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Nicotinic acetylcholine receptor-mediated neuroprotection by donepezil against glutamate neurotoxicity in rat cortical neurons

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Footnotes

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Running title: Nicotinic receptor-mediated neuroprotection by donepezil

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Abbreviations

AChE, acetylcholinesterase; AD, Alzheimer's disease; amyloid β protein, Aβ; CNS, central nervous system; DHβE, dihydro-β-erythroidine; donepezil, ((±)-2-[(1-benzylpiperidin-4-yl)methyl]- 5,6-dimethoxy-indan-1-one monohydrochloride; PI3K, phosphatidylinositol 3-kinase; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NO, nitric oxide; NOS-1, nitric oxide synthase-1.

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Abstract

Donepezil is a potent and selective acetylcholinesterase (AChE) inhibitor developed for the treatment of Alzheimer's disease (AD). To elucidate whether donepezil shows neuroprotective action in addition to amelioration of cognitive deficits, we examined the effects of donepezil on glutamate-induced neurotoxicity using primary cultures of rat cortical neurons. A 10-min exposure of cultures to glutamate followed by a 1-h incubation with glutamate-free medium caused a marked loss of viability, as determined by Trypan blue exclusion. Glutamate neurotoxicity was prevented by 24-h pretreatment of donepezil in a concentration-dependent manner. Among AChE inhibitors examined, donepezil and certain AChE inhibitors such as tacrine and galanthamine showed potent neuroprotective action, although physostigmine did not affect glutamate neurotoxicity. Neuroprotective action of donepezil was antagonized by mecamylamine, a nicotinic acetylcholine receptor (nAChR) antagonist, but not by scopolamine, a muscarinic acetylcholine receptor antagonist. Furthermore, both dihydro-β-erythroidine, an α4β2 neuronal nAChR antagonist, and methyllycaconitine, an α7 nAChR antagonist, each also significantly antagonized the effect of donepezil. Next, we examined the effects of donepezil on glutamate-induced apoptosis. Exposure of 100 μM glutamate to cortical neurons for 24 h induced apoptotic neuronal death and nuclear fragmentation. Donepezil for 24 h prior to and 24 h during glutamate exposure prevented nuclear

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fragmentation and glutamate-induced apoptosis. These results suggest that donepezil not only protects cortical neurons against glutamate neurotoxicity via α4β2- and α7-nAChRs but also prevents apoptotic neuronal death.

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Alzheimer's disease (AD) is a progressive neurodegenerative disease. AD is characterized by the presence of two types of abnormal deposit, senile plaques and neurofibrillary tangles, and by extensive neuronal loss (Giannakopoulos *et al*., 1996). Although the pathogenesis of AD remains unknown, recent studies suggested that amyloid β protein (Aβ) plays an important role in the neurodegeneration by the progress of the disease (Hardy and Selkoe, 2002). Involvement of glutamate cytotoxicity in various neurodegenerative diseases was suggested in previous studies (Brensnick, 1989; Choi *et al*., 1987). It was reported that Aβ increases the vulnerability of the cultured cortical neurons to glutamate cytotoxicity (Mattson *et al*., 1992; Koh *et al*., 1990). Thus, glutamate may play an important role in Aβ-induced cytotoxicity in the cerebral cortex.

Marked loss of the central cholinergic neurons is one of the hallmarks of the pathogenesis of AD brain (Whitehouse *et al*., 1982). Moreover, loss or down-regulation of the neuronal nicotinic acetylcholine receptors (nAChRs) has also been observed (Shimohama *et al*., 1986; Perry *et al*., 1995). Remarkably, nicotine was reported to inhibit amyloid β protein (Aβ) toxicity (Kihara *et al*., 1997). Based on those findings, we previously examined the effects of nicotine and related compounds on neurotoxicity induced by glutamate. Nicotine protected cortical neurons against glutamate neurotoxicity via neuronal nAChRs including α4β2- and α7-nAChRs (Shimohama *et al*., 1996; Kaneko *et al*., 1997). These findings suggest that activation of neuronal

nAChRs is effective in preventing neuronal loss in AD brain.

This study was carried out to examine the neuroprotective effects of acetyl cholinesterase (AChE) inhibitors developed for the treatment of AD. Donepezil is a potent inhibitor of AChE, and the first agent utilized in the treatment of AD because donepezil shows modest improvement with minimal side-effects among other drugs enhancing central cholinergic functions (Kosaka *et al*., 1999, 2000; Sugimoto *et al*., 2000; Ogura *et al*., 2000). Thus, special attention was paid to investigate the effect of donepezil on glutamate neurotoxicity, and we found that donepezil protected cultured cortical neurons against not only neurotoxicity induced by brief exposure to glutamate but also apoptosis induced by long exposure.

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Materials and methods

Materials

Eagle's minimal essential medium (Eagle's MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Drugs and sources were as follows: L-glutamic acid monosodium salt (Nacalai Tesque, Kyoto, Japan); dihydro-β-erythroidine-HBr and methyllycaconitine citrate (Reseach Biochemicals Interational, Natick, MA); galanthamine-HBr, tacrine, metrifonate, neostigmine bromide, physostigmine (eserine sulfate), pyridostigmine bromide, mecamylamine-HCl, (–)-scopolamine-HBr and (+)-MK-801 (Sigma, Saint Louis, MO); DEVD-CHO, 2-[2-(4-Hydroxyphenyl)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl)-benzimidazole-3H Cl (Hoechst 33258) (Carbiochem, Darmstadt, Germany); and donepezil hydrochloride ((±)-2-[(1-benzylpiperidin-4-yl) methyl]-5,6- dimethoxy-indan-1-one monohydrochloride: E2020) was supplied by Eisai Co. Ltd. (Tsukuba-shi, Japan).

Cell culture

Primary cultures were obtained from the cerebral cortex of fetal Wistar rats (17–19 days of gestation) according to the procedures described previously (Kume *et al*., 1997, 2000). Briefly, single cells dissociated from the whole cerebral cortex of fetal rats were

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plated on plastic coverslips placed in Falcon 60-mm dishes and 12-well plates (5.1×10^6 cells per dish).

Cultures were incubated in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum (1–7 days after plating) or 10% heat-inactivated horse serum (8–12 days after plating), glutamine (2 mM), glucose (total 11 mM), $NaHCO_3$ (24 mM) and HEPES (10 mM). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Six days after plating, non-neuronal cells were removed by adding cytosine arabinoside (10 μM). Only mature cultures (10–13 days *in vitro*) were used for experiments.

Measurement of neurotoxicity

Neurotoxicity induced by glutamate was quantitatively assessed by examining cultures under Hoffman modulation microscopy according to the methods described previously (Kume *et al*., 1997, 2000). All experiments were performed in Eagle's MEM at 37°C. Cell viability was assessed by Trypan blue solution for 10 min at room temperature, fixed with isotonic formalin (pH 7.0, 2–4°C), and rinsed with physiological saline. In each experiment, the cells on five coverslips were counted to obtain the means ± S.E.M. of cell viability.

Hoechst staining

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To reveal the nuclear morphological changes in cultured neurons, cells were stained with a nuclear dye Hoechst 33258 according to the methods described previously (Osakada et al., 2003). Briefly, after glutamate treatment, cultures were fixed with neural formaldehyde for 30 min and incubated with 1 mg/ml Hoechst 33258 for 30 min at room temperature. Cells were visualized and photographed under ultraviolet illumination using fluorescent microscopy (Olympus, Tokyo, Japan).

Statistics

Values are shown as means ± S.E.M. The statistical significance was evaluated by Dunnett's two-tailed test, and defined as a probability value of less than 5%.

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Results

Effects of donepezil on glutamate neurotoxicity.

The exposure of cultured cortical neurons to glutamate markedly reduced cell viability (Fig. 1). Trypan blue exclusion revealed that the cell viability was decreased by 10-min treatment with 1 mM glutamate, whereas most of the cells without drug treatment had nonstained, bright cell bodies. We examined the effects of donepezil on this glutamate neurotoxicity. Treatment of cultures with donepezil (1 μM) for 24 h prior to, 10 min during and 1 h following glutamate exposure significantly reduced the number of cells stained with Trypan blue (Fig. 1C). As shown in Fig. 2, the long-term exposure of donepezil significantly inhibited glutamate neurotoxicity. However, the neuroprotection was not observed when donepezil and glutamate were concomitantly added to the cultures without pretreatment (Fig. 2). These findings suggest that the long-term exposure of donepezil is necessary to exhibit a protective action against glutamate neurotoxicity.

The magnitude of the protective effect of donepezil against glutamate neurotoxicity increased in a concentration-dependent manner when the long-term exposure was employed (Fig. 3). Cultures were treated with donepezil at concentrations ranging from 0.001 to 10 μM. Donepezil at concentrations greater than 0.1 μM significantly protected

cortical neurons against glutamate neurotoxicity.

Effects of AChE inhibitors on glutamate neurotoxicity.

Table 1 summarizes IC_{50} values of AChE inhibitors in previous studies. IC_{50} values of donepezil for AChE were reported to be 6.7–13.6 nM (Ogura *et al*., 2000; Tang, 1996). That the neuroprotective effect of donepezil was observed at higher concentrations than IC_{50} values for AChE suggested that donepezil protected cortical neurons against glutamate neurotoxicity through mechanisms other than AChE inhibition.

Among other drugs enhancing central cholinergic functions, donepezil is one of the most potent and selective anti-dementia drugs which show modest improvement with minimal side-effects (Sugimoto *et al*., 2000; Ogura *et al*., 2000). We compared the effect of donepezil with that of various AChE inhibitors. We tested the effects of four groups of AChE inhibitors at the same concentration (1 μM). The first group includes tacrine and galanthamine, which are approved for AD treatment. The second group was physostigmine, a potent AChE inhibitor but is not approved for AD treatment. The third including neostigmine and pyridostigmine, quaternary ammonium compounds unable to pass through the membrane. The fourth one was metrifonate, an irreversible AChE inhibitor. As shown in Fig. 4, all drugs, except for physostigmine, showed significant protective effects against glutamate neurotoxicity. Statistical analysis revealed that

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donepezil and galanthamine of the first group showed more potent neuroprotective effects than the drugs of the other groups. By contrast, tacrine showed more potent effect than physostigmine but not other drugs of the third and fourth groups.

Effects of nicotinic receptor antagonists on the neuroprotection by donepezil.

Previously, we reported that nicotine protected cortical neurons against Aβ-, or glutamate-induced neurotoxicity via nAChRs (Shimohama *et al*., 1996; Kihara *et al*., 1997; Kaneko *et al*., 1997). To determine the involvement of nAChRs in the neuroprotective effects of donepezil, we examined the effects of mecamylamine, a nAChR antagonist, and scopolamine, a muscarinic acetylcholine receptor (mAChR) antagonist, on the neuroprotective action of donepezil. Each antagonist was added to donepezil-containing media. As a result, mecamylamine (10 μM), but not scopolamine (10 μM), significantly antagonized the protection by 24-h pretreatment with 10 μM donepezil (Fig. 5), suggesting that nAChRs play a crucial role in the neuroprotective effect of donepezil.

To elucidate the nicotinic receptor subtypes mediating the neuroprotective action of donepezil, we utilized the following antagonists: dihydro-β-erythroidine (DHβE), an α4β2 nAChR antagonist, and methyllycaconitine (MLA), an α7 nAChR antagonist. Each antagonist was added to donepezil-containing media. Both DHβE (10 nM) and

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MLA (10 nM) significantly antagonized the protective effect of donepezil (Fig. 6).

Effects of donepezil on glutamate-induced apoptosis.

Next, we investigated whether donepezil could prevent apoptosis. Exposure of 100 μM glutamate to cortical neurons for 24 h induced nuclear fragmentation (Fig. 7) and neuronal death (Fig. 8). To address whether caspase-3 activation contributes to 24 h glutamate neurotoxicity, we utilized DEVD, a caspase-3 inhibitor. Treatment of cultures with DEVD (1 μM) for 24 h prior to and 24 h during glutamate exposure prevented nuclear fragmentation (Fig. 7) and neuronal death (Fig. 8). Cycloheximide, a protein synthesis inhibitor, and actinomycin D, an RNA synthesis inhibitor, also prevented 24 h glutamate neurotoxicity (data not shown). Take together with these results, we considered 24 h glutamate neurotoxicity was mediated through apoptosis. Donepezil (1 μM) for 24 h prior to and 24 h during glutamate exposure prevented nuclear fragmentation (Fig. 7) and glutamate-induced apoptosis (Fig. 8).

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Discussion

In the present study, we demonstrated that donepezil protected cultured cortical neurons against glutamate neurotoxicity. The neuroprotective action of donepezil was prominent among various types of AChE inhibitors examined. In addition, the neuroprotective effect of donepezil was inhibited by selective antagonists for α4β2- and α7-nAChRs. Furthermore, donepezil prevented glutamate-induced apoptosis.

Donepezil prevented glutamate neurotoxicity when the cultures were treated with glutamate 24 h prior to, 10 min during and 1 h following glutamate exposure. In contrast, concomitant addition of donepezil and glutamate did not exhibit a protective action. This suggests that a prolonged donepezil treatment prior to glutamate exposure is necessary to elicit a neuroprotective effect. Since the effect of donepezil was antagonized by nAChR antagonists, some possible hypotheses could be proposed. First, donepezil may induce neuroprotective factors by stimulating nAChRs. Greenberg et al. demonstrated the regulation of c-*fos* transcript levels following nAChRs activation in PC12 cells (Greenberg *et al.*, 1986). The regulation of gene expression by nAChRs stimulation suggests that donepezil can induce the transcription factor-regulated synthesis of neuroprotective factors via nAChRs. Second, long-time exposure of donepezil may up-regulate the expression of nAChRs, which would potentiate nAChR-mediated neuroprotective effects. In addition to nicotine, donepezil or certain

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AChE inhibitors approved for AD treatment were reported to up-regulate the expression of nAChRs (Barnes *et al*., 2000; Svensson, 2000; Woodruff-Pak *et al*., 2001). Although it was known that α7 nAChRs were inactivated by nicotine, Kawai et al. recently showed that the expression of nAChRs containing the α7 subunit was up-regulated by nicotine exposure without undergoing inactivation (Kawai and Berg, 2001). These previous findings suggest that the long-term exposure of donepezil at higher concentration than IC_{50} value of AChE would elicit up-regulation of neuronal nAChRs, which may lead to potentiate neuroprotective effects against glutamate neurotoxicity.

Among AChE inhibitors examined in the present study, donepezil and galanthamine, which are currently available for AD treatment (Stahl, 2000), showed more potent neuroprotective effects than physostigmine, neostigmine, pyridostigmine and metrifonate. Donepezil and galanthamine have similar neuroprotective effects on glutamate neurotoxicity although IC_{50} of these inhibitors for AChE were very different (Table 1). Thus, it is likely that donepezil and galanthamine protect cortical neurons against glutamate neurotoxicity through mechanisms other than AChE inhibition. Interestingly, galanthamine was reported to interact with nAChRs directly and sensitize nAChRs by increasing the probability of channel opening and by slowing down receptor desensitization (Maelicke *et al.*, 2001; Coyle and Kershaw, 2001). These allosterically potentiating ligand actions of galanthamine were observed in both α4β2-

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and α7-nAChRs (Maelicke, 2000; Samochocki *et al*., 2000). However, a recent study showed that galanthamine, but not donepezil, acting primarily as an allosteric potenciating ligand at presynaptically located nAChRs, potentiated glutamatergic transmission in rat hippocampal slice (Santos *et al*., 2002). However, as various experimental conditions including culture preparation and drug administration are different, further investigations are required to explain the nAChRs-mediated neuroprotection by donepezil.

α4β2- and α7-nAChRs are involved in cognitive processes in the brain in addition to neuroprotection (Changeux *et al*., 1998). α7 nAChRs were reported to bind Aβ 1-42 (Wang *et al*., 2000), and this binding might inhibit α7 nAChRs-dependent learning and memory. The reduction of α7 nAChRs activation would cause neurons vulnerable to various toxic insults such as glutamate. Recent findings suggested that α7 nAChRs activation led to the neuroprotection against glutamate neurotoxicity via the Ca^{2+}-dependent phosphatidylinositol 3-kinase (PI3K) pathway, and that nicotine protected neurons by activating PI3K, which activated Akt and up-regulated Bcl-2 (Kihara *et al*., 2001). Furthermore, Ca^{2+} influx through α7 nAChRs could activate Ca^{2+}/calmodulin-dependent protein kinase 2 in PC12 cells (Macnicol *et al*., 1992), resulting in nitric oxide synthase-1 (NOS-1) inactivation by its phosphorylation (Nakane *et al*., 1991). We previously reported that the neuroprotective effect of nicotine

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via α4β2- and α7-nAChRs was effective against glutamate neurotoxicity (Kaneko *et al*., 1997). Thus,

these findings suggest that the neuroprotective action of donepezil via α4β2- and α7-nAChRs may be mediated by the similar mechanisms to nicotine.

Finally, we investigated the effects of donepezil on apoptotic neuronal death. Twenty-four hours exposure of glutamate induced apoptotic neuronal death. Donepezil prevented glutamate-induced apoptosis when the cultures were treated with donepezil for 24 h prior to and 24 h during glutamate exposure. A recent study showed that donepezil did not prevent apoptosis induced by staurosporine in NG108-15 and Hela cell lines (Zhang *et al*., 2002). However, effects of donepezil on apoptosis in neuronal cells did not understood. In the present study, we showed donepezil prevented glutamate-induced apoptosis in cortical neurons. To our knowledge, this is the first report that donepezil prevented neuronal apoptosis.

In conclusion, it was shown that donepezil protected cortical neurons against glutamate neurotoxicity via neuronal α4β2- and α7-nAChRs. In addition, donepezil prevented glutamate-induced apoptosis. The present findings suggest that donepezil shows neuroprotective action in addition to amelioration of cognitive deficits.

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Figure Legends

Fig. 1. Microphotographs showing effects of donepezil on glutamate neurotoxicity. Culture fields were photographed after Trypan blue staining followed by formalin fixation. A shows the cells with sham treatment. B shows the cells treated with glutamate (1 mM) for 10 min and further incubated with glutamate-free medium for 1 h. C shows the cells exposed to donepezil (1 μM) for 24 h prior to glutamate treatment. Scale bar = 50 μm.

Fig. 2. Effects of 24-h pretreatment (pre) and simultaneous treatment (sim) of donepezil on glutamate neurotoxicity. Cultures were exposed to 1 mM glutamate for 10 min then incubated with glutamate-free medium for 1 h. Cultures were treated with donepezil for 24 h before, 10 min during and 1 h after glutamate exposure (pre) or for 10 min during and 1 h after glutamate (sim). $^{**}P < 0.01$, compared with glutamate alone. Data represent the means ± S.E.M. of n=5 independent observations.

Fig. 3. Concentration-dependent effect of donepezil on glutamate neurotoxicity. Cultures were pretreated by donepezil (0.001-10 μM) for 24 h prior to glutamate exposure and were exposed to 1 mM glutamate for 10 min then incubated with glutamate-free medium for 1 h. $^{**}P < 0.01$, compared with glutamate alone. Data

represent the means ± S.E.M. of n=5 independent observations.

Fig. 4. Effects of AChE inhibitors on glutamate neurotoxicity. Cultures were pretreated with AChE inhibitors (1 μM) in the same manner as donepezil application. $^{**}P < 0.01$, compared with glutamate alone. Data represent the means ± S.E.M. of n=5 independent observations.

Fig. 5. Effects of nicotinic and muscarinic ACh receptor antagonists on donepezil-induced neuroprotection against glutamate neurotoxicity. We utilized the following antagonists; mecamylamine (10 μM) as a nAChR antagonist, scopolamine (10 μM) as a mAChR antagonist. Donepezil plus mecamylamine (A), and donepezil plus scopolamine (B) were added for 24 h prior to glutamate treatment. $^{**}P < 0.01$, compared with glutamate alone. $^{\#\#}P < 0.01$. *NS* : not significant. Data represent the means ± S.E.M. of n=5 independent observations.

Fig. 6. Effects of α4β2- and α7-nAChRs antagonists on donepezil-induced neuroprotection against glutamate neurotoxicity. We utilized dihydro-β-erythroidine (DHβE ; 10 nM) as an α4β2 nAChR antagonist, and methyllycaconitine (MLA; 10 nM) as an α7 nAChR antagonist. Donepezil and antagonists were added for 24 h prior to application of glutamate. $^{**}P < 0.01$, compared with glutamate alone. $^{\#\#}P < 0.01$. Data

represent the means ± S.E.M. of n=5 independent observations.

Fig. 7. Microphotographs showing effects of donepezil and caspase-3 inhibitor on glutamate-induced apoptosis. We utilized DEVD as a caspase-3 inhibitor. Culture fields were photographed after Hoechst33258 staining followed by formalin fixation. A shows the cells with sham treatment. B shows the cells treated with glutamate (100 μM) for 24 h. C shows the cells exposed to donepezil (1 μM) for 24 h prior to glutamate treatment. D shows the cells exposed to DEVD (1 μM) for 24 h prior to glutamate treatment. Arrowheads indicate nuclear fragmentation. Scale bar = 10 μm.

Fig. 8. Effects of donepezil and caspase-3 inhibitor on glutamate-induced apoptosis. Cultures were pretreated by donepezil (1 μM), DEVD (1 μM), MK-801 (1 μM) for 24 h prior to 100 μM glutamate application for 24 h. $^{**}P < 0.01$, compared with glutamate alone. Data represent the means ± S.E.M. of n=5 independent observations.

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TABLE 1 Comparison of IC_{50} of AChE inhibitors

Drug	IC_{50} [a] (nM)	IC_{50} [b] (nM)	IC_{50} [c] (nM)	IC_{50} [d] (nM)
Donepezil	6.7±0.35	–	13.6	–
Tacrine	77±1.4	450	93.0	80
Galanthamine	–	–	1,995	–
Physostigmine	0.67±0.015	18	251	60
Neostigmine	–	25	–	15
Pyridostigmine	–	–	–	300
Metrifonate	–	1080	–	–

[a] IC_{50} values for AChE in the rat brain are taken from Ogura et al., 2000.

[b] IC_{50} values for AChE in human cortex are taken from Rakonczay et al., 1998.

[c] IC_{50} values for AChE in the rat cerebral cortex are taken from Tang, 1996.

[d] IC_{50} values for AChE in the mouse brain are taken from Arnal et al., 1990.

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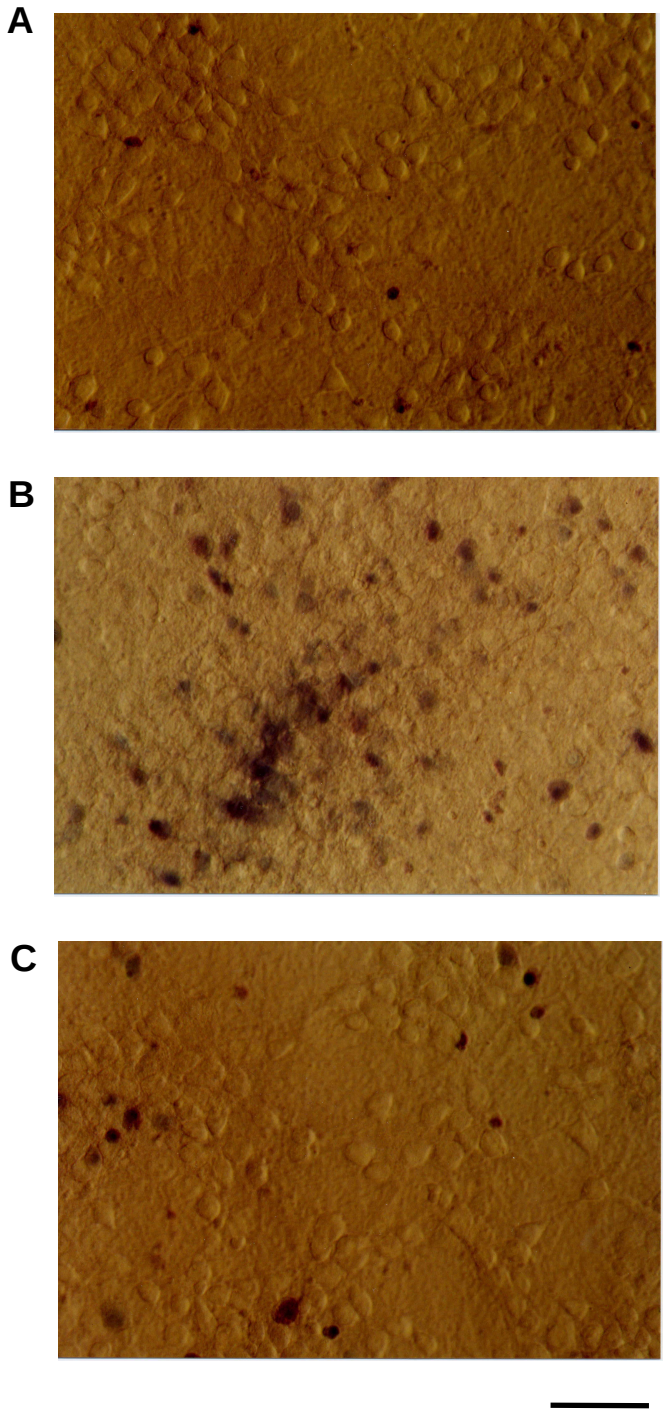

Fig. 1 Takada et al.

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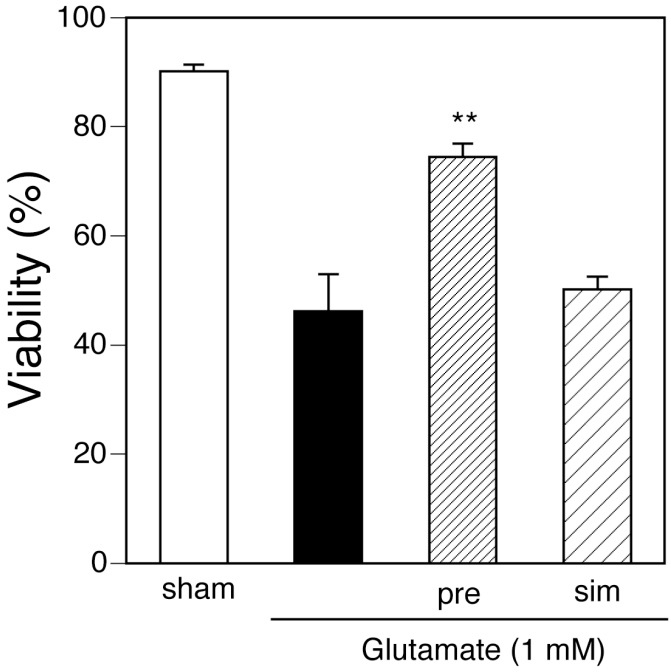

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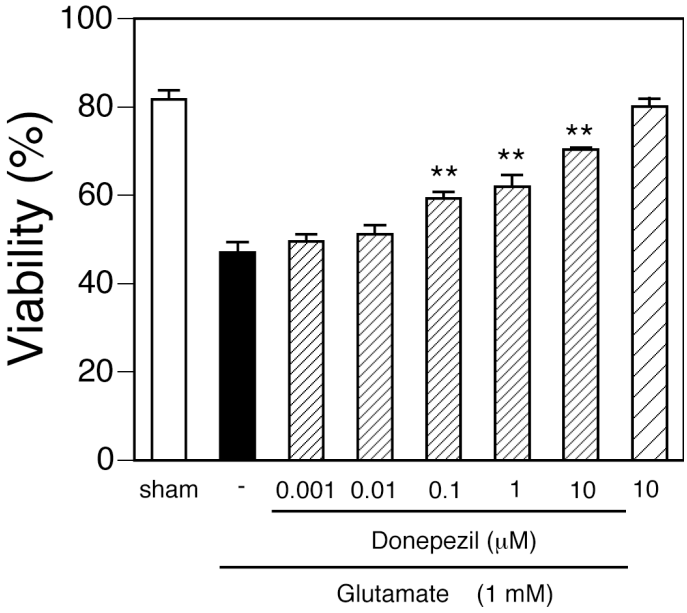

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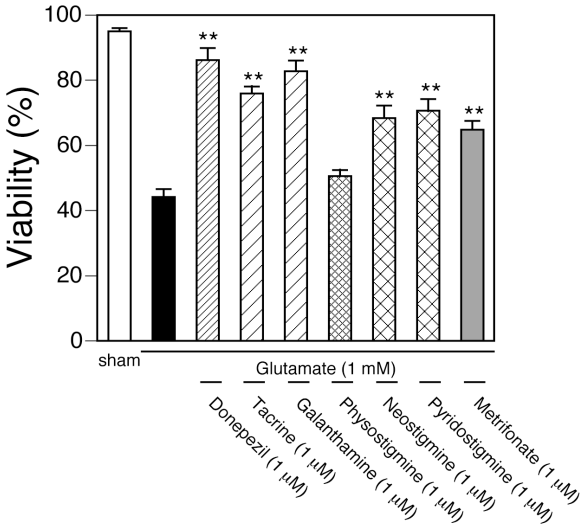

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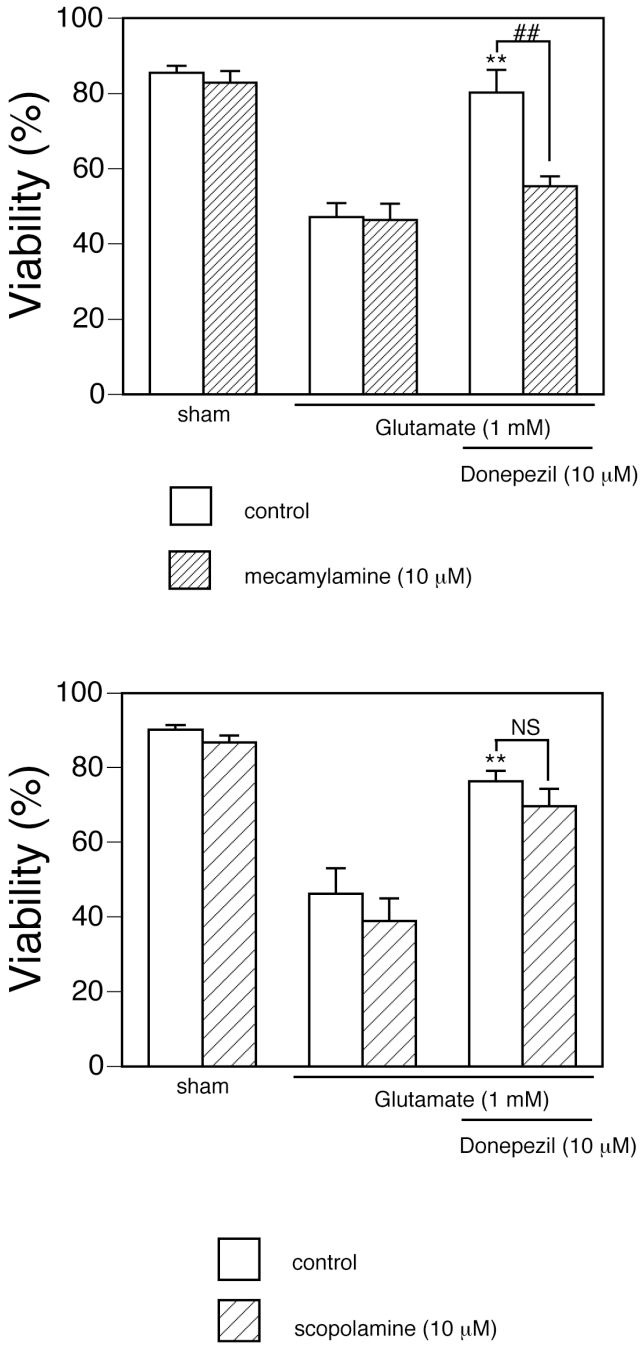

Fig. 5 Takada et al.

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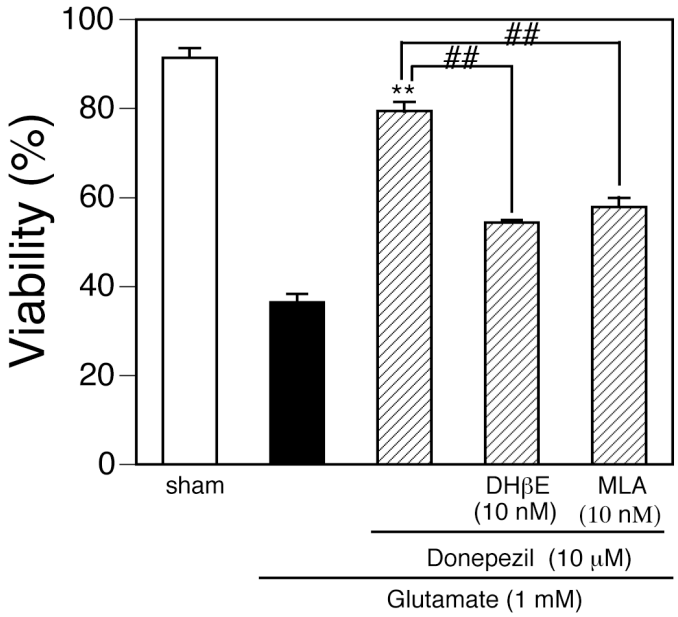

Fig. 6 Takada et al.

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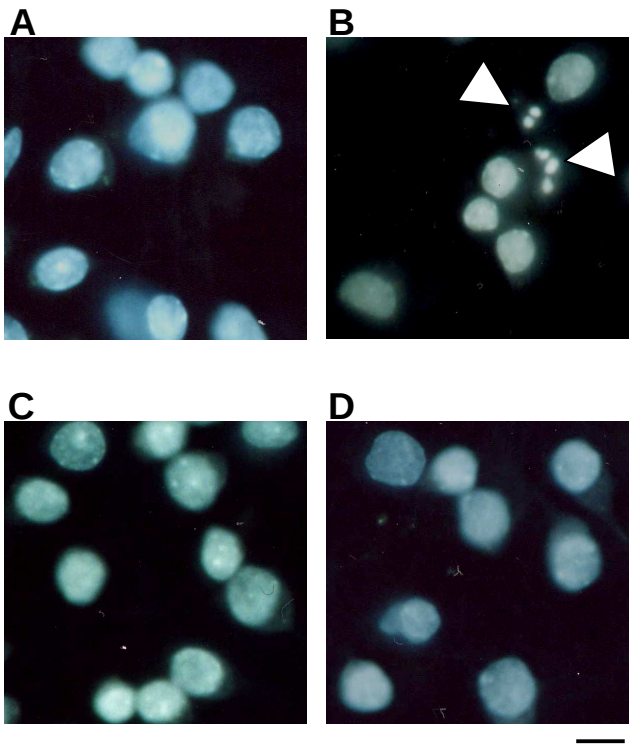

Fig. 7 Takada et al.

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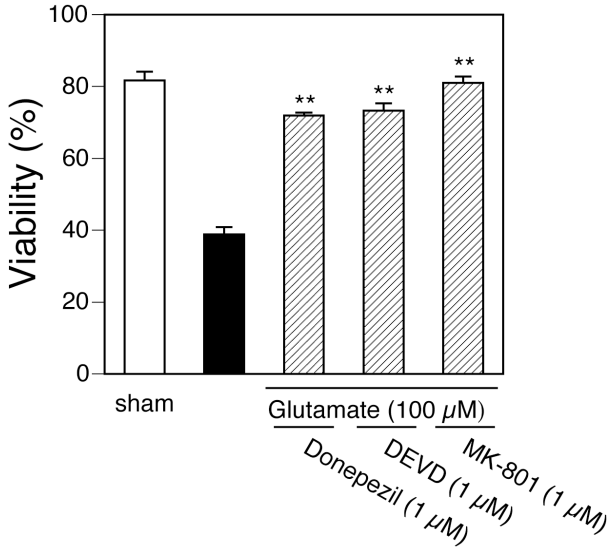

Fig. 8 Takada et al.