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Potentiation of NMDA-mediated Toxicity on Nigrostriatal Neurons by a Low Dose of 7-Nitro Indazole

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Summary—Recent evidence suggests that an excitotoxic mechanism may play a role in the etiology of Parkinson's disease. Previously, we have shown that the nigrostriatal dopaminergic neurons are sensitive to focal infusions of an *N*-methyl-D-aspartate (NMDA) receptor agonist; this toxicity was potentiated by systemic administration of the nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester. The present investigation was undertaken to assess the role of the selective neuronal NOS inhibitor, 7-nitro indazole (7-NI) on the neurotoxicity elicited by NMDA receptor activation *in vivo*. Single injections of 7-NI (0–125 mg/kg, i.p.) into male Sprague–Dawley rats resulted in a dose-dependent decrease in both nigral and cerebellar NOS activity measured 30 min post-injection. Maximal NOS inhibition was obtained with 20 mg/kg 7-NI (nigra: 90.2 ± 3.7%, cerebellum: 86.7 ± 6.3%). In addition, it was found that 7-NI (80 mg/kg, i.p.) did not cause an increase in mean arterial blood pressure over a 48 hr period. Vehicle pretreatment of animals prior to stereotaxic infusion of NMDA (15 nmol) into the substantia nigra compacta resulted in a 56.1 ± 5.1% decrease in striatal tyrosine hydroxylase (TH) activity from the contralateral side. Pretreatment with 7-NI (5 and 80 mg/kg) produced a 76.9 ± 3.2% and 49.8 ± 5.6% decrease, respectively, in striatal TH activity. Thus, a significant increase in NMDA toxicity was observed at the lower but not higher dose of 7-NI. It was also observed that 7-NI (20 and 80 mg/kg) produced a decrease in locomotor activity over a 2 hr period. This effect of 7-NI did not occur at the 5 mg/kg dose which produced a significant but submaximal inhibition of nigral and cerebellar NOS activity. Although, both 20 and 80 mg/kg 7-NI produced maximal inhibition of NOS activity, the low dose produced partial sedation (a decrease in upper but not lower locomotor activity) while the higher dose produced a more complete sedative effect (a decrease in both upper and lower locomotor activity). The failure of 80 mg/kg 7-NI to augment NMDA toxicity may be due to its significant sedative effect.

Keywords—Parkinson's disease, substantia nigra, excitotoxicity, 7-nitro indazole, *N*-methyl-D-aspartate (NMDA), nitric oxide synthase.

Parkinson's disease (PD) is characterized by the relatively selective degeneration of the dopaminergic nigrostriatal pathway. Recently, it has been postulated that glutamate-mediated excitotoxicity may be involved in this degeneration (Kopin, 1993; Zeevalk *et al.*, 1994). Autoradiographic studies have revealed the presence of glutamate receptor binding sites on the dopaminergic neurons of the substantia nigra compacta (SNc) (Difazio *et al.*, 1992). It has also been reported that cultured mesencephalic dopaminergic neurons are sensitive to glutamate toxicity mediated through *N*-methyl-D-aspartate (NMDA) receptors (Kikuchi and Kim, 1993). Previously, we have reported that the nigral dopaminergic neurons are sensitive to focal infusions of the NMDA receptor agonist, quinolinic acid (QUIN) *in vivo* (Beninger *et al.*, 1994; Connop *et al.*, 1993).

It has been postulated that nitric oxide (NO) is an intermediary in NMDA receptor-mediated excitotoxicity (Buisson *et al.*, 1992; Dawson *et al.*, 1991; Izumi *et al.*, 1992; Kollegger *et al.*, 1993; Reif, 1993). In contrast, other studies using similar protocols have provided conflicting evidence with respect to the role of NO in this neurotoxicity (Demerle-Pallardy *et al.*, 1991; Haberny *et al.*, 1992; Hewett *et al.*, 1993; Pauwels and Leysen, 1992; Rondouin *et al.*, 1993; Weissman *et al.*, 1992; Yamamoto *et al.*, 1992). In a recent report, we have shown that not only are the nigral neurons sensitive to focal infusions of QUIN, but that this toxicity was potentiated by pretreatment with the nitric oxide

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synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Connop *et al.*, 1993). Since L-NAME inhibits all NOS isozymes, it is possible that this potentiation could have been secondary to changes in local cerebral blood flow mediated by inhibition of endothelial NOS. Selective inhibition of neuronal NOS *in vivo* can be achieved with 7-nitro indazole (7-NI) (Babbedge *et al.*, 1993; Moore *et al.*, 1993a, b). Therefore, the goal of the present study was to investigate whether (7-NI) could also modulate NMDA neurotoxicity of nigrostriatal neurons *in vivo*.

METHODS

Inhibition of brain nitric oxide synthase

Prior to determining the role of NOS inhibition of NMDA toxicity, the ability of systemic administration of 7-NI to inhibit NOS activity was investigated. To determine the relationship between 7-NI and brain NOS activity, male Sprague–Dawley rats (250–275 g) were given a single i.p. injection of the vehicle (arachis oil) or 7-NI (1, 3, 5, 20, 60, 80, 100, 125 mg/kg). 7-NI (ICN, Toronto, Ontario) was sonicated in arachis oil. Thirty minutes post-injection, animals were sacrificed and both the substantia nigra (SN) and cerebellum removed and placed in ice cold saline for assay of NOS activity. The SN from each hemisphere was dissected from a 3 mm thick coronal section which was removed 4.5 mm posterior to the optic chiasm. The SN from both hemispheres was pooled for each nigral sample. The respective tissue samples were homogenized with a Teflon pestle in a solution containing 1 mM DTT, 10 μg/ml leupeptin, 1 mM EDTA and 50 mM HEPES buffer at pH 7.4. Each nigral and cerebellar sample was homogenized in 175 μl and 1 ml, respectively. The homogenates were centrifuged at 20,000 ***g*** for 30 min at 4°C and the supernatant retained for the NOS assay.

Nitric oxide synthase assay

NOS activity was assayed using a modification of the method described by Bredt and Snyder (1990) which measures the amount of ^{14}C-labelled arginine converted to ^{14}C-labelled citrulline. Tissue samples (25 μl) were incubated for 1 hr at 37°C with 25 μl of 100 μM [^{14}C]arginine (0.319 Ci/mmol, 50 μCi/ml; Amersham, Oakville, Ontario) and 100 μl of reaction buffer. The reaction buffer consisted of 1 mM DTT, 1.25 mM $CaCl_2$, 1 mM valine, 2 mM NADPH, 1 mM EDTA and 50 mM HEPES buffer pH 7.4. The reaction was terminated by the addition of 2 ml ice cold stop buffer which contained 2 mM EDTA and 20 mM HEPES buffer at pH 5.5. Each sample was passed through a 1 ml AG50W-X8 cation exchange column (Na^+ form) which was washed with 2 ml of distilled water and the eluate collected and counted using a Beckman scintillation counter. Protein was determined by the method of Bradford (1976) and the enzyme activity was expressed as nmol citrulline formed/mg protein/hr.

Measurement of mean arterial blood pressure

The surgical techniques and measurement of baseline mean arterial blood pressure (MABP) were performed using the methods of Thompson *et al.* (1992). MABP measurements were performed in conscious animals. The treatment procedure for 7-NI was the same as for the NMDA pretreatment experiments (see below) and a 250 mg/kg dose of L-NAME was examined for comparison. The blood pressure was monitored for 48 hr for both L-NAME and 7-NI.

Intranigral infusion of NMDA

Male Sprague–Dawley rats (250–275 g) were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a Narashige stereotaxic apparatus. NMDA (15 nmol) or 0.9% saline was unilaterally infused through a cannula into the SNc according to the following coordinates: 5.3 mm posterior to bregma, 2.2 mm lateral to the midline and 7.7 mm ventral to bregma (Paxinos and Watson, 1982). NMDA (Sigma, St Louis, MO) was dissolved in 0.9% saline and titrated to pH 7.4 with 1 N NaOH. Each solution was infused for 75 sec through the cannula (30 gauge) connected to a Hamilton syringe to deliver a total volume of 0.5 μl. The cannula was left in place for an additional 2 min and then withdrawn, bone wax applied and the scalp incision sutured. Four days after infusion, the animals were decapitated and each brain was rapidly removed and placed in ice cold saline. Each of the ipsilateral and contralateral striata were dissected and homogenized with a glass hand-homogenizer in 150 μl of buffer consisting of 0.3 M sucrose in 0.01 M Tris–HCl at pH 7.3. The samples were subsequently centrifuged at 12,000 ***g*** for 6 min at 4°C and the supernatant retained for the assay of tyrosine hydroxylase (TH) activity.

In the 7-NI pretreatment experiments, animals were injected with 7-NI (5 and 80 mg/kg, i.p.) or vehicle 30 min prior to stereotaxic infusion of NMDA (15 nmol) into the SNc. Animals were also given a second injection of 7-NI 30 min following stereotaxic infusion of NMDA (1 hr after the initial 7-NI injection) due to the proposed short half-life of 7-NI (Moore *et al.*, 1993b).

Tyrosine hydroxylase assay

TH activity was assayed using a modification of the method described by Yamauchi and Fujisawa (1978). The modifications included a 1.5 hr incubation period at 37°C as well as centrifugation at 2000 ***g*** for 10 min following each vortex after addition of alumina. The amount of L-dopa formed from L-tyrosine was determined by the trihydroxyindole fluorometric method (Yamauchi and Fujisawa, 1978). Protein was determined by the method of Bradford (1976) and the enzyme activity was expressed as nmol L-dopa formed/mg protein/hr. For each animal, the results were expressed as the percent decrease from the contralateral (uninjected) side.

Determination of sedative effects

Eleven male Sprague–Dawley rats purchased from Charles River Canada, initially weighing 225–250 g, were housed in groups of four in hanging wire mesh cages. They were kept in a temperature controlled ($20 \pm 1°C$) colony on a 12 hr light (0600–1800 hr)/dark cycle with free access to food (Purina Rat Chow) and water for the duration of the study, except during activity sessions.

Activity was monitored in six Plexiglas chambers ($41 \times 50 \times 37$ cm), each enclosed in a Styrofoam insulated outer box painted flat black. Light was provided in each chamber by a 2.5 W bulb mounted on the ceiling and a fan behind the back wall provided ventilation and constant background noise. The floor was comprised of wire rods, 0.5 cm apart, approx. 5 cm above the bottom of the outer box. Each chamber was equipped with two sets of seven infrared emitters and detectors spaced 10 cm apart at 5 and 15 cm above the floor which detected horizontal (lower) and ventrical (upper) activity, respectively. A more detailed description of the system can be found in Beninger *et al.* (1985).

Initially all rats received one 60 min habituation session; no injections were administered prior to this session. The four test sessions were 2 hr in duration and occurred every third day. Upper and lower beam breaks were recorded separately every 15 min throughout the 2 hr test session. Every rat received four treatments, the order being counterbalanced across rats. The treatments were: vehicle (arachis oil), 5, 20 and 80 mg/kg 7-NI. Each animal was injected (1 ml/kg, i.p.) twice, the first injection being 1 hr before and the second immediately before each test session.

Statistical analyses

Data were compared using analyses of variance (ANOVA) with either a Newman–Keuls or Tukey post-hoc test used for statistical significance at $P < 0.05$. From 3 to 8 animals were used to determine each point and values were expressed as mean $\pm$ SEM.

RESULTS

Inhibition of brain NOS

The effect of 7-NI on nigral and cerebellar NOS activity is shown in Fig. 1. The dose–response curve represents the percent decrease in enzyme activity observed in these two brain regions 30 min following i.p. injection of 7-NI (0, 1, 3, 5, 20, 60, 80 and 125 mg/kg). Maximal nigral and cerebellar NOS inhibition of $90.2 \pm 3.7\%$ and $86.7 \pm 6.3\%$, respectively, was obtained following a dose of 20 mg/kg 7-NI. These dose–response relationships proved to be very steep at the lower doses of 7-NI with IC_{50} values of approx. 3.5 mg/kg for each curve.

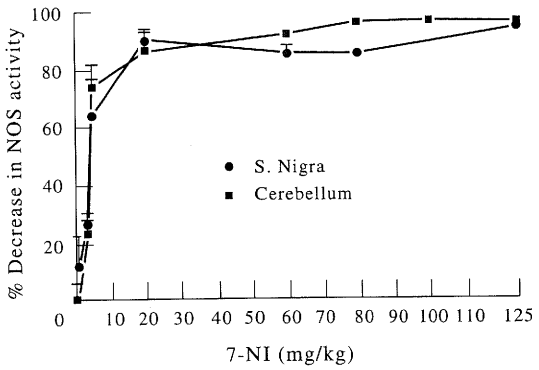

Fig. 1. Percent decrease in nigral (●) and cerebellar (■) NOS activity following a single i.p. injection of vehicle or varying doses of 7-NI with dissection of brain regions 30 min post-injection. Values are expressed as mean $\pm$ SEM with $n = 4$ for each point except the 80 mg/kg dose where $n = 3$.

Measurement of MABP

A single injection of 7-NI (80 mg/kg, i.p.) did not produce a significant increase in MABP over a 64 min period (Fig. 2). For comparison, it can be seen that a dose of L-NAME (250 mg/kg, i.p.; Connop *et al.*, 1993) which gave a similar inhibition of cerebellar NOS produced a marked increase in MABP of approx. 30% over basal pressure (Fig. 2). This L-NAME-induced hypertension persisted over a period of at least 48 hr.

Effects of NMDA infusions

The striatal TH activity in the contralateral (unlesioned side) striatum of vehicle pretreated animals was found to be 570 ± 40 pmol/mg/hr which is in agreement with values reported by Vrana *et al.* (1992) in which an HPLC assay was used to measure TH activity. A saline infusion into the SNc of animals pretreated with 7-NI (80 mg/kg, i.p.) resulted in a $2.7 \pm 2.9\%$ decrease in ipsilateral striatal TH activity (Fig. 3). An infusion of 15 nmol NMDA into vehicle (arachis oil) pretreated animals resulted in a $56.1 \pm 5.1\%$ decrease in striatal TH activity (Fig. 3). Animals pretreated with 5 mg/kg 7-NI prior to 15 nmol NMDA infusions produced a

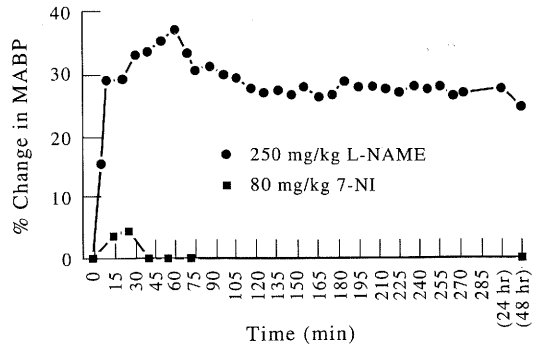

Fig. 2. Percent change in mean arterial blood pressure (MABP) following systemic administration of 7-NI (80 mg/kg, i.p.) (■) and L-NAME (250 mg/kg, i.p.) (●). $n = 3$ for 7-NI and $n = 8$ for L-NAME.

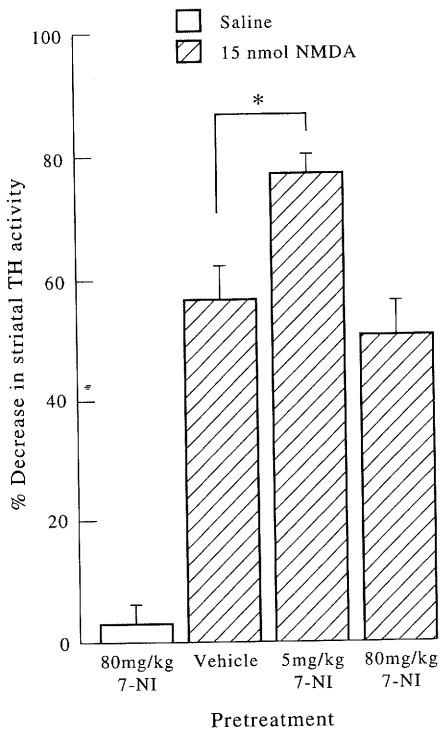

Fig. 3. Percent decrease in striatal TH activity following intranigral infusions of saline and 15 nmol NMDA into vehicle or 7-NI pretreatment animals. Values are expressed as % decrease in striatal TH activity from the contralateral (unlesioned) side. Data are mean ± SEM with $n = 3$ to 8 for each point. *$P < 0.05$.

76.9 ± 3.2% decrease in ipsilateral striatal TH activity which represents a statistically significant increase in NMDA toxicity over vehicle pretreatment ($P < 0.01$) (Fig. 3). However, when 15 nmol NMDA was infused into the SNc of animals pretreated with 80 mg/kg 7-NI, a 49.8 ± 5.6% decrease in striatal TH activity was obtained. This was not significantly different from a 15 nmol NMDA infusion into vehicle pretreated animals (Fig. 3).

Sedative effect of 7-NI

As shown in Fig. 4, 7-NI produced a dose-dependent decrease in both upper and lower activity. An ANOVA of the upper activity measure, using the Box corrected degrees of freedom to reduce type I error associated with repeated measures, revealed a significant dose effect [$F(2.37, 21.33) = 22.88$, $P < 0.0001$]. Similarly, an ANOVA of the lower activity measure found a significant dose effect [$F(2.13, 19.17) = 4.87$, $P < 0.02$]. Post-hoc Tukey tests were performed on both activity measures to determine which treatments differed from each other. For upper activity, both the 20 and 80 mg/kg treatments were significantly different ($P < 0.01$) from the vehicle and 5 mg/kg treatments. For lower activity, the 80 mg/kg dose was significantly different ($P < 0.05$) from the vehicle and 5 mg/kg treatments. Planned t-test comparisons between the 20 and 80 mg/kg doses revealed a significant difference [$t(10) = 2.06$, $P < 0.05$] for upper but not lower activity.

DISCUSSION

In an effort to resolve some of the recent discrepancies concerning the role of NO in NMDA receptor-mediated neurotoxicity, the selective brain NOS inhibitor, 7-NI was examined to determine its effect on focal infusions of NMDA into the SNc of experimental animals. This model of excitotoxicity was used based on previous findings that the nigrostriatal neurons are sensitive to QUIN infusions *in vivo* (Beninger *et al.*, 1994; Connop *et al.*, 1993). The sensitivity of nigral neurons to NMDA agonists is important if an excitotoxic mechanism is involved in the etiology of Parkinson's disease as has been suggested by others (Kopin, 1993; Zeevalk *et al.*, 1994).

In the present study, it was found that 7-NI produced a marked inhibition of rat nigral and cerebellar NOS *in vivo*. The kinetics of both nigral and cerebellar NOS inhibition following systemic administration of 7-NI were not significantly different from each other. The amount of cerebellar NOS inhibition obtained in this study is larger than those reported by Moore *et al.* (1993a, b). This discrepancy may be due to the relatively

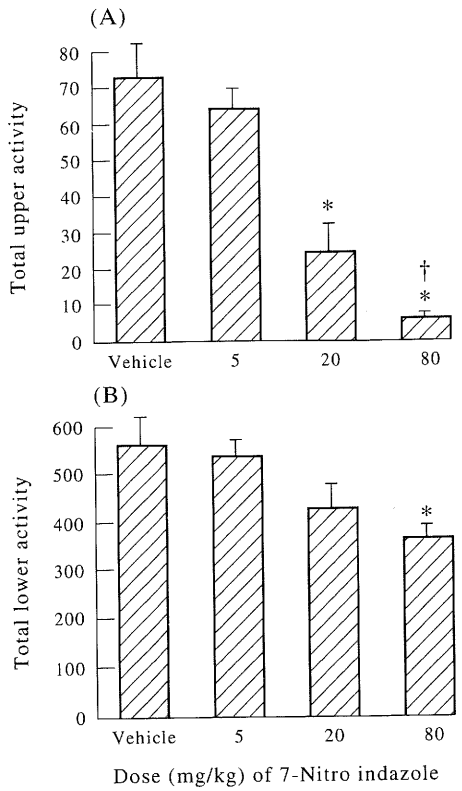

Fig. 4. Mean (±SEM) total activity (beam breaks) for each treatment for both upper (A) and lower (B) activity. *A significant difference from the vehicle and 5 mg/kg treatments. †A significant difference from the 20 mg/kg treatments. Note that the scale on the vertical axis differs on the two graphs.

brief and competitive nature of 7-NI inhibition (Babbedge *et al.*, 1993) resulting in a maximal effect occurring before the 60 min interval used by Moore *et al.* (1993a, b). We also report that 7-NI (80 mg/kg, i.p.) does not cause an increase in MABP when administered systemically which is in agreement with the findings of Moore *et al.* (1993a, b) and suggests that 7-NI is selective for brain NOS *in vivo*.

Recently, with the use of a NO detecting microelectrode, it has been shown that 7-NI (40 mg/kg, i.p.) can decrease the amount of NO directly released in the cat cortex (Kovách *et al.*, 1994). These researchers reported that the basal cortical NO level decreased by over 50%, 30 min following an injection of 7-NI. In addition, NOS catalytic activity was decreased in various brain regions without any effect on blood vessel NOS activity which, with our results on MABP, supports the hypothesis that 7-NI acts selectively on neuronal NOS.

Our neurotoxicity experiments indicate that an enhancement of NMDA toxicity occurs following pretreatment with 5 mg/kg 7-NI. At least two mechanisms can be presented to explain why a potentiation of excitotoxicity may occur following inhibition of NO synthesis *in vivo*. Firstly, it has been suggested that endogenously synthesized NO may inhibit the NMDA receptor as an autoregulatory response (Fujimori and Pan Hou, 1991; Manzoni and Bockaert, 1993; Manzoni *et al.*, 1992). Loss of this negative control would exacerbate NMDA toxicity. Secondly, it has been shown that inhibition of NO synthesis leads to a loss of autoregulation of local cerebral blood flow (LCBF) (Tanaka *et al.*, 1991). In addition, it has been suggested that this autoregulation of LCBF may be directly regulated by NOS containing neurons (NADPH diaphorase neurons) in the brain (Iadecola, 1993). Kovách *et al.* (1994) have shown that systemic administration of 7-NI (40 mg/kg, i.p.) can cause a large decrease in LCBF and that neuronally released NO may play a major role in cerebrovascular regulation. Therefore, increased neuronal activity in a particular brain region may result in the local release of NO due to the activation of NADPH diaphorase neurons. This would produce an accompanying vasodilation in order to accommodate an increase in neuronal energy requirements. Rigaud *et al.* (1993) reported that focal infusions of kainate into the hippocampus resulted in a 400% increase in LCBF which was blocked by systemic administration by L-NAME. In the present study, inhibition of neuronal NOS may lead to a loss of the activity-dependent autoregulation of LCBF which, in turn, would cause the metabolic compromise of a brain region exposed to the NMDA excitatory stimulus. It has been found that NOS inhibition can cause an "uncoupling" of LCBF and metabolic demand in the CNS (Dirnagl *et al.*, 1993; Goadsby *et al.*, 1992; Macrae *et al.*, 1993). The idea that metabolic compromise can increase the susceptibility of neurons to excitotoxins has been reported previously and that sufficient impairment of oxidative phosphorylation may in itself lead to "excitotoxic" lesions (Beal *et al.*, 1993; Greene *et al.*, 1993).

In the present study, it was observed that there was a significant decrease in motor activity, suggesting a sedative effect at 20 and 80 but not 5 mg/kg 7-NI even though nigral and cerebellar NOS were largely inhibited. However, at 80 mg/kg 7-NI the sedation was more complete than at 20 mg/kg since both upper and lower locomotor activity was reduced by the higher dose. At this dose, the NMDA-induced toxicity was not altered although there was maximal inhibition of NOS activity. The failure of the higher dose to influence toxicity was probably due to the sedation seen at this dose. The 20 mg/kg dose was not tested since it produced a decrease in upper but not lower locomotor activity and thus, only a partial sedative effect. The sedative effect of 7-NI appears to be, at least in part, independent of brain NOS inhibition. Various indazole derivatives have been investigated as lipoxygenase inhibitors (Foster *et al.*, 1989) as well as leukotriene and 5-HT_3 antagonists (Robertson *et al.*, 1990; Yee *et al.*, 1990). These mechanisms may be responsible for the sedative effect observed at the higher doses of 7-NI in the present study. Sedation at the 80 mg/kg dose of 7-NI may have reversed the potentiation of NMDA toxicity mediated through NOS inhibition.

In conclusion, the sensitivity of nigral dopaminergic neurons to NMDA *in vivo* was examined in the presence and absence of the selective brain NOS inhibitor, 7-NI. It was found that inhibition of neuronal NO synthesis by a low dose of 7-NI potentiated excitotoxicity.

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