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Inhibition of *N*-methyl-D-aspartate receptor function appears to be one of the common actions for antidepressants

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Abstract

In order to explore the possible common action mechanisms of three kinds of classical antidepressants, inhibition of drugs on the *N*-methyl-D-aspartate (NMDA)–Ca^2–nitric oxide synthase (NOS) signal pathway was observed. With 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and lactic dehydrogenase (LDH) assay, classical antidepressants, desipramine (1, 10 μM), fluoxetine (0.625–10 μM) or moclobemide (2.5, 10 μM) antagonized NMDA 300 μM induced-lesion in PC12 cells. Using fura-2/AM (acetoxymethyl ester) labelling assay, desipramine or fluoxetine at doses 1, 5 μM attenuated the intracellular Ca^{2+} overload induced by NMDA 200 μM for 24 h in PC12 cells. Meanwhile, using confocal microscope, it was also found that desipramine 5 μM, fluoxetine 2.5 μM or moclobemide 10 μM decreased the NMDA 20 μM induced intracellular Ca^{2+} overload in primarily cultured rat hippocampal neurons. Furthermore, desipramine (1, 5 μM), fluoxetine (1, 5 μM) or moclobemide (2.5, 10 μM) significantly inhibited NOS activity in NMDA (300 μM) treated PC12 cells for 4 h. In summary, we suggest that inhibition on the function of NMDA–Ca^{2+}–NOS signal pathway appears to be one of the common actions for antidepressants despite their remarkably different structures, which is expected to have great implication for the evaluation and screening *in vitro* of new antidepressants.

Keywords

antidepressants, PC12 cells, N-methyl-D-aspartate, Ca^{2+}, nitric oxide synthase, hippocampal neurons

Introduction

There are three main kinds of antidepressants (tricyclic antidepressants (TCAs, dual reuptake inhibitors), such as desipramine; selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine; monoamine oxidase inhibitors (MAOIs), such as moclobemide) that have been used in clinic for several decades. Although these antidepressants with remarkable structural diversity have certain therapeutic effects, their mechanisms (especially the common action pathways) remain unknown, which causes some difficulty in the development of new drugs.

Since the mid-1980s, the *N*-methyl-D-aspartate (NMDA) receptors have been implicated in various neurological disorders because of their mediated role in excitotoxicity. The 'excitotoxic hypothesis' proposes that excessive stimulation of NMDA receptors, such as excessive stimulation or release of glutamic acid, can result in cell death. This process may be responsible for several neurodegenerative diseases, including Alzheimer's disease, ischaemic brain injury, epilepsy, and schizophrenia. Radioligand-binding studies indicate that NMDA receptors are found in high density in the cerebral cortex, hippocampus, striatum and amygdala (Petrie *et al.*, 2000). Damage to these areas will result in cognitive and emotional defect. In fact, accumulative evidences over a decade demonstrate that the brain glutamic acid accumulation as well as its excitotoxicity also play an important role in depressive disorder (Skolnick, 1999).

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In fact, there is a close relationship between antidepressant action and NMDA receptors. TCAs can selectively block the NMDA receptor current in cultured rat hippocampal neurons in a time- and dose-dependent manner, while SSRIs have no such direct action. Amitriptyline attenuates NMDA or kainate-induced Ca^{2+} overload in cerebellar granule cells (Petrie *et al.*, 2000). Furthermore, NMDA receptor antagonists, such as MK801 (dizocilpine, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo [*a*,*d*] cyclohepten-5,10-imine), amantadine or Zn^{2+}, possess antidepressant-like effects in animal models (Kroczka *et al.*, 2000; Petrie *et al.*, 2000).

NMDA receptors are ligand-gated Ca^{2+} channels with varying subunits, whose activation leads to Ca^{2+} influx. The overloading intracellar Ca^{2+} ($[Ca^{2+}]_i$) will activate downstream nitric oxide synthase (NOS), which catalyses L-arginine into nitric oxide (NO). Ca^{2+} and NO are both important signal messages responsible for NMDA receptor function. It has been demonstrated that NOS inhibitors possess antidepressant-like effect in animal models (Harkin *et al.*, 1999). But now, whether the inhibition on the NMDA–Ca^{2+}–NOS function is one of the common actions for antidepressants is not well demonstrated, which is very significative for new drug screening and evaluation *in vitro*. The rat adrenal pheochromocytoma cell line, designated PC12 cells, possesses typical features of neurons. In this study, PC12 cells were incubated with high concentration of NMDA and the inhibition of three kinds of classical antidepressants on the NMDA–Ca^{2+}–NOS signal pathway was observed.

Materials and methods

Reagents and drugs

Desipramine, MK801 and fluoxetine were Sigma Chemical Co. (USA) products. Moclobemide was provided by the Chemical Synthesis Lab. of our institute (white power with purity > 98%). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were from Gibco BRL (USA). NMDA was from ACROS ORGANICS (USA); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Merck Chemical Co. (USA); Fura-2/AM (acetoxymethyl ester) was obtained from Fluka (Sweden); Fluo-3/AM was bought from Molecular Probes Co. (USA); the NOS activity assay kit was from Biotinge-Tech Co. (Beijing, China) and lactic dehydrogenase (LDH) assay kit was bought from Promega (USA).

PC12 cells culture and evaluation of cell viability

PC12 cells were seeded into 96-well plates at a density of 2×10^5/mL and cultured in the medium consisting of 90% DMEM, 5% heat-inactivated horse serum, 5% fetal calf serum, benzylpenicillin 200 kU/L, and streptomycin 100 mg/L in a humidified incubator with 5% CO_2, 37 °C for 3–4 days. The cells were incubated with serum-free low-glucose DMEM containing NMDA 300 μM and desipramine (1, 10 μM), fluoxetine (0.156, 2.5, 10 μM), moclobemide (2.5, 10 μM) or NMDA receptor non-competitive antagonist, MK801 5 μM, for 24 h. The medium was collected and LDH activity which was quantified as an index of cell death was determined with a LDH assay kit. The reaction mixture was monitored at 340 nm with a spectrophotometer (Vital Scientific Co., the Netherlands) and the LDH activity was calculated from the decrease of NADH absorbance resulting from the conversion of pyruvate to lactate. In the MTT assay, after two washes with D-Hanks, the cells were incubated with DMEM containing 0.5 mg/mL MTT for another 4 h at 37 °C. Sodium dodecyl sulfate (10%) 100 μL was subsequently added to each well to dissolve formazan crystals (about 12–16 h). Absorbance at 570 nm (A_{570nm} value) was detected with a Versamax spectrophotometer (Molecular Devices, USA).

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells

The $[Ca^{2+}]_i$ in PC12 cells was monitored by fluorometry, using the Ca^{2+}–sensitive dye, fura-2/AM. The cells were seeded in a 24-well plate at the density 2×10^5/mL and cultured for 3–4 days. The medium was replaced by serum-free DMEM containing desipramine (1, 5 μM), fluoxetine (1, 5 μM) in the presence of 200 μM NMDA, and the cells were then cultured for another 24 h. For fura-2/AM loading, cells were collected and incubated with the complete medium containing 5 μM fura-2/AM at 37 °C for 45 min before the cells were washed and resuspended again with cold balanced salt solution (BSS) buffer (NaCl 130 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, glucose 5.5 mM, HEPES 20 mM, pH 7.4) containing 0.2% bovine serum albumin. The cells were incubated at 37 °C for another 5 min just prior to measurement. $[Ca^{2+}]_i$ was determined by alternating excitation wavelengths of between 340 nm and 380 nm with emission at 510 nm, using a fluorescence spectrophotometer (F-4500, HITACH, Japan) and the data were analysed with customized software provided by F-4500. The ratio of fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluorescence.

Measurement of $[Ca^{2+}]_i$ in primarily cultured rat hippocampal neurons

Hippocampal neurons were isolated from 1-day-old fetal Wistar rat's brain of either sex provided by the Experimental Animal Centre of our institute. Briefly, dissected hippocampi were mechanically dissociated, spun down, and resuspended in DMEM supplemented with 10% fetal bovine serum, 10% horse serum, 2 mM glutamine, benzyl penicillin 100 kU/L, and streptomycin 100 mg/L. The cell suspension was seeded on poly-D-lysine-coated plastic dishes (Nunc, Germany) at a density 2×10^5/mL. After 24 h, the medium was replaced by high-glucose DMEM medium plus 10% horse serum, 2 mM glutamine, 1% N_2 and 2% B_{27}. After 3 days, cytosine arabinoside 3 μg/mL was added to inhibit the proliferation of non-neuronal cells. Neurons were fed and in a 5% CO_2 incubator at 37 °C and half of the culture medium was changed per 2–3 days. Experiments were performed on 10-d (post-isolation) old neurons. For the measurement of $[Ca^{2+}]_i$, the medium was replaced by low-glucose and serum-free DMEM con-

taining NMDA 20 μM, and indicated antidepressants (desipramine 5 μM, fluoxetine 2.5 μM or moclobemide 10 μM) respectively, and then cultured for another 24 h. After being washed with PBS, the neurons were loaded with fluo-3/AM 10 μM in a 8% CO_2 incubator at 37 °C for 45 min. Having been washed again with PBS twice, the $[Ca^{2+}]_i$ was measured on a laser confocal microscope (Radiance 2100, Bio-rad Co. USA).

NOS activity assay

The cells were seeded in a 10 cm diameter plastic dish at the density 2×10^5/mL and cultured for 3–4 days. The medium was replaced by serum-free low-glucose DMEM containing desipramine (1, 5 μM), fluoxetine (1, 5 μM) or moclobemide (2.5, 10 μM) in the presence of 300 μM NMDA, and the cells were then cultured for another 4 h before being collected and washed twice with 0.9% saline. The cells were resuspended in 300 μL cold saline following with homogenization. And then, the homogenates were centrifuged at 750 × g for 10 min at 4 °C. The supernatants 50 μL were collected for assay. The protein concentration of the supernatant was determined by Coomassie brilliant blue method. NO concentration was measured colourimetrically at a wavelength of 530 nm as instructed by the kit. The NOS activity was calculated from the increase of absorbance resulting from the conversion of L-arginine to NO. The activity of NOS was expressed as 1 nM NO production per microgram protein in 1 min.

Statistical analysis

The results were expressed as means ± SD. One-way ANOVA and dunnett t-test were used for overall analyses and comparisons between groups, respectively.

Results

Inhibition of antidepressants on the NMDA-induced lesion in PC12 cells.

After the treatment of PC12 cells with NMDA 300 μM for 24 h, the A_{570nm} values decreased and LDH release increased markedly compared with the corresponding control, indicating that the cells were impaired or that some were dead. Desipramine (1, 10 μM), fluoxetine (0.156, 2.5, 10 μM), moclobemide (2.5, 10 μM) or NMDA receptor non-competitive antagonist, MK801 (5 μM), reversed the changes of cell viability. Compared with the corresponding NMDA treatment group, the drugs as mentioned above increased the A_{570nm} values and decreased LDH release significantly, indicating that desipramine, fluoxetine or moclobemide could inhibit the high-concentration NMDA induced lesion in PC12 cells (Figs 1–3).

Effect of antidepressants on the NMDA-induced $[Ca^{2+}]_i$ overload in PC12 cells

After the treatment of PC12 cells with NMDA 200 μM for 24 h, $[Ca^{2+}]_i$ was significantly elevated compared with the control, while in the presence of desipramine or fluoxetine at doses 1, 5 μM, the NMDA-induced $[Ca^{2+}]_i$ overload was attenuated (Fig. 4). These results suggest that the cytoprotective action of antidepressants may be associated with their reduction of $[Ca^{2+}]_i$ overload.

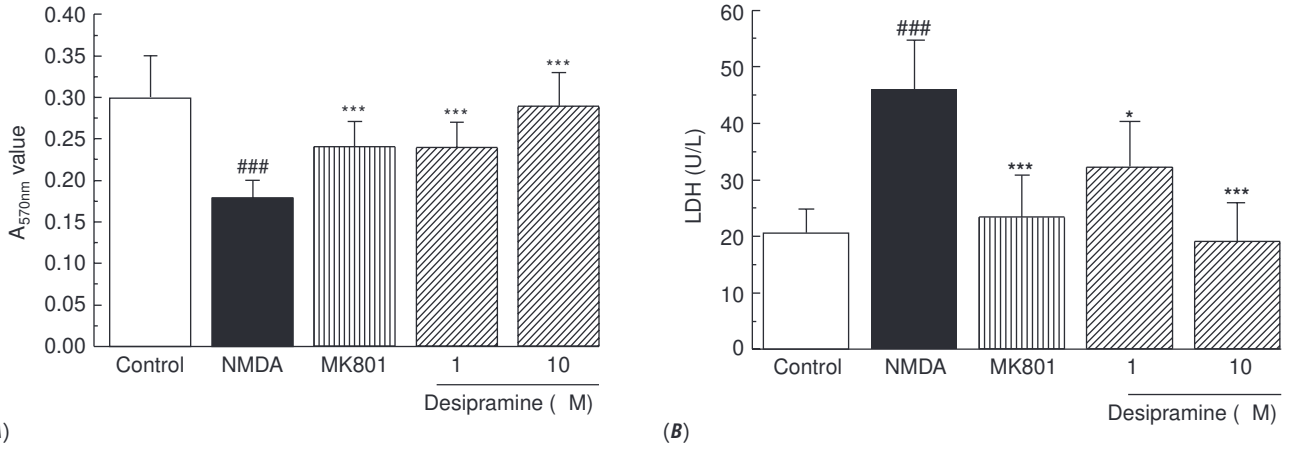

Figure 1 Inhibition of desipramine or MK801 on the NMDA-induced lesion in PC12 cells.

Cells were exposed to NMDA 300 μM in the absence or presence of desipramine or MK801 for 24 h. Cell viability was measured using a colourimetric MTT assay (***A***) and LDH assay (***B***). Data were expressed as means ± SD.

###$p < 0.001$ vs. corresponding control. *$p < 0.05$ and ***$p < 0.001$ vs. corresponding NMDA-treated groups.

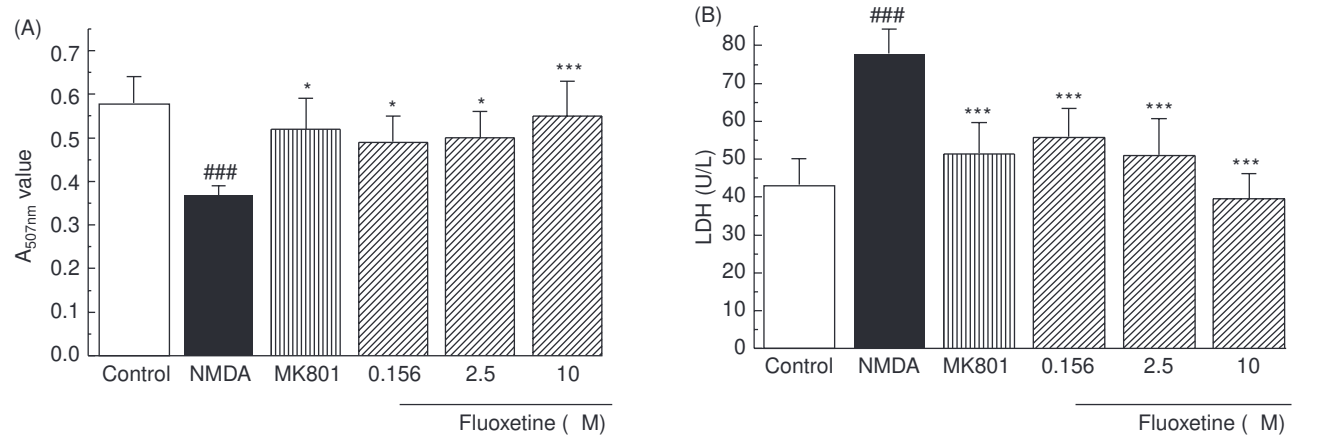

Figure 2 Inhibition of fluoxetine or MK801 on the NMDA-induced lesion in PC12 cells.

Cell viability was measured using a colourimetric MTT assay (***A***) and LDH assay (***B***). Data were expressed as means ± SD. ###$p < 0.001$ vs. corresponding control. *$p < 0.05$ and ***$p < 0.001$ vs. corresponding NMDA-treated groups.

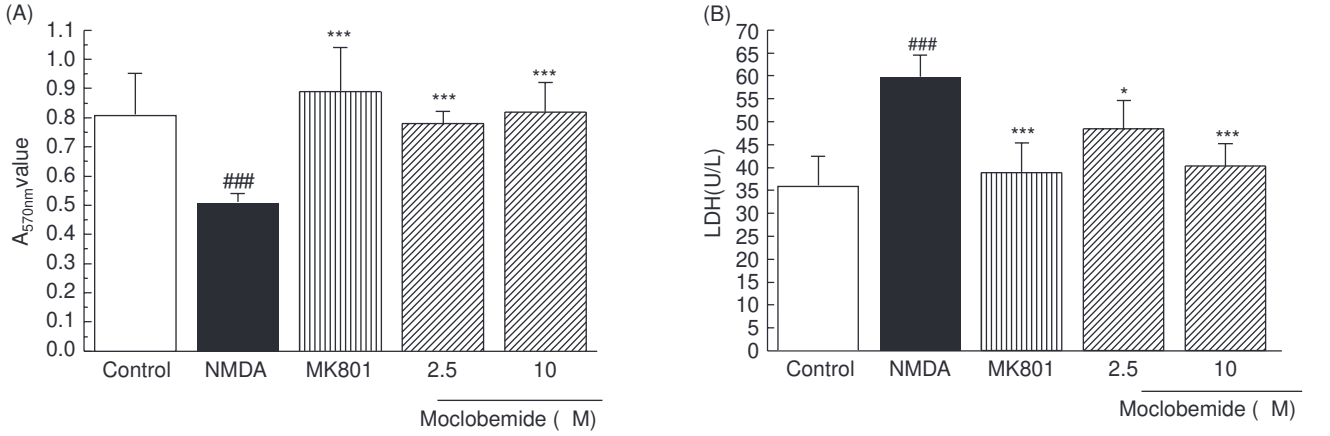

Figure 3 Inhibition of moclobemide or MK801 on the NMDA-induced lesion in PC12 cells.

Cell viability was measured using a colourimetric MTT assay (***A***) and LDH assay (***B***). Data were expressed as means ± SD. ###$p < 0.001$ vs. corresponding control. *$p < 0.05$ and ***$p < 0.001$ vs. corresponding NMDA-treated groups.

Effect of antidepressants on the NMDA-induced $[Ca^{2+}]_i$ overload in hippocampal neurons

After the treatment of cultured hippocampal neurons with NMDA 20 μM for 24 h, $[Ca^{2+}]_i$ was obviously elevated compared with the control, while in the presence of desipramine 5 μM or fluoxetine 2.5 μM or moclobemide 10 μM, the NMDA-induced $[Ca^{2+}]_i$ overload was attenuated (Fig. 5 and Fig. 6). This adds to the evidence that the cytoprotection of antidepressants is associated with their reduction of $[Ca^{2+}]_i$ overload.

Effect of antidepressants on the NOS activity in NMDA-treated PC12 cells

After the treatment of PC12 cells with NMDA 300 μM for 4 h, NO production was remarkably elevated compared with the control, indicating that the NOS activity increased. While in the presence of desipramine (1, 5 μM), fluoxetine (1, 5 μM) or moclobemide (2.5, 10 μM), the NMDA-induced NOS activation was attenuated (Fig. 7). Given all the findings, it is demonstrated that the three kinds of classical antidepressants can all inhibit the NMDA–Ca^{2+}–NOS function, which may be responsible for their common cytoprotective action.

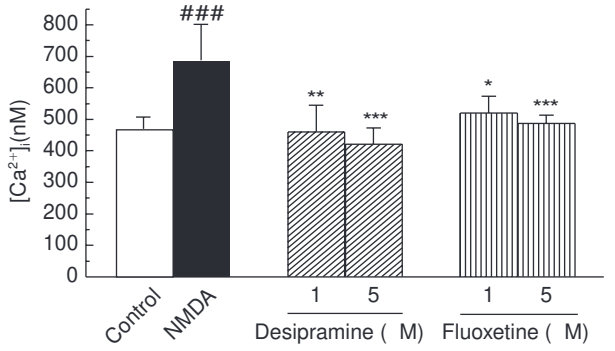

Figure 4 Effect of desipramine or fluoxetine on the NMDA-induced $[Ca^{2+}]_i$ overload in PC12 cells.

Cells were exposed to NMDA 200 μM in the absence or presence of antidepressants for 24 h and $[Ca^{2+}]_i$ was detected using the sensitive indicator dye, fura-2/AM. Data were expressed as means ± SD.
$^{###}p < 0.001$ vs. control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NMDA-treated group.

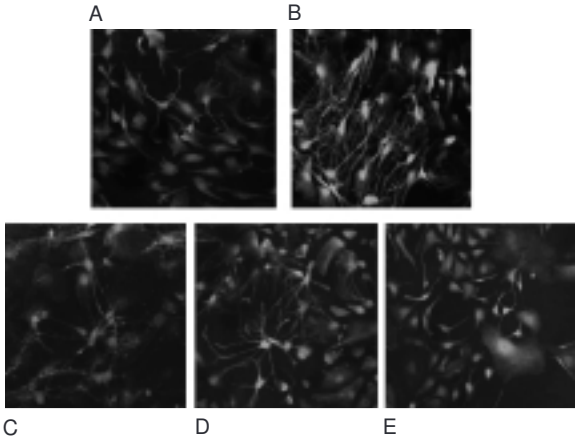

Figure 5 Photographs of NMDA treated hippocampal neurons in the absence or presence of antidepressants.

Neurons were exposed to NMDA 20 μM in the absence or presence of antidepressants for 24 h and $[Ca^{2+}]_i$ was detected using fluo-3/AM on laser confocal microscope. ***A:*** Control; ***B:*** NMDA 20 μM;
C: NMDA + desipraming 5 μM; ***D***: NMDA + fluoxetine 2.5 μM;
E: NMDA + moclobemide 10 μM.

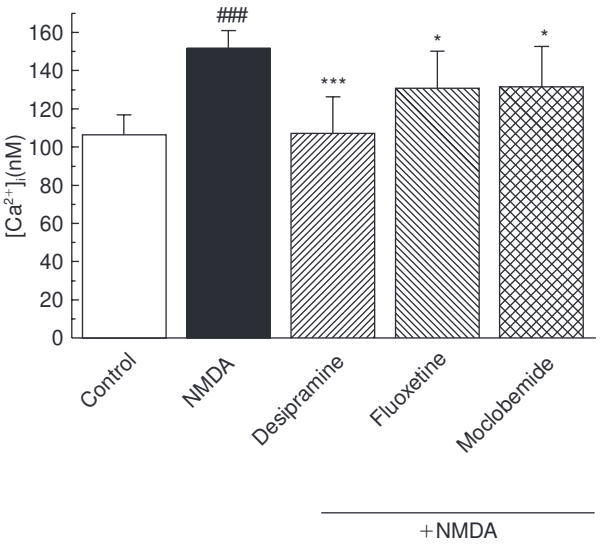

Figure 6 Effect of antidepressants on the NMDA-induced $[Ca^{2+}]_i$ overload in primarily cultured hippocampal neurons.

Data were expressed as means ± SD.
$^{###}p < 0.001$ vs. control. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. NMDA-treated group.

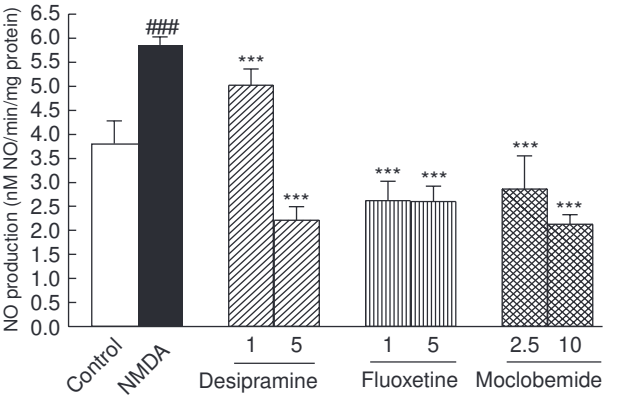

Figure 7 Effect of antidepressants on the NOS activity in NMDA-treated PC12 cells.

Cells were exposed to NMDA 300 μM in the absence or presence of antidepressants for 4 h and the NO production was detected. Data were expressed as means ± SD.
$^{###}p < 0.001$ vs. control. $^{***}p < 0.001$ vs. NMDA-treated group.

Discussion

It is reported that the volume of hippocampus is reduced in patients with major depression compared to healthy controls, and there is a positive correlation between hippocampus atrophy and the time course of the depression (Sapolsky, 2000). Chronic psychosocial stress caused apical dendritic atrophy of hippocampal CA3 pyramidal neurons, which may be mediated by activation of the hypothalamic–pituitary–adrenal axis acting in concert with the endogenous excitatory amino acid release (Magarinos *et al.*, 1996; Sapolsky, 2000). Since the hippocampus is a major site for emotional processes, a role for excessive/long-lasting plasma glucocorticoid levels has been suggested in case of mental impairment. Our previous studies found that high concentration corticosterone can not only result in lesion of PC12 cells or cultured rat hippocampal neurons, but also elevate the glutamic acid content in PC12 cells. Moreover, NMDA receptor antagonist, MK801, antagonize the corticosterone-induced lesion in PC12 cells (Huang *et al.*, 2003). Based on corresponding reports (Gould and Tanapat, 1999), we hypothesize that endogenous glutamic acid release and its NMDA receptors mediate the corticosterone-induced lesion in hippocampus.

Our previous results also showed that the three kinds of classical antidepressants all protected the PC12 cells from the lesion induced by corticosterone, while antipsychotic chlorpromazine or anxiolytic diazepam had no such effect (Li *et al.*, 2003a). Supported by other studies (Li *et al.*, 2003b, 2004), we hold that the cytoprotection is one of the common action pathways for antidepressants. In fact, the excitatory amino acids accumulate in the brain of depressive patients and are capable of ultimately leading to the lesion of the brain (especially hippocampus). It is reported that PC12 cells express functional NMDA receptors (Casado *et al.*, 1996). This study, in more detail, demonstrated that three main kinds of antidepressants not only antagonized the NMDA-induced lesion in PC12 cells, but also inhibited the function of NMDA–Ca^{2+}–NOS signal pathway, indicating that inhibition on NMDA–Ca^{2+}–NOS pathway also appears to be one of the common actions for antidepressants despite remarkable structural diversity. And also, this action may be at least partly underlying their cytoprotective effects.

The therapeutical plasma concentrations of desipramine, fluoxetine, moclobemide are about 0.5–1 μM, 1–2 μM, >0.4 μM (even up to 50 μM (Fu *et al.*, 1999)) respectively. The drug concentrations used in these studies correspond to plasma levels that produce pharmacological effects in humans. All these results offer more evidence for the hypothesis that region-specific damping of NMDA receptor function becomes a strategy for discovering novel antidepressants (Skolnick, 1999).This view is also supported by the antidepressant-like effect of agmatine and its inhibition on NMDA receptor function (Li *et al.*, 2003b).

It is reported that TCAs can selectively block the NMDA receptor current in rat hippocampal neurons, while SSRIs have no such direct blockage (Petrie *et al.*, 2000). Our studies find that the three kinds of antidepressants all inhibit NMDA receptor function, suggesting that besides direct blockage, antidepressants may have other indirect inhibition on NMDA receptors, such as the involvement of the monoamine system, because the three kinds of the antidepressants can increase the brain levels of monoamine that can also antagonize NMDA or corticosterone induced lesion in PC12 cells (Huang *et al.*, 2003; Li *et al.*, 2003b). Furthermore, antidepressants or monoamines also attenuate the $[Ca^{2+}]_i$ overload induced by NMDA or corticosterone, which also suggests that monoamines may be involved in the inhibition of NMDA receptor function caused by antidepressants (Huang *et al.*, 2003; Li *et al.*, 2003b). Another possibility is that the NMDA receptor inhibition causes the increase of monoamine contents in PC12 cells and monoamines subsequently cascade the cytoprotective effect of antidepressants. Further studies are needed.

Recent studies demonstrated that stress decreases hippocampal neurogenesis, while the three kinds of antidepressants up-regulate it, which is regarded as an important action mechanism for antidepressants (Duman *et al.*, 2001). Furthermore, NMDA receptor antagonists up-regulate hippocampal neurogenesis, and conversely, activation of NMDA receptors inhibits hippocampal neurogenesis (Gould and Tanapat, 1999), suggesting that up-regulation of hippocampal neurogenesis by antidepressants may be related to their inhibition on NMDA receptors.

In summary, despite a remarkable structural diversity, inhibition on the function of NMDA receptor–Ca^{2+}–NOS, and therefore the cytoprotective action, appears one of the common actions for antidepressants. Thus, region-specific damping of NMDA receptor function becomes an attractive strategy for discovering novel antidepressants.

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