhibitor, N^G-monomethyl-L-arginine (NMMA), partially prevented the NMDA-induced release of glutamate and elevation of $[Ca^{2+}]_i$ and completely blocked the elevation of cGMP. These effects of NO on glutamate release and $[Ca^{2+}]_i$ elevation were unlikely to be secondary to cGMP as the cGMP analogue, dibutyryl cGMP (dBcGMP), did not suppress the effects of NMDA. Rather, dBcGMP slightly augmented the NMDA-induced elevation of $[Ca^{2+}]_i$ with no change in the basal level of glutamate or $[Ca^{2+}]_i$. The extracellular NO scavenger hydroxocobalamine prevented the NMDA-induced release of glutamate providing indirect evidence that the effect of NO may act on the NMDA receptor. These results suggest that low concentration of NO has a role in maintaining the NMDA receptor activation in a cGMP-independent manner.

Key words : NMDA, N^G-Monomethyl-L-arginine, Dibutyryl-cGMP, Ca^{2+} Influx, Hydroxocobalamin, Glutamate release

INTRODUCTION

The NMDA receptor plays a key role in synaptic plasticity which is thought to underlie memory, learning and development of the nervous system. Triggers for nitric oxide (NO) generation in the central nervous system (CNS) were first shown to be glutamate (Garthwaite *et al.*, 1989). However, NO is not a classical neurotransmitter since it is not released by exocytosis (Garthwaite and Garthwaite, 1991). Nitric oxide does not interact with a receptor protein but rather diffuses rapidly across the membrane of target neurons where it binds with the iron in heme-containing proteins (Gally *et al.*, 1990). NO has been linked with memory formation (Holscher and Rose, 1992; Snyder, 1992) and both long-term potentiation (LTP) occurring in the hippocampus (Schuman and Madison, 1991) and long-term depression (LTD) occurring in the cerebellum (Shibuki and Okada, 1991). However, the mechanism of NO on these processes is unclear.

The production of NO in the CNS is most closely linked with stimulation of the NMDA receptor, a glutamate receptor subtype. High concentrations of nitric oxide synthase (NOS) have been immunolocalized in the cerebellum to the granule cell layer (Bredt *et al.*, 1990), and a functional role of NO is thought to be the activation of guanylyl cyclase. Consistent with this role is the fact that both NOS and cGMP levels are higher in the cerebellum than in any other brain region. Elevation of cGMP induced by NMDA is enhanced by L-arginine and inhibited by NOS inhibitors (Garthwaite *et al.*, 1989; Bredt and Snyder, 1989). NO can act on molecular targets through cGMP such as cGMP-dependent protein kinase (Lincoln and Corbin, 1983; Zhuo *et al.*, 1994) which can mediate phosphorylations. The cGMP analogue, 8-bromo-cyclic GMP, produced neurotrophic and neuroprotective effects that

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were quite similar to the effects produced by NMDA in cerebellar granule cells (Pantazis *et al.*, 1998). Also cGMP can act on cyclic nucleotide dependent ion channels (Ligut *et al.*, 1990: Distler *et al.*, 1994) which can modulate membrane potential and signal transduction.

In an attempt to determine the effect of the physiological level of NO on NMDA receptor-mediated functions, the NOS inhibitor, N^G-monomethyl-L-arginine (NMMA) was used, and to determine whether NO-mediated effects on NMDA receptor functions are secondary to cGMP elevations, dBcGMP was used. In the present study, we found that the NMDA-induced elevations of extracellular glutamate and $[Ca^{2+}]_i$ levels were inhibited by NOS inhibitor but not changed by dBcGMP.

MATERIALS AND METHODS

Materials

N-methyl-D-aspartate, dibutyryl-cGMP, hydroxocobalamin and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Cyclic GMP kit was purchased from Amersham (Arlington Height, IL).

Cerebellar granule cell culture

Cerebellar granule cells were cultured as described previously (McCaslin and Morgan, 1987) with slight modifications. Briefly, 8-day-old rat pups (Sprague-Dawley) were decapitated, and the heads were sterilized by immersion in 95% ethanol. The cerebellum was dissected from the tissue and placed in Dulbecco's modified Eagle's medium (DMEM) without serum and bicarbonate. Neurons were separated from the greater bulk of tissue by mechanical trituration using serial sucking and pushing with syringe. Dissociated cells were applied at a volume of 1 ml over the slide glass which was cut properly to be fitted in 60 mm culture dish. Dissociated cells were collected at a density of about 2×10^6 cells/ml. After 20 min, the DMEM medium was aspirated from the culture dish and growth medium was added (5 ml/dish, 300 mOsm). Growth medium was DMEM supplemented with sodium pyruvate (1 mM), glutamine (4 mM), sodium bicarbonate (44 mM), glucose (25 mM), 6% conditioned bovine serum (Hyclone, Logan, UT) and 6% fetal bovine serum (JRH Bioscience, Lenexa, KS). After 2 days of incubation (37°C, 10% CO_2), growth medium was aspirated from the cultures and new growth medium (5 ml/dish) containing 25 mM KCl was added with 5 (M cytosine arabinoside to prevent proliferation of glia cells. Postmitotic granule neurons can be readily maintained in vitro in their fully differentiated state for several weeks if depolarized with high concentrations of K^+ (Gallo *et al.*, 1987). The cells were then incubated for 14 more days.

Glutamate measurement

Experiments were performed using cultures grown for 10~14 days after plating. After growing cells, medium was removed and after washing, the cells were placed in a physiological saline HEPES (PSH) buffer containing the following mM concentrations: 135 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 40 bicarbonate, 10 glucose and 5 HEPES (pH 7.4, 300 mOsm). After a 30 min equilibriation period in PSH buffer (37°C, 10% CO_2), the glass slides containing attached cells were washed by serial dipping in HEPES buffer and re-incubated in the presence of compound in 60 mm culture dish at 37°C for 1 h. The amount of glutamate secreted into the buffer (3 ml/dish) was determined by HPLC (Ellison *et al.*, 1987) as described below. A small amount of buffer (500 µl) is collected from the culture dish, and the glutamate concentration was quantified by HPLC (Bioanalytical Systems) with an electrochemical detector after precolumn derivatization of sample aliquots (50 µl) with 20 µl of *o*-phthaldialdehyde/2-mercaptoethanol reagent. The C18, 5 mm, 4.6×150 mm, reverse phase column (Rainin, CA) was eluted with mobile phase (pH 5.2) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a rate of 1.0 ml/min.

Cyclic GMP measurement

Cyclic GMP levels were determined via radioimmunoassay as described (Harper and Brooker, 1975). Briefly, after stimulating cells with an excitatory amino acid and inactivating enzymes with hot (90°C) 1 N $HClO_4$, cells were sonicated and then centrifuged at 5000 g for 5 min. Cyclic GMP levels are determined in the supernatant after neutralization (pH 7.4) with 2 M $KHCO_3$ using a radioimmunoassay kit (Amersham, IL). cGMP levels were determined in units of pmol/mg protein. The protein content was determined according to Bradford (Bradford, 1976) using bovine serum albumin as the standard.

Intracellular Ca^{2+} measurement

Intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by ratio fluorometry as described (Grynkiewicz *et al.*, 1985; Cai and McCaslin, 1992). The cells were grown on glass cover slides, loaded with 10 µM fura-2 AM for 1 hr in PSH buffer at 37°C, and washed with PSH buffer. Cell culture slides were cut with a diamond pencil and mounted into spectrophotometer cuvettes containing 2.5 ml PSH buffer (without bicarbonate). Fluorescence was measured with a FLUOROLOG-2 spectrophotometer (SPEX Ind. Inc., Edison, NJ) by exciting cells at 340 and 380 nm and measuring light emission at 505 nm. Baseline of $[Ca^{2+}]_i$ was measured for 60 sec before the addition of various experimental

compounds. Rmax and Rmin were determined by addition of ionomycin (10 μM) and EGTA (20 mM), respectively. Calcium concentrations were calculated by using TM 3000 software (SPEX).

Statistics

Data were analyzed by either Student's *t*-test (2 groups) or an analysis of variance (ANOVA) followed by a Newman-Keuls and values are given as the mean ±SEM.

RESULTS

In preliminary experiments, NMDA-induced elevated extracellular glutamate in a dose-dependent manner (25 to 200 μM) for 60 min incubation without cell damage. The NOS inhibitor, NMMA (300 μM), strongly blocked NMDA-induced cGMP elevation and at the same time decreased NMDA-induced extracellular glutamate accumulation (Fig. 1). The inhibitory effects of NMMA on the NMDA-induced accumulation of glutamate and elevation of cGMP were completely reversed by pretreatment with 1 mM L-arginine. Also nitric oxide scavenger (Rajanayagam *et al.*, 1993), hydroxocobalamin (HCOB, 50 μM) blocked NMDA-induced cGMP elevation and glutamate accumulation. However, a low dose of NMMA (100 μM) did not show a significant blocking effect on NMDA-induced glutamate accumulation although this concentration significantly blocked NMDA-induced cGMP elevations, and the other NOS inhibitor nitroarginine methylester showed the same pattern as NMMA did to NMDA-induced glutamate release and cGMP elevation (data not shown). To determine whether a NOS inhibitor has a blocking effect on the kainate- or KCl-induced accumulation of glutamate, cells were activated by kainate (25 and 50 μM) or KCl (20 mM) with or without NMMA (300 μM) and accumulation of extracellular glutamate was measured. However, NMMA did not affect the kainate- or KCl-induced accumulation of glutamate (Fig. 2).

For comparative purposes, we also investigated the involvement of cGMP on the NMDA receptor-mediated glutamate accumulation using the cGMP analogue, dibutyryl cGMP (dBcGMP). dBcGMP (100 μM) failed to affect the basal or NMDA (25-200 μM)-induced glutamate accumulation (Fig. 3). However, this dose of dBcGMP (100 μM) significantly augmented kainate-induced Ca^{2+} influx (Oh *et al.*, 1996).

To characterize the effect of NO or cGMP on NMDA-induced $[Ca^{2+}]_i$ levels, NMMA or dBcGMP was applied and the change of $[Ca^{2+}]_i$ was measured. After

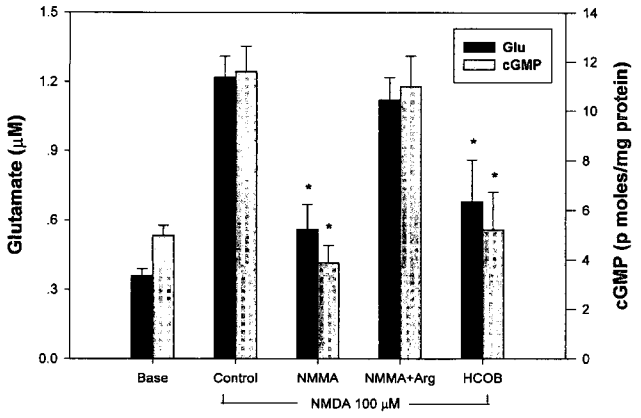

Fig. 1. Effects of NOS inhibitor and NO scavenger on NMDA-induced elevations of extracellular glutamate and intracellular cGMP levels. Cerebellar granule cells were incubated with NMDA 100 μM for 1 h in the presence of NMMA (300 μM), NMMA (300 μM)+L-arginine (1 mM) or HCOB (hydroxocobalamin, 50 μM). Values present the mean±SEM (n=7-10). *p<0.05 from the respective NMDA alone group.

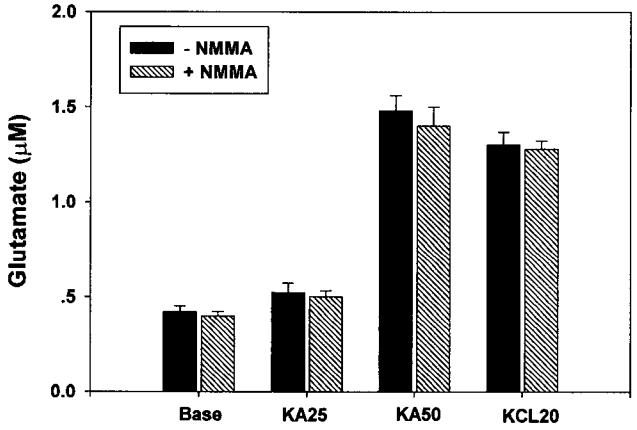

Fig. 2. Effects of NMMA on kainate- or KCl-induced elevation of extracellular glutamate levels. Cerebellar granule cells were incubated with or without NMMA (300 μM) in the presence of kainate (25 or 50 μM) or KCl (20 mM) for 1 h. Values represent the mean±SEM (n=6).

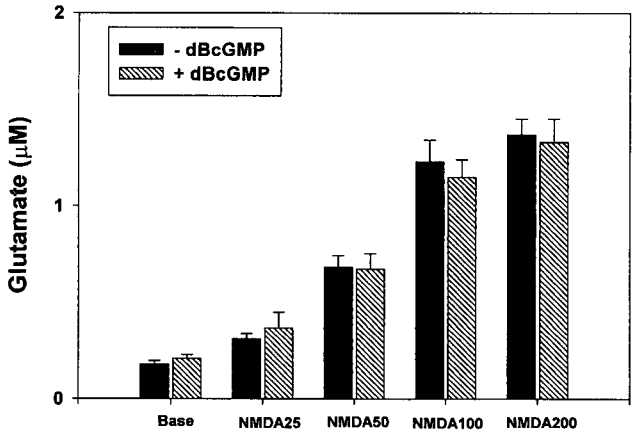

Fig. 3. Effects of dBcGMP on NMDA-induced elevation of extracellular glutamate levels. Cerebellar granule cells were incubated with or without dBcGMP (100 μM) in the presence of variable dose of NMDA (25 to 200 μM) for 1 h. Values represent the mean±SEM (n=5-8).

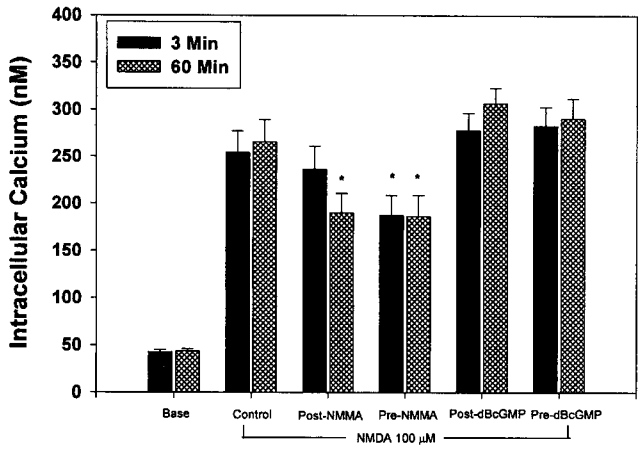

Fig. 4. Effects of NMMA or dBcGMP on NMDA-induced $[Ca^{2+}]_i$. Cells were loaded with fura-2 AM for 60 min and washed with PSH buffer. Intracellular Ca^{2+} levels were measured after stimulation with NMDA (100 μM). After the cells were activated with NMDA and maintained plateau level of $[Ca^{2+}]_i$, NMMA (300 μM) or dBcGMP (100 μM) (denoted as post-NMMA and post-dBcGMP, respectively) was applied and ratios were determined at 3 and 60 min after the addition. Cells were pretreated with NMMA or dBcGMP for 1 h (denoted as pre-NMMA and pre-dBcGMP, respectively), the NMDA-induced $[Ca^{2+}]_i$ elevation was measured for 1 h. Values present the mean±SEM (n=4-6) of the level of $[Ca^{2+}]_i$ at each incubation times. *p<0.05 from the each NMDA alone group.

cells were activated with NMDA and the $[Ca^{2+}]_i$ level had plateaued, NMMA (300 μM) or dBcGMP (100 μM) was applied into PSH buffer and level of $[Ca^{2+}]_i$ continuously measured for 60 min. The NMDA-induced $[Ca^{2+}]_i$ elevation was not changed by NMMA in short time (up to 3 min), but the NMDA-induced $[Ca^{2+}]_i$ level was gradually reduced and significantly decreased (around 20%) at 60 min (Fig. 4). In addition, the inhibitory effect of NMMA on NMDA-induced $[Ca^{2+}]_i$ was also shown by pretreatment with NMMA. To define whether the down-regulating effect of NMMA on NMDA-induced $[Ca^{2+}]_i$ elevation is due to decreasing of cGMP level, membrane permeable cGMP analogue, dBcGMP was used. dBcGMP (100 (M) did not change NMDA-induced $[Ca^{2+}]_i$ elevation in short time (up to 3 min) but the NMDA-induced $[Ca^{2+}]_i$ level was slightly elevated (around 10%) over the plateau without statistic significance at 60 min incubation. Also pretreatment with dBcGMP failed to decrease NMDA-induced elevation of $[Ca^{2+}]_i$, and dBcGMP recovered the blocking effect of NMMA on NMDA-induced release of glutamate or elevation of $[Ca^{2+}]_i$ (Table I). Neither NMMA nor dBcGMP alone (i.e. without activation with NMDA) modulated the level of $[Ca^{2+}]_i$ for 60 min incubation (data not shown).

Table 1. Effects of NMMA and/or dBcGMP on NMDA-induced glutamate release and intracellular Ca^{2+} elevation

	Glutamate (μM)	$[Ca^{2+}]_i$ (nM)
NMDA	1.40±0.09	242.27±20.22
NMDA+dBcGMP	1.44±0.21	250.42±27.44
NMDA+dBcGMP+NMMA	0.71±0.21*	178.57±23.21*

Cerebellar granule cells were incubated with 100 μM of NMDA for 1 h in the presence of NMMA (300 μM) or dBcGMP (100 μM) prior to measurement of glutamate release. In case of measurement of $[Ca^{2+}]_i$, cells were pretreated with NMMA or dBcGMP for 1 h before adding NMDA.
*p<0.05 from the respective NMDA only group.

DISCUSSION

These experiments showed that NMDA receptor functions positively maintained with NO generation evidenced by down-regulating effect of a NOS inhibitor on NMDA receptor-mediated elevations of extracellular glutamate accumulation and $[Ca^{2+}]_i$ level. The NOS inhibitor, NMMA, decreased NMDA-induced elevations of extracellular glutamate. Also, the NO scavenger HCOB showed the decreasing effect as NOS inhibitor did (Fig. 1). This result raises the intriguing possibility that once diffused NO affects the NMDA receptor activity via its action at the extracellular level. Indeed, our observations are in agreement with other evidence for the involvement of NO in the NMDA-induced neurotrasmitter release (Montague *et al.*, 1994; Jones *et al.*, 1994; Nei *et al.*, 1996; McNaught and Brown, 1998). These authors showed that NOS inhi- bitors strongly abolished NMDA-stimulated release of [^{3}H] noradrenaline, [^{3}H]aspartate, and endogenous glutamate from hippocampal slices, and [^{3}H]noradrenaline and endogenous glutamate from synaptosomal preparations from cerebellar cortex.

It has been known that NMDA receptors are necessary for the induction of LTP. In principle, there are two broad categories of the induction of LTP: postsynaptic level, reflecting an increase in the receptor (NMDA) functions; presynaptic level, reflecting a sustained increase in neurotransmitter (glutamate) release. Bliss and co-worker have shown, both *in vivo* and *in vitro,* that the release of the main excitatory neurotransmitter, glutamate, was still increased between 60 and 120 min after induction of LTP in hippocampal tissue (Lynch *et al.*, 1985; Bliss *et al.*, 1986). Such a persistent increase in glutamate release could lead to long-term modifications of NMDA receptor activities after LTP induction (Davies *et al.*, 1989; Bashir *et al.*, 1991). There are several evidences that nitric oxide is required for LTP as a possible retrograde messenger (Schuman and Madiosn, 1991; Bohme *et al.*, 1991; O'Dell *et al.*, 1991), and NO has a role in glutamate release in cerebral cortex (Montague *et al.*, 1994) and hippocampal synaptosomes (Meffert *et al.*, 1994). Interestingly, NO has been recently been proposed to play a role as an intraneuronal messenger in LTP (Schuman and Madison, 1994). In our results, NOS

inhibitor NMMA down-regulated the NMDA-induced $[Ca^{2+}]_i$ elevation (Fig. 3). Although our experimental model may not be able to differentiate the action of NO at the presynaptic or postsynaptic level, our results suggest a diverse role of NO in cell signalling; NO might also contribute to the regulation of NMDA receptor activity.

Since NO stimulates cGMP synthesis, many physiological activities of NO were attributed to its effects on cGMP in target cells. Even though the functions of cGMP are not still clear, cGMP can act through several mechanisms in the CNS (Garthwaite and Garthwaite, 1991): directly on ionic channels, on cGMP dependent protein kinase, on phosphodiesterase either to increase or to decrease cyclic AMP and cGMP levels. Nitric oxide can stimulate cGMP which results in phosphorylation of some proteins that may participate in releasing neurotransmitter at synaptic vesicles or in maintaining NMDA receptor activation. There is an interesting experimental result demonstrating that NO as well as dBcGMP could enhance the release of a number of neurotransmitters in rat striata *in vivo* (Guevara-Guzman *et al.*, 1994). However, in our present study dBcGMP neither induced extracellular glutamate elevation nor augmented NMDA-induced glutamate elevations (Fig. 3), although it slightly augmented NMDA-induced $[Ca^{2+}]_i$ elevation (Fig. 4). It may be difficult to investigate the basis of this discrepancy since Guevara-Guzman *et al.* (1994) determined the effect of dBcGMP on dopamine release in rat striata in vivo but our experiment was done in cultured cells. It is noteworthy that there was no positive control for the action of dBcGMP in our present experiment. However, it was confirmed that dBcGMP was taken into the cells after 1 h incubation with 100 μM dBcGMP as evidenced by the measurement of intracellular levels of dBcGMP with the RIA kits where nanomole versus the usual picomole levels were measured after dBcGMP application. In addition, 100 μM dBcGMP augments the kainate-induced elevation of $[Ca^{2+}]_i$ using the same experimental conditions (Oh *et al.*, 1996). Nevertheless, dBcGMP did not elevate basal or NMDA-induced accumulation of extracellular glutamate. These results suggest that the down-regulating effect of NMMA on NMDA receptor was not secondary to blocking cGMP elevation but endogenous NO (i.e. generated by NMDA receptor activation) was involved in maintaining NMDA receptor activation. At this point, it is not clear how NO affects NMDA receptor activation. One possibility is an indirect effect of released glutamate on NMDA receptor: NOS inhibitor decreased NMDA-induced glutamate release, therefore less glutamate accessed to NMDA receptor. However low concentration of glutamate (1~3 μM) did not augment 50 μM NMDA-induced $[Ca^{2+}]_i$ elevations (data not shown). The other possibility is that NMMA may disrupt L-arginine dependent synthesis or metabolic pathway which can induce cell damage. However, we found no evidence of cell damage with NMDA and NMMA for 60 min incubation, as evidenced by LDH release or trypan blue exclusion tests in our system. In addition, NO scavenger HCOB also decreased elevation of NMDA-induced extracellular glutamate (Fig. 1). It has been known that NO downregulates the NMDA receptor by an S-nitrosylation at the redox-sensitive site on the receptor. Of the 11 cysteine residues in a functional NMDA receptor, 9 appears to residue extracellularly, 2 of which are paired (Moriyoshi *et al.*, 1991). It is likely that NO affects the thiol groups of the redox-sensitive site on the receptor, not directly but from the transfer or reaction of NO^+ or $ONOO^-$ (Lipton *et al.*, 1993). However, in our previous report, NO producing compound SNAP does not affect NMDA-induced glutamate release although iron-containing NO producing compound sodium nitroprusside blockes NMDA-induced glutamate release (Oh and McCaslin, 1995). The effect of NMMA is selective for the NMDA receptor. It had no effect on the kainate- or KCl-induced accumulation of extracellular glutamate. Further study is needed to define the action of NO on NMDA receptor activation.

In summary, NOS inhibition decreases NMDA-induced glutamate accumulation, cGMP elevation, and $[Ca^{2+}]_i$ elevation by blocking NO generation while cGMP analogue, dBcGMP, does not augment NMDA-induced elevations of extracellular glutamate or $[Ca^{2+}]_i$ level. These results suggest that NO is one of the major factors involved in maintaining NMDA receptor activation via a cGMP independent mechanism.

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