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Role of brain nitric oxide in (±)3,4-methylenedioxymethamphetamine (MDMA)-induced neurotoxicity in rats [1]

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Abstract

The role of nitric oxide (NO) in the long-term serotoninergic neurotoxicity induced by (±)3,4-methylenedioxymethamphetamine (MDMA) in rats was investigiated. Pretreatment with N^{ω}-nitro-L-arginine (L-NOARG) (10 mg kg^{-1}), a nitric oxide synthase (NOS) inhibitor, partially protected against long-term serotonin (5-HT) depletion induced by MDMA (40 mg kg^{-1}) in frontal cortex and parietal cortex, but not in other brain regions examined. Brain NOS activities in these two regions were significantly elevated at 6 h after MDMA administration. Moreover, L-NOARG pretreatment caused significant inhibition of brain NOS activity but did not affect the acute 5-HT and dopamine (DA) changes or the hyperthermia induced by MDMA. These results suggest that it is the NOS inhibitory properties of L-NOARG, rather than its effects on the acute monoamine changes or the hyperthermia induced by MDMA, that are responsible for the prevention of neurotoxicity. The regional differences on the protection of L-NOARG and on the activation of NOS by MDMA indicate the unequal role that NO may play in MDMA-induced neurotoxicity in different brain regions.

Keywords: (±)3,4-methylenedioxymethamphetamine; Nitric oxide; Nitric oxide synthase; N^{ω}-nitro-L-arginine; Neuroprotection

1. Introduction

(±)3,4-methylenedioxymethamphetamine (MDMA) is a popular recreational drug which has been widely abused but is considered safe by many users. However, there is extensive evidence that MDMA is a fairly selective neurotoxin for serotonin (5-HT) pathways in rats and nonhuman primates, leaving dopamine (DA) pathways spared [17,30,33], while in mice, it produces neurotoxic damage to both 5-HT and DA systems [19,20,26]. The long-term neurotoxicity induced by MDMA on brain serotoninergic system is demonstrated by a long-lasting depletion of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), a reduction of tryptophan hydroxylase activity and a decrease in synaptosomal [^{3}H] 5-HT uptake [7,20,30,31,36]. There is also histological evidence that 5-HT neurodegeneration occurs following the administration of MDMA [7]. However, the mechanism(s) by which MDMA produces neurotoxicity is not clear.

In the CNS, nitric oxide (NO) has been implicated both as a neuromodulator and a mediator of neurotoxicity [4,14,38]; immunochemical studies have shown a widespread distribution of nitric oxide synthase (NOS) in the brain [2,13]. Recently, the linkage between NO production and excitotoxicity has been studied [4,14]. For example, glutamate-induced cytotoxicity was blocked by MK-801, a noncompetitive NMDA receptor antagonist, and attenuated by treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, in vitro [14]. Using primary cortical cultures from transgenic neuronal NOS null mice, neurotoxicity elicited by NMDA was markedly attenuated [9]. Accumulating evidence supports the view that an overstimulation of NMDA receptors results in a massive influx of Ca^{2+} and as a consequence of the stimulation of Ca^{2+}/calmodulin-dependent NOS, an excessive formation of NO may occur. The reaction of NO with superoxide can yield a variety of highly toxic molecules which may mediate the neuronal damage (see reviews by Moncada et al. [23] and Vincent [38])

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The involvement of NMDA activation in the long-term serotoninergic neurotoxicity induced by MDMA is shown by the protective effects of both competitive and noncompetitive NMDA antagonists on the neurotoxic effects of MDMA on 5-HT terminals in rat brain [5,12,19]. It is also reported that MDMA- and methamphetamine (METH)-induced neurotoxicity on dopaminergic and serotoninergic cells in primary cultures were attenuated by inhibitors of NOS [4]. Most recently, two independent papers reported similar results of the protective effect of neuronal NOS inhibitor, 7-nitroindazole, on the neurotoxicity induced by METH in vivo [10,16]. Thus, it is of interest whether NO is also involved in the long-term serotoninergic neurotoxicity induced by MDMA in vivo.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (6 weeks old, Animal Breeding Station, Otago University) were housed 2 per cage, in a temperature-controlled room (24 ± 1°C) with a 12-h alternating light/dark cycle. Animals were allowed free access to standard laboratory food and water.

2.2. Drug administration and sample collecting

For the long-term neuroprotective effect of L-NOARG, two groups of rats ($n = 8$) received either a single dose of MDMA (40 mg kg^{-1}, i.p.) or normal saline and another group ($n = 16$) was injected twice with L-NOARG (10 mg kg^{-1}) 16 h and 30 min before MDMA. Rats were sacrificed by decapitation at 7 days. The brains were rapidly removed and dissected into frontal cortex, parietal cortex, occipital cortex, hippocampus, striatum and cerebellum, frozen on dry ice and stored at −80°C for monoamine assays. In a separate experiment, rats were given the same treatment and sacrificed at 1 h, 6 h and 24 h after MDMA. The tissue from one hemisphere was used for monoamine assays and the tissue from the other hemisphere was used for NOS activity assay. In the third experiment, two groups of rats ($n = 8$) received either a single dose of MDMA (40 mg kg^{-1}, i.p.) or sterilized normal saline and were killed at 1, 3, 4, 6, 7 and 24 h after MDMA. The tissue was used for NOS activity assay.

2.3. Measurement of body temperature

In the long-term neurotoxicity experiment, body temperature in all the experimental groups was measured rectally at a depth of 1.5 cm using a thermistor probe, at 0.5, 1, 2, 4, 6 and 24 h after administration of MDMA or saline.

2.4. Determination of monoamine and monoamine metabolite concentrations

Tissue concentrations of 5-HT, 5-HIAA, DA and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined by HPLC with electrochemical detection (ECD) as in a previous study [39]. Briefly, samples were homogenised in 0.4 M $HClO_4$ and centrifuged ($12{,}000 \times g$, 10 min) at 4°C. The supernatant (20 μl) was injected onto a 100×4.6 mm, 5 μm, C_8, MOS column and eluted with a mobile phase containing 9% (v/v) acetonitrile, 100 mM NaH_2PO_4, 0.46 mM octylsulphonic acid, 19.5 mM sodium acetate and 0.3 mM Na_2EDTA at pH 2.6. The flow rate was set to 1.5 ml min^{-1}. The ESA model 5100 A Coulochem ECD system consisted of a model 5020 guard cell (detector setting, +1.04 V), followed in sequence by a model 5010 analytical cell (detector 1, +0.3 V, detector 2, +0.45 V). Components were quantified by comparison with a curve generated from external standards.

2.5. Measurement of brain NOS activity

NOS activity was measured according to Bredt and Snyder [3] with slight modifications, by monitoring the conversion of [^{3}H] L-arginine to [^{3}H] citrulline. Briefly, tissues were homogenized in 5 volumes of 20 mM Tris–HCl buffer (containing 2 mM EDTA, pH 7.4) and centrifuged ($12{,}000 \times g$ for 10 min at 4°C). 25 μl of supernatant were incubated for 1 min at 37°C with 0.5 μCi [^{3}H] L-arginine (concentration, 150 nM), NADPH (1 mM) and $CaCl_2$ (0.75 mM) in a total volume of 100 μl. The reaction was started by the addition of supernatant and stopped by the addition of 1 ml of 1: 1 (v/v) H_2O/Dowex-50 W (200–400, 8% cross-linked, Na^+-form) and transferred onto ice. H_2O (3 ml) was added to the mixture and allowed to stand for 10 min. [^{3}H] citrulline in the supernatant was quantified by liquid scintillation spectroscopy.

2.6. Chemicals and drugs

MDMA was synthesised in the School of Pharmacy, University of Otago. L-[2,3-^3H]-arginine (40.0 Ci mmol^{-1}) was purchased from Life Technologies, USA. BCS (Biodegradable Counting Scintillant for aqueous) was obtained from Amersham Canada. All other chemicals were purchased from Sigma.

2.7. Statistics

The results are presented as the mean ± S.E.M. of the tissue contents of the treatment groups expressed as a percentage of corresponding saline-injected controls for ease of comparison. The statistical significance of differ-

ences between groups was determined using a one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test.

3. Results

3.1. Effect of L-NOARG on the long-term neurotoxicity of MDMA

In this experiment, a single high dose of MDMA (40 mg kg^{-1}) produced significant decreases of 5-HT and 5-HIAA levels in all the brain regions examined when the rats were killed at 7 days after the administration, indicating long-term damage to the 5-HT terminals. Pretreatment with two doses of L-NOARG (10 mg kg^{-1}) significantly protected against the 5-HT depletion in frontal and parietal cortex and the 5-HIAA depletion in frontal cortex (Fig. 1A and B). Protection was almost complete in frontal cortex, less complete in parietal cortex. Levels of 5-HT in other regions in L-NOARG/MDMA group were higher than that in MDMA alone, but the differences were not statistically significant. Studies in our laboratory have shown that 5-HT, 5-HIAA levels in L-NOARG alone group were not significantly different from that in saline control group at 7 days (data not shown).

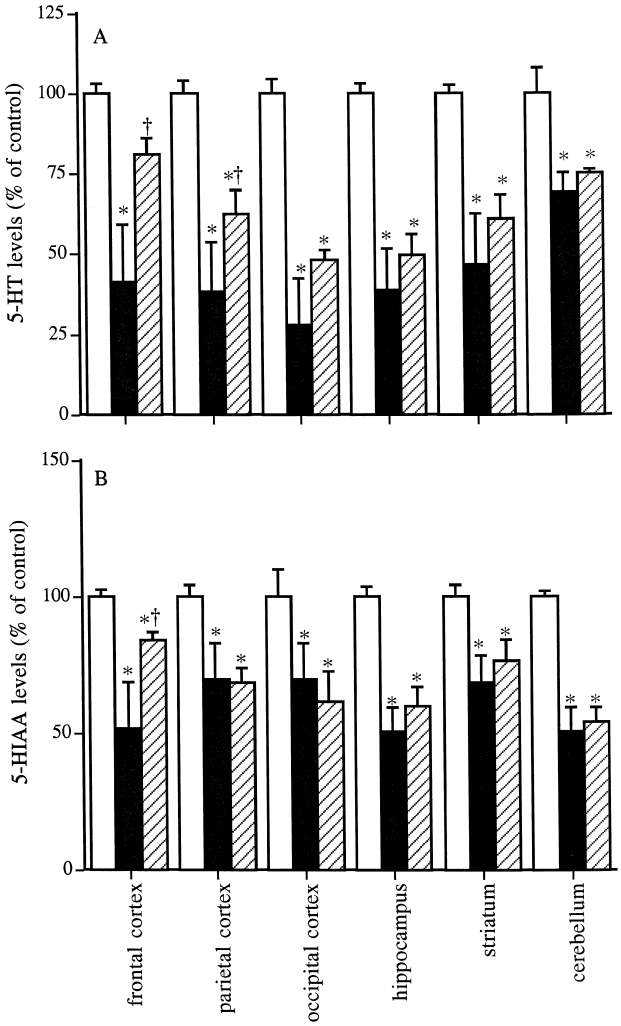

Fig. 1. Effect of L-NOARG on 5-HT and 5-HIAA levels in different brain regions 7 days after administration of MDMA. Rats which received a single dose of MDMA (40 mg kg^{-1}, i.p.) were pretreated with two doses of either saline (dark bars) or L-NOARG (10 mg kg^{-1}, i.p.) (striped bars) at 16 h and 30 min before MDMA. Control group received saline (open bars). Rats were killed 7 days after the last injection. 5-HT (A) and 5-HIAA (B) were measured in different brain regions. Data are expressed as percentage of control levels. $* p < 0.05$ vs. the saline control; $\dagger p < 0.05$ vs. the MDMA treatment.

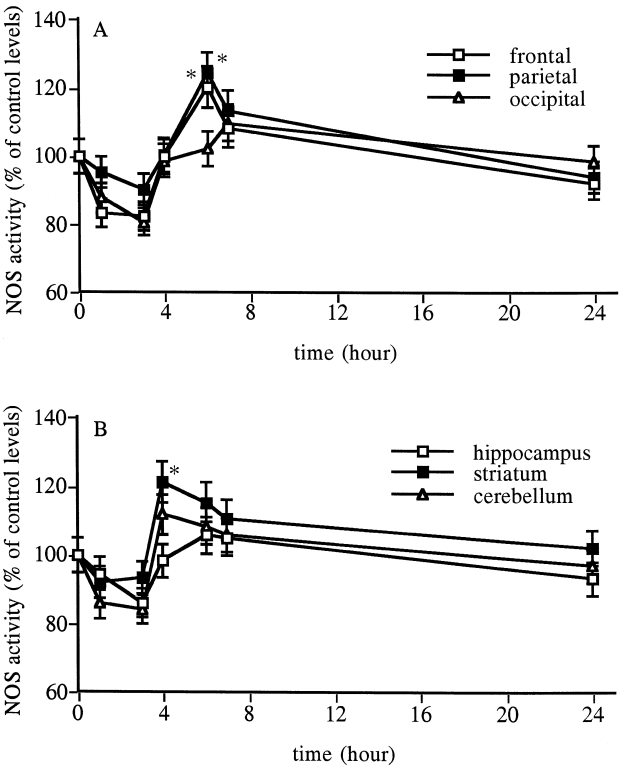

Fig. 2. Time course of acute NOS activity changes in different brain regions after a single dose of MDMA (40 mg kg^{-1}, i.p.). Control group received sterilized saline. Rats were killed at 1, 3, 4, 6, 7 and 24 h respectively after the injection and NOS activity were measured in frontal cortex, parietal cortex and occipital cortex (A) and in hippocampus, striatum and cerebellum (B). Data are expressed as percentage of control levels at each time point. $* p < 0.05$ vs. the saline control.

3.2. Acute effect of MDMA on brain NOS activity

The time course of NOS activity changes was measured in different brain regions after a single dose of MDMA (40 mg kg^{-1}). As shown in Fig. 2, slight decreases of NOS activity were found at 1 and 3 h after MDMA in all the brain regions examined. After 3 h, time dependent increases were observed and statistically significant increases were obtained from frontal (120.1%) and parietal (124.4%) cortex at 6 h (Fig. 2A) and from striatum (121.1%) at 4 h (Fig. 2B). The levels returned to control

values at 24 h. Similar but non-significant changes were seen in the other brain regions studied. The validity of the NOS assay was checked by using L-NOARG to block the ^{3}H-citrulline production in vitro. A concentration-dependent inhibition of the activity was produced by L-NOARG in the tissue from either saline or MDMA treated animals with a maximum of 95% inhibition obtained.

3.3. Effect of L-NOARG on acute monoamine and NOS activity changes induced by MDMA

Rats received the single high-dose MDMA (40 mg kg^{-1}) regime and the tissues were collected as described before. Since a significant protective effect of L-NOARG on the long-term 5-HT depletion induced by MDMA was achieved only in frontal and parietal cortex, as was the significant increase of NOS activity induced by MDMA, the time course of 5-HT and 5-HIAA changes in this experiment was only measured in these two regions. 5-HT and 5-HIAA levels were not significantly different in L-NOARG pretreated group compared with that in MDMA alone at 1 h, 6 h and 24 h after MDMA in both frontal cortex (Fig. 3) and parietal cortex (Fig. 4). There were no significant differences on striatal DA levels between MDMA group and L-NOARG/MDMA group at different time points though

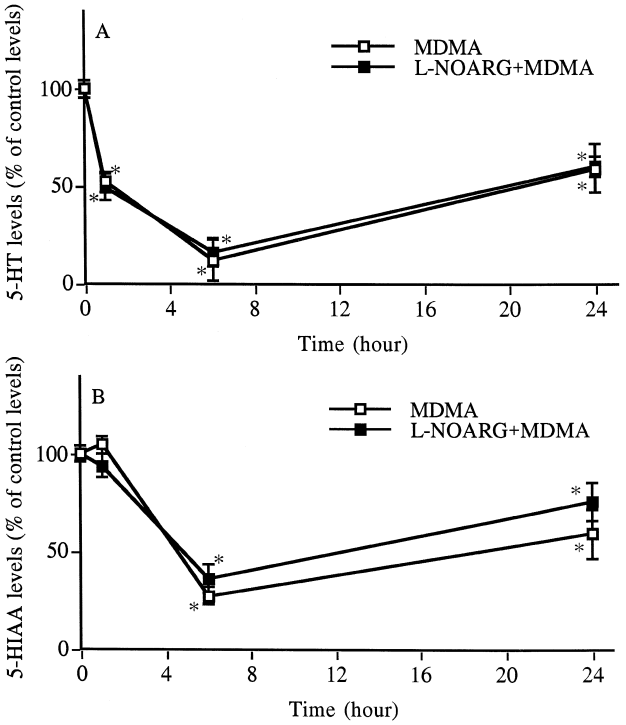

Fig. 3. Time course of brain 5-HT (A) and 5-HIAA (B) levels in frontal cortex after the acute administration of MDMA. Rats which received a single dose of MDMA (40 mg kg^{-1}, i.p.) were pretreated with two doses of either saline or L-NOARG (10 mg kg^{-1}, i.p.) at 16 h and 30 min before MDMA. Control group received saline. Rats were killed at 1, 6 and 24 h after MDMA. Data are expressed as percentage of control levels. $*$ $p < 0.05$ vs. the saline control.

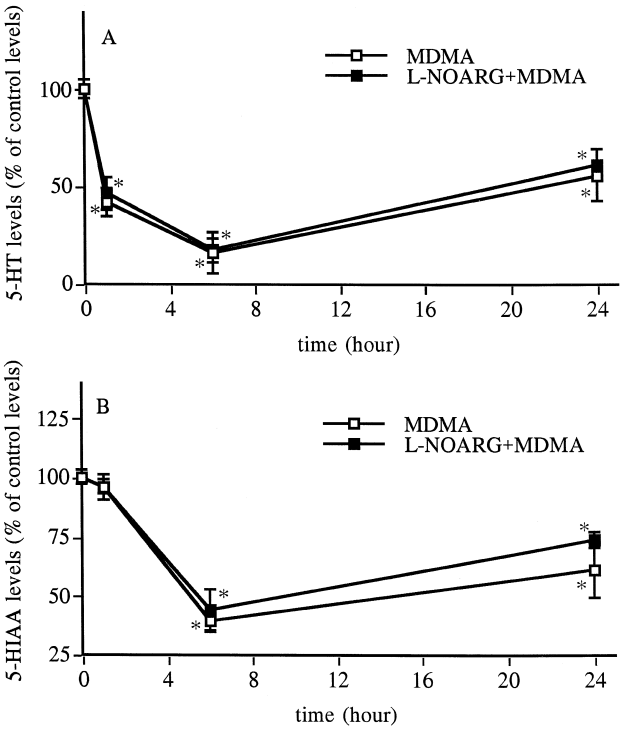

Fig. 4. Time course of brain 5-HT (A) and 5-HIAA (B) levels in parietal cortex after the acute administration of MDMA. Rats which received a single dose of MDMA (40 mg kg^{-1}, i.p.) were pretreated with two doses of either saline or L-NOARG (10 mg kg^{-1}, i.p.) at 16 h and 30 min before MDMA. Control group received saline. Rats were killed at 1, 6 and 24 h after MDMA. Data are expressed as percentage of control levels. $*$ $p < 0.05$ vs. the saline control.

the DA concentration in L-NOARG/MDMA group was significantly higher than control value at 6 h (data not shown).

NOS activity was measured in all the brain regions collected from the other hemisphere in this experiment and the results showed that an extensive inhibition of brain NOS by the two prior doses of L-NOARG was obtained at 1 h (18.8%–27.7% of control values) after MDMA injection in all the brain regions. The inhibitory effect of L-NOARG was maintained at 6 h (14.3%–24.8% of control values) and 24 h (25.6%–50.4% of control values) after MDMA. There were no significant regional differences in NOS inhibition induced by L-NOARG (data not shown).

3.4. Effect of L-NOARG on acute body temperature changes induced by MDMA

A single dose of MDMA (40 mg kg^{-1}) significantly increased the body temperature at 30 min after the injection. The temperature went up 3°C and remained at that level until 4 h and then returned towards the normal level. Pretreatment with L-NOARG (2×10 mg kg^{-1}) as before, did not alter the hyperthermia induced by MDMA.

4. Discussion

It is well accepted that MDMA acts as a selective serotoninergic neurotoxin in rats [29]. Pretreatment with L-NOARG significantly protected against the 5-HT depletion induced by a single dose of MDMA in frontal and parietal cortex though the protection was not complete, as the levels were still lower than that in saline control group. 5-HT levels in other brain regions showed some protection but the differences were not statistically significant (Fig. 1A). A protective effect was observed on 5-HIAA depletion in frontal cortex, but not in other brain regions examined (Fig. 1B).

The dose regimen of L-NOARG was designed to inhibit brain NOS prior to and during MDMA treatment since it is not clear what is the critical time for NO involvement in such neurotoxicity. As the half life of plasma L-NOARG in rats is about 20 h [37], the two doses were given 16 h apart in order to inhibit brain NOS evenly before and throughout the whole course of MDMA's action; NOS activity at 6 h was approximately 20% of control values and remained at 40% as long as 24 h. This almost complete inhibition of brain NOS by L-NOARG only partially blocked the serotoninergic neurotoxicity induced by MDMA and only in some of the brain regions, namely frontal and parietal cortex.

The formation of NO has been suggested to play a key role in excitotoxicity in the cortex. For example, NMDA-induced cell death in cortical cultures was attenuated by NOS inhibitors or by lesions of NOS neurones in the culture [8]. It is generally believed that stimulation of NMDA receptors activates the calmodulin-dependent neuronal NOS by causing an influx of Ca^{2+} in NOS containing neurones and increases the formation of intracellular NO [8,21]. Although MDMA, unlike METH, does not produce a delayed increase in extracellular glutamate [25], both competitive and noncompetitive NMDA receptor antagonists prevented the serotoninergic neurotoxicity induced by MDMA [5,12,19]. Thus, on the one hand, the NMDA receptor has been implicated in long-term serotonin depletion induced by MDMA or METH [12,27]. On the other hand, inhibition of NO formation attenuates MDMA- and METH-induced neurotoxicity on dopaminergic and serotoninergic cells in primary cultures [4]. Furthermore, dopaminergic neurotoxicity induced by METH was prevented by 7-nitroindazole, the neuronal NOS inhibitor, in vivo [10,16]. Our results in this experiment extend the observations above by linking NO formation to at least some of the serotoninergic neurotoxicity induced by MDMA in vivo.

Pretreatment with L-NOARG provided similar degrees of inhibition on brain NOS in different regions, however the degrees of protection on MDMA-induced 5-HT depletion varied between brain regions. To seek why these regional differences occur, we studied brain NOS activity at different times after MDMA injection. As shown in Fig. 2, NOS activities were at first slightly reduced at 3 h after MDMA and then increased during the period of 3–6 h. Although a similar tendency was shown in different brain regions, statistically significant increases were only found in frontal and parietal cortex at 6 h and in striatum at 4 h after MDMA. Therefore, our results demonstrated that MDMA caused an increase of brain NOS activity in most regions but this increase varied both in degree and times in different brain regions. This may partly explain the regional difference in the protective effect of L-NOARG. Some regions where NOS activity was significantly activated by MDMA, such as frontal and parietal cortex, were well protected by L-NOARG, while in other regions where NOS activity showed nonsignificant increases after MDMA, only limited protection was obtained, such as in occipital cortex and hippocampus. This suggests that inhibition of the activated NOS, not the baseline NOS activity, may be essential for the protective effect of L-NOARG. However, a significant increase of NOS activity was also detected at 4 h after MDMA in striatum, but only slight protection was provided by L-NOARG. Thus, there must be other mechanisms other than NO to account for MDMA-induced neurotoxicity in other regions.

Brain NOS shows a distinct regional distribution, with the highest levels being present in the cerebellum, lower levels in hypothalamus, striatum and hippocampus [13]. NOS inhibitors do not prevent glutamate neurotoxicity in cerebellar granule cells [28] which are enriched in NOS [2] and NOS containing neurones are uniquely resistant to NMDA toxicity [8]. Our results are consistent by showing that the long-term 5-HT level in cerebellum was less affected by MDMA, and L-NOARG did not prevent the neurotoxicity in cerebellum, possibly due to high levels of SOD in NOS neurones.

Acute depletion of 5-HT may cause the long-term MDMA neurotoxicity since pretreatment with a serotonin precursor or a 5-HT uptake inhibitor both provided protection from such neurotoxicity [31,35]. However, pretreatment with L-NOARG had no effect on the acute 5-HT and 5-HIAA changes induced by MDMA showing that L-NOARG protected against long-term 5-HT depletion induced by MDMA in frontal and parietal cortex without altering MDMA-induced acute depletion of 5-HT.

Administration of MDMA is believed to increase DA synthesis and release acutely in striatum [24,34], and decreasing DA function by inhibiting synthesis or release appears to provide protection against the serotoninergic neurotoxicity induced by MDMA [32,33]. Furthermore, NO has been reported to increase the release of DA from striatal slices [18,40]. Thus, L-NOARG might act by blocking the DA changes after MDMA. However, pretreatment with L-NOARG did not change the time course of DA changes induced by MDMA. Our results are also compatible with the finding in mice that 7-NI consistently counteracted dopaminergic neurotoxicity even when it did not prevent the initial release of DA caused by METH [10].

Therefore, it appears that L-NOARG protected cortical serotoninergic neurotoxicity induced by MDMA without altering the acute DA changes after MDMA and further suggests that formation of NO may have a more direct role in the process of MDMA-induced neurotoxicity.

The relationship between drug-induced hyperthermia, ambient temperature and drug-induced long-term neurotoxicity has attracted more and more attention [1,15,22]. It has been demonstrated that lowering ambient temperature blocked D-MDMA- or severely attenuated D-METH-induced DA depletion in the mouse [22]. Some protective drugs, such as chlormethiazole, haloperidol and MK-801, also blocked the hyperthermia induced by both drugs [1,15]. However, there are opposing results. Fenfluramine, another substituted amphetamine, caused hypothermia itself in rats, but produced long-term 5-HT depletion [11]. Moreover, coadministration of chlormethiazole which abolished the hyperthermia of *p*-chloroamphetamine was ineffective in protecting against the 5-HT depletion [5]. Pretreatment with L-NOARG did not alter the hyperthermia induced by MDMA in this experiment. The similar results were observed by Di Monte et al. [10]. Therefore, our results support those previous studies indicating that hyperthermia is probably not associated with the long-term neurotoxicity induced by MDMA or other amphetamine analogues [5,6,10].

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