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σ Receptor ligands attenuate *N*-methyl-D-aspartate cytotoxicity in dopaminergic neurons of mesencephalic slice cultures

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Abstract

We investigated the potential neuroprotective effects of several σ receptor ligands in organotypic midbrain slice cultures as an excitotoxicity model system. When challenged with 100-μM *N*-methyl-D-aspartate (NMDA) for 24 h, dopaminergic neurons in midbrain slice cultures degenerated, and this was prevented by (5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*b*]-cyclohepten-5,10-imine (MK-801; 1–10 μM). Concomitant application of ifenprodil (1–10 μM) or haloperidol (1–10 μM), both of which are high-affinity σ receptor ligands, significantly attenuated the neurotoxicity of 100 μM NMDA. The σ_1 receptor-selective ligand (+)-*N*-allylnormetazocine ((+)-SKF 10047; 1–10 μM) was also effective in attenuating the toxicity of NMDA. The effect of *R*(−)-*N*-(3-phenyl-1-propyl)-1-phenyl-2-aminopropane hydrochloride ((−)-PPAP), a σ receptor ligand with negligible affinity for the phencyclidine site of NMDA receptors, was also examined. (−)-PPAP (3–100 μM) caused a concentration-dependent reduction of NMDA cytotoxicity, with significant protection at concentrations of 30 and 100 μM. In contrast, (+)-SKF 10047 (10 μM) and (−)-PPAP (100 μM) showed no protective effects against cell death induced by the Ca^{2+} ionophore ionomycin (1–3 μM). These results indicate that σ receptor ligands attenuate the cytotoxic effects of NMDA on midbrain dopaminergic neurons, possibly via inhibition of NMDA receptor functions.

Keywords: σ Receptor; Organotypic slice culture; Mesencephalon; *N*-methyl-D-aspartate (NMDA); Dopaminergic neuron

1. Introduction

Parkinson's disease is a progressive neurodegenerative disorder that is estimated to affect about 2% of the population. The most prominent feature of the brain pathology of this disorder is selective degeneration of dopaminergic neurons in the substantia nigra, and the progression of Parkinson's disease is correlated with the progressive degeneration of dopaminergic neurons.

Overactivation of glutamatergic excitatory neurotransmission may cause damage and death of central nervous system (CNS) neurons. Lipton and Rosenberg (1994) proposed that neurodegeneration in various neurological disorders may involve excitotoxicity as the final common pathway. Indeed, accumulating evidence suggests that excitotoxic injury is at least partly involved in the progressive degeneration of dopaminergic neurons in Parkinson's disease (Blandini et al., 1996). For example, in vivo studies have shown that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin, stimulates glutamate release (Carboni et al., 1990), and dopaminergic neuron death caused by MPTP is reduced by NMDA receptor antagonists (Turski et al., 1991). Moreover, mesencephalic dopaminergic neurons are vulnerable to *N*-methyl-D-aspartate (NMDA) excitotoxicity (Kikuchi and Kim, 1993; Sawada et al., 1996a), and 1-methyl-4-phenylpyridium ion, an active metabolite of MPTP, potentiates NMDA receptor-mediated cytotoxicity (Sawada et al., 1996b). Therefore, prevention of excitotoxic injury is an important strategy to protect dopaminergic neurons from degeneration and to prevent progression of Parkinson's disease.

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σ Receptors, originally postulated to explain the psychotomimetic effects of *N*-allylnormetazocine (SKF 10047) (Martin et al., 1976), are defined as high-affinity haloperidol-sensitive binding sites that exist as molecular entities distinct from opioid receptors and the phencyclidine (PCP) binding site of NMDA receptors. Biochemical and pharmacological studies have suggested that there are at least three classes of σ receptors designated as σ_1–σ_3 (Quirion et al., 1992; Booth et al., 1993). A protein with the radioligand binding profile corresponding to σ_1 has been cloned and identified as a 223-amino acid protein that has a single transmembrane segment anchored in the endoplasmic reticulum (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1998).

σ Receptor ligands exhibit a wide variety of actions in the CNS, including potent neuroprotective actions. In previous in vitro studies, σ receptor ligands have been shown to attenuate hypoxia/hypoglycemia-mediated neurotoxicity in rat primary neuronal cultures (Lockhart et al., 1995) and glutamate-induced neuronal injury in rat cortical cultures (DeCoster et al., 1995). In vivo, σ receptor ligands reduce CA1 neuronal loss in gerbil global brain ischemia (Lysko et al., 1992), protect against hypoxia-induced lethality in mice (Pontecorvo et al., 1991), and reduce acute injury in the cortex and caudate nucleus after transient focal ischemia in rats (Takahashi et al., 1996).

Several studies using radiolabeled ligands suggested the abundance of σ receptors in the substantia nigra pars compacta (Graybiel et al., 1989; Walker et al., 1992; Okuyama et al., 1995). Moreover, the reduction of σ_1 binding sites following destruction of dopamine neurons by 6-hydroxydopamine provided anatomical evidence for potential interactions between σ_1 receptors and dopaminergic neurons (Gundlach et al., 1986). However, although the significant effects of σ receptor ligands on the activities of dopaminergic neurons (Gronier and Debonnel, 1999; Minabe et al., 1999) suggest the functional importance of σ receptors, possible neuroprotective actions of σ receptor ligands on dopaminergic neurons have not been reported.

We have previously established an excitotoxicity model using in vitro organotypic slice cultures of the neonatal rat mesencephalon (Maeda et al., 1998). Using this model, we investigated the effects of several σ receptor ligands on NMDA cytotoxicity in dopaminergic neurons.

2. Materials and methods

2.1. Preparation of slice cultures

The method of Stoppini et al. (1991) for the preparation of organotypic slice cultures was adapted for use with the slight modification described previously (Maeda et al., 1998; Shimazu et al., 1999). Briefly, postnatal days 2–3 Wistar rats were anesthetized by hypothermia. The mesencephalon was dissected under sterile conditions and cut into slices 350-μm thick with a tissue chopper (Narishige, Tokyo, Japan). Slices were transferred onto insert membranes (Millicell-CM; Millipore, Bedford, MA, USA) in 6-well plates (Corning Costar, Tokyo, Japan) supplied with 700 μl/well of culture medium of the following composition: 50% Earle's minimum essential medium/HEPES, 25% Hank's balanced salt solution and 25% heat-inactivated horse serum (Gibco BRL, Rockville, MD, USA) supplemented with 6.5 g/l glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. The 6-well plates were placed in a humidified atmosphere of 5% CO_2 and 95% air in an incubator at 34°C. Culture medium was replaced with fresh medium every 2 days. Slices cultured for 17 days were used in the cytotoxicity experiments. For cytotoxic insult by NMDA, slices were submerged for 24 h in 2 ml of culture medium containing NMDA (Nacalai Tesque, Kyoto, Japan), then processed for immunohistochemistry. For ionomycin cytotoxic insult, slices were submerged for 24 h in 2 ml of serum-free culture medium containing ionomycin (Nacalai Tesque), because we found in preliminary experiments that ionomycin showed no significant cytotoxicity in the presence of serum. The organotypic culture tolerated the serum-free conditions for at least 24 h. Serum-free medium consisted of 67% Earle's minimum essential medium/HEPES and 33% Hank's balanced salt solution (Gibco BRL) supplemented with 6.5 g/l glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. The test substances *R*-(−)-*N*-(3-phenyl-1-propyl)-1-phenyl-2-aminopropane hydrochloride ((−)-PPAP), (+)-SKF 10047, ifenprodil, haloperidol (RBI, Natick, MA, USA) and (5*R*, 10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*b*]-cycloheptene-5,10-imine hydrogen maleate (MK-801 hydrogen maleate, Nacalai Tesque) were applied concomitantly with NMDA or ionomycin. Slices were submerged in culture medium only during application of toxic insults.

2.2. Tyrosine hydroxylase immunohistochemistry

Dopaminergic neurons in the slice cultures were identified by tyrosine hydroxylase immunohistochemistry using the avidin–biotin peroxidase method. The cultures were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde and 4% sucrose for 2 h, rinsed with 10-mM phosphate-buffered saline (PBS) and exposed for 30 min to 0.02% H_2O_2 in 100% methanol to eliminate endogenous peroxidase activity. The cultures were then exposed to PBS containing 0.2% Triton X-100 for 15 min. Nonspecific antibody binding was reduced by exposure to 10% fetal calf serum for 30 min. Cultures were incubated overnight at 4°C with rabbit anti-tyrosine hydroxylase–polyclonal antibody (1:500 dilution; Chemicon International, Temecula, CA, USA). After being rinsed with three changes of PBS, cultures were incubated for 1 h at room temperature with biotinylated anti-rabbit immunoglobulin

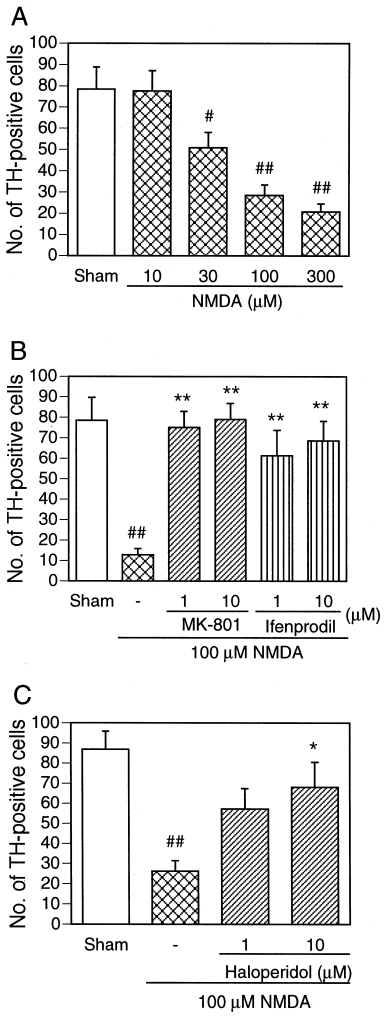

Fig. 1. NMDA-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures. (A) Concentration dependence of NMDA cytotoxicity. (B) Protective effects of MK-801 and ifenprodil on 100-μM NMDA-induced dopaminergic neuronal death. (C) Protective effects of haloperidol on 100 μM NMDA-induced dopaminergic neuronal death. The ordinate shows the number of tyrosine hydroxylase (TH)-positive cells. NMDA was added for 24 h. Drugs were concomitantly added with NMDA. $^{\#}P < 0.01$ and $^{\#\#}P < 0.01$ compared with sham control. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with NMDA alone ($n = 10$–18 per group).

G (1:200 dilution). Following a further rinse in PBS, the cultures were treated for 1 h at room temperature with avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Lab., Burlingame, CA, USA). After further washing in 0.05 M Tris-buffered saline (TBS), peroxidase was visualized with 0.07% diaminobenzidine and 0.018% H_2O_2 in TBS. The cultures were dehydrated with ethanol and mounted on slides.

2.3. Evaluation of cell viability and statistical analysis

The viability of tyrosine hydroxylase-positive cells was evaluated following tyrosine hydroxylase immunostaining. Tyrosine hydroxylase-positive cells with developed dendrites were considered as viable, surviving dopaminergic neurons. The numbers of tyrosine hydroxylase-positive cells in an area of 520×670 μm^2 were counted in individual slices. The surviving cell number is expressed as the mean ± SEM. Statistical significance of differences was first determined by one-way analysis of variance that included all data sets in each figure, then comparisons between groups were made by Dunnett's two-tailed test. Comparisons were made between the NMDA-alone group and the other groups. Percentage of neuroprotection was calculated using the following equation: protection (%) = $[(D - N)/(S - N)] \times 100$, where D is the number of cells in cultures treated with test substance and NMDA, N is the number of cells in NMDA-treated cultures, and S is the number of cells in sham-treated cultures.

3. Results

Consistent with our previous findings (Maeda et al. 1998; Shimazu et al., 1999), application of NMDA (10–300 μM) for 24 h resulted in a marked, concentration-dependent decrease in the number of surviving dopaminergic neurons (Fig. 1A). Simultaneous application of MK-801

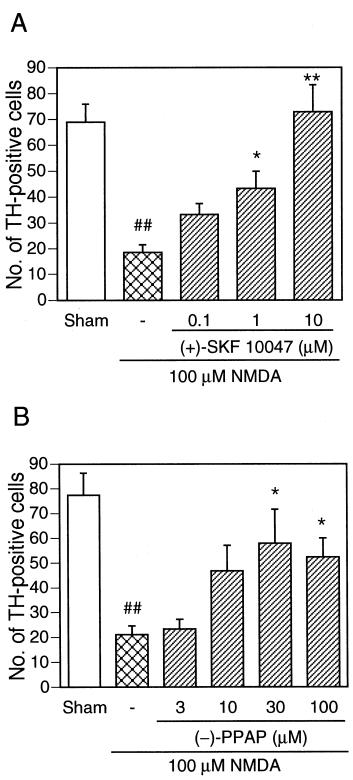

Fig. 2. (A) Effects of (+)-SKF 10047 on 100 μM NMDA-induced dopaminergic neuronal death. (B) Effects of (−)-PPAP on 100 μM NMDA-induced dopaminergic neuronal death. The ordinate shows the number of tyrosine hydroxylase (TH)-positive cells. NMDA was added for 24 h. Drugs were concomitantly added with NMDA. $^{\#\#}P < 0.01$ compared with sham control. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with NMDA alone ($n = 10$–19 per group).

(1–10 μM), a non-competitive antagonist of NMDA receptors, virtually abolished the neurotoxic effect of NMDA (Fig. 1B).

First, we examined the effect of ifenprodil on NMDA neurotoxicity. Ifenprodil is an *N*-substituted 4-benzylpiperidine compound that binds with high affinity to both σ_1 and σ_2 receptors (Whittemore et al., 1997). Ifenprodil, at concentrations of 1 and 10 μM, almost completely blocked the cytotoxic effect of 100-μM NMDA (Fig. 1B). We also tested the effect of haloperidol, another compound that is recognized with comparable high affinity by both σ_1 and σ_2 receptors (Quirion et al., 1992). When concomitantly applied with 100 μM NMDA, haloperidol at concentrations of 1–10 μM markedly reduced the cytotoxic effect of NMDA (Fig. 1C)

Next, we examined the effect of (+)-SKF 10047. In contrast to ifenprodil and haloperidol, (+)-SKF 10047 has a low affinity for σ_2 receptors and binds selectively to σ_1 receptors (Quirion et al., 1992). When (+)-SKF 10047 was applied concomitantly with NMDA, this compound also showed a significant protective effect (Figs. 2A and 3C). The protective effect of (+)-SKF 10047 on dopaminergic neurons was concentration-dependent over the range of 0.1–10 μM. Percentages of neuroprotection by 0.1 and 1 μM (+)-SKF 10047 were 28% and 48%, respectively, whereas, (+)-SKF 10047 at the highest concentration of 10 μM completely inhibited the NMDA insult (Fig. 2A).

Compounds with σ receptor-binding affinity have been shown to exhibit neuroprotective actions against NMDA cytotoxicity on midbrain dopaminergic neurons. However, because these ligands also have binding affinities for NMDA receptors (Williams, 1993; Ilyin et al., 1996; Whittemore et al., 1997), their neuroprotective effects may be explained by direct inhibition of NMDA receptor activity and may not involve σ receptors. Therefore, it is difficult to draw definitive conclusions regarding whether σ receptors mediate the neuroprotective actions of σ receptor ligands. To circumvent this problem, we used yet another ligand, (−)-PPAP (Glennon et al., 1990). In the rat, (−)-PPAP has been reported to bind [^{3}H]haloperidol-labeled σ sites with high affinity (IC_{50} = 24 nM) but does not bind [^{3}H]*N*-[1-(2-thienyl)cyclohexy]piperidine (TCP)-labeled PCP sites of NMDA receptors (IC_{50} > 75 μM).

Various concentrations of (−)-PPAP were applied to midbrain slice cultures concomitantly with 100 μM NMDA. As shown in Figs. 2B and 3D, (−)-PPAP showed a concentration-dependent neuroprotective effect. Significant protection was observed with 30 and 100 μM (−)-PPAP, although these concentrations of this drug did not completely inhibit the cytotoxic effect of NMDA. Applica-

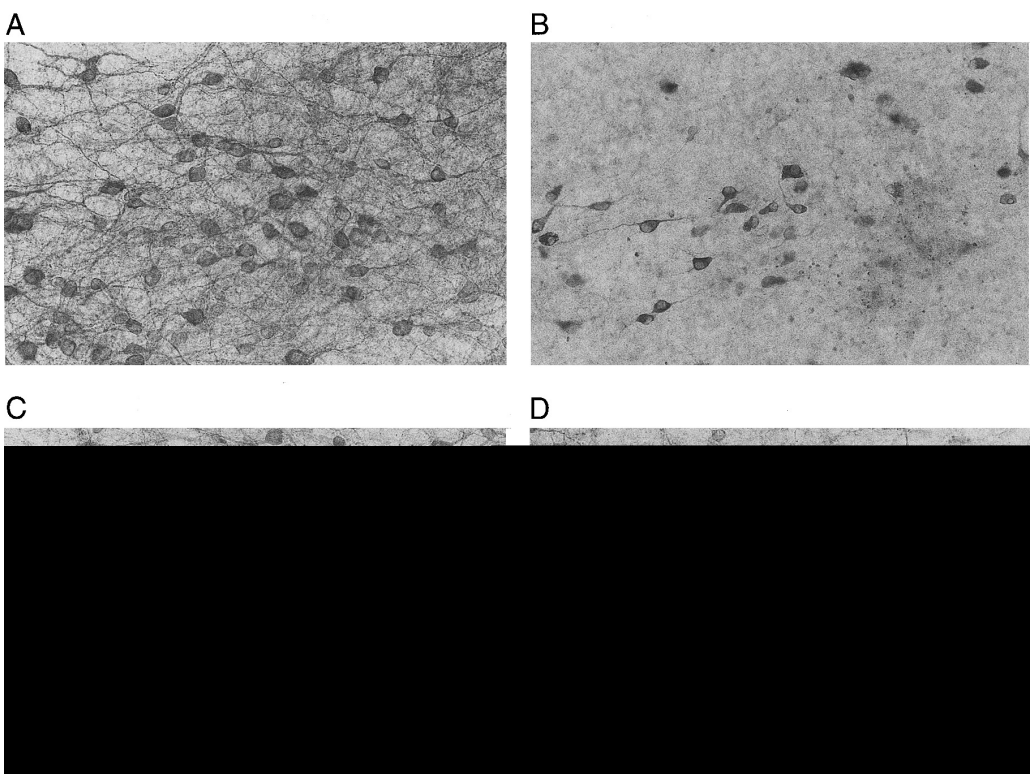

Fig. 3. Photographs showing the effects of (+)-SKF 10047 and (−)-PPAP on NMDA-induced neurotoxicity. All mesencephalic slice cultures were immunostained with anti-tyrosine hydroxylase antibody. Representative examples of tyrosine hydroxylase-positive cells in the slice cultures showing cytotoxic effects of 24-h exposure to 100 μM NMDA, and its reversal by test substances. (A) Sham-treated culture. (B) Culture treated with 100 μM NMDA alone. (C) Culture treated with 100 μM NMDA and 10 μM (+)-SKF 10047. (D) Culture treated with 100 μM NMDA and 30 μM (−)-PPAP. (+)-SKF 10047 or (−)-PPAP was applied concomitantly with NMDA. Scale bar indicates 100 μm (A–D).

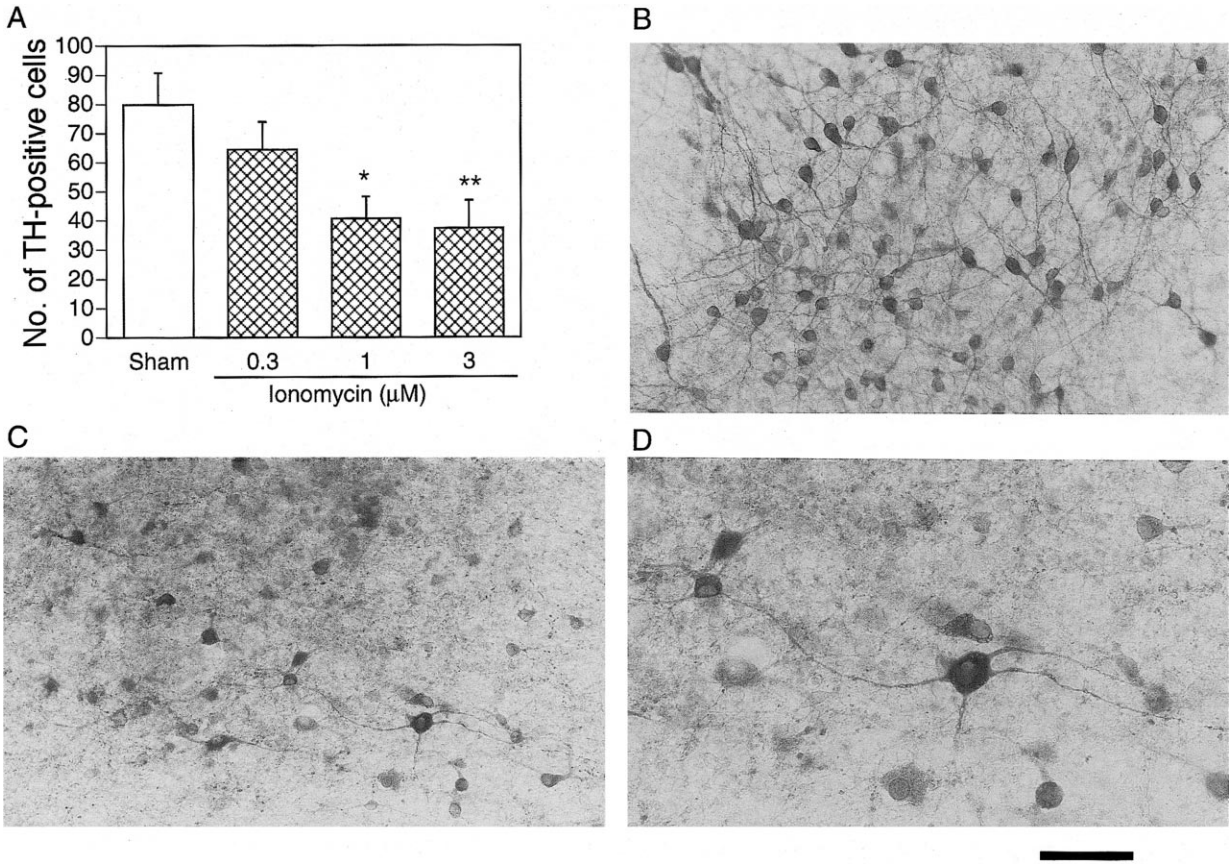

Fig. 4. Effects of ionomycin-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures. (A) Ionomycin induced dopaminergic neuronal death in a dose-dependent manner. The ordinate shows the number of tyrosine hydroxylase (TH)-positive cells. Ionomycin was added for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with sham control. ($n = 12$ per group). (B) Photograph of sham control, and (C,D) ionomycin-treated (3 μM) slices. Scale bar indicates 100 μm (B,C) or 50 μm (D).

tion of (−)-PPAP at 3, 10, 30 and 100 μM resulted in 4%, 46%, 65% and 56% neuroprotection, respectively. These results strongly suggest that σ receptors are indeed involved in these neuroprotective actions.

Table 1
Effects of MK-801, (+)-SKF 10047 and (−)-PPAP on 1 or 3 μM ionomycin-induced dopaminergic neuronal death in mesencephalic organotypic slice cultures
ns; Not significant vs. ionomycin alone.

Treatment	*N*	No. of tyrosine hydroxylase-positive cells (mean ± SEM)
Sham	12	69.7 ± 9.1
1 μM Ionomycin	12	40.4 ± 7.3[a]
1 μM Ionomycin + 10 μM MK-801	11	41.8 ± 5.4 ns
3 μM Ionomycin	12	33.2 ± 6.8[a]
3 μM Ionomycin + 10 μM MK-801	11	16.4 ± 3.8 ns
Sham	12	71.2 ± 8.7
1 μM Ionomycin	12	39.5 ± 7.4[a]
1 μM Ionomycin + 10 μM (+)-SKF 10047	12	47.1 ± 5.5 ns
3 μM Ionomycin	12	35.5 ± 2.1[a]
3 μM Ionomycin + 10 μM (+)-SKF 10047	12	38.3 ± 5.9 ns
Sham	11	76.7 ± 9.1
1 μM Ionomycin	12	50.1 ± 8.2[a]
1 μM Ionomycin + 100 μM (−)-PPAP	6	37.2 ± 6.9 ns
3 μM Ionomycin	8	43.0 ± 6.7[a]
3 μM Ionomycin + 100 μM (−)-PPAP	6	31.4 ± 6.8 ns

[a] $P < 0.05$ vs. sham control.

Ca^{2+} influx is thought to be a primary trigger that induces NMDA receptor-mediated excitotoxic neuronal cell death. Accordingly, we examined whether σ receptor ligands are also effective in attenuating neurotoxic actions of the calcium ionophore ionomycin. If σ ligands afford neuroprotection against ionomycin, their targets for exerting these neuroprotective effects are considered to be located downstream of Ca^{2+} influx.

Ionomycin applied for 24 h induced dopaminergic neuronal death in a concentration-dependent manner (Fig. 4A). Neurites of surviving dopaminergic neurons after ionomycin insult appeared swollen, and contained intracellular granules (Fig. 4C and D). MK-801 (10 μM) failed to block the neurotoxicity of 1–3 μM ionomycin, suggesting that ionomycin-induced cell death does not involve NMDA receptor activation (Table 1). (+)-SKF 10047 and (−)-PPAP, at concentrations that were effective in preventing NMDA neurotoxicity, were applied concomitantly with ionomycin. Neither compound protected dopaminergic neurons from neurotoxicity induced by 1–3 μM ionomycin (Table 1).

4. Discussion

The present study demonstrated that four compounds with characteristics of σ receptor ligands can prevent the excitotoxic effects of NMDA on dopaminergic neurons in midbrain slice cultures. In contrast, the σ receptor ligands failed to prevent neuronal death induced by ionomycin, an agent that increases the intracellular Ca^{2+} level by acting as a Ca^{2+} ionophore. Based on the assumption that NMDA excitotoxicity is triggered by Ca^{2+} influx from NMDA receptor channels, these results suggest that σ receptor ligands exert their neuroprotective actions by regulating the activity of NMDA receptors.

A frequently encountered issue in studies using σ receptor ligands is the interaction of these ligands with receptors other than σ. Particularly, a major problem is the direct interaction with NMDA receptors. For instance, a σ_1 receptor ligand (+)-SKF 10047 shows affinity for the PCP site of NMDA receptors (Whittemore et al., 1997). Ifenprodil acts as a subtype-selective NMDA receptor antagonist that exhibits about 400-fold higher affinity for receptors comprised of NR1/2B than for those comprised of NR1/2A hetero-oligomers (Williams, 1993). Haloperidol also shows properties of an NMDA receptor antagonist with selectivity for NR1/2B receptors (Ilyin et al., 1996). Therefore, we cannot exclude the possibility that direct blockade of NMDA receptors is involved in the neuroprotective effects of these ligands. However, (−)-PPAP, a selective σ receptor ligand with negligible affinity for NMDA receptors (Glennon et al., 1990), showed neuroprotective effects in the present study, suggesting that σ receptor ligands can provide neuroprotection without direct interactions with NMDA receptors. Consistent with this view, recent studies have demonstrated that specific σ_1 receptor ligands exert neuroprotective effects on primary neuronal cultures (Nakazawa et al., 1998; Senda et al., 1998).

Although the mechanisms by which σ receptors exert protective actions against NMDA cytotoxicity are not clear, indirect inhibition of NMDA receptor activity could be involved because σ ligands failed to show protective effects against ionomycin-induced injury. Indirect mechanisms of inhibition of NMDA receptors by σ ligands were proposed previously. Yamamoto et al. (1995) demonstrated a close correlation between the affinity of σ receptor ligands for σ_1 receptors and their potency for inhibition of TCP binding. Hayashi et al. (1995) reported that inhibition of NMDA-induced Ca^{2+} influx in cultured cortical neurons by σ receptor ligands is independent of the PCP site but is correlated well with the affinity of these ligands for σ receptors. These results suggested that inhibition of NMDA receptors is due to indirect modulation mediated by σ receptors, although several studies have provided evidence to the contrary (Fletcher et al., 1995; Whittmore et al., 1997). In this context, stimulation of σ_1-like receptors reduces Ca^{2+} uptake by nicotinic receptor stimulation in PC12 cells (Sagi et al., 1996) and reduces KCl-induced Ca^{2+} uptake in cortical neurons (Klette et al., 1995), indicating that σ ligands have the potential to modulate the activities of various types of ion channels.

σ_1 Receptors may modulate NMDA receptor function via second messenger systems. Several reports have indicated that the σ_1 receptor is coupled to pertussis toxin-sensitive GTP-binding proteins (Beart et al., 1989; Itzhak, 1989; Monnet et al., 1994). Morin-Surun et al. (1999) recently demonstrated that, in guinea pig brainstem neurons, the activation of σ_1 receptors results in their translocation from the cytosol to the vicinity of the cell membrane and induces a robust and rapid decrease in neuronal activity, which is mediated by phospholipase C. However, σ_1 sites have been reported to negatively regulate phosphatidylinositol turnover stimulated by muscarinic acetylcholine receptor activation (Bowen et al., 1988). As NMDA receptor activity is regulated by protein kinase C (Courtney and Nicholls, 1992; Logan et al., 1999), interactions with the phosphatidylinositol–phospholipase C pathway may play an important role in σ_1 receptor-mediated regulation of NMDA receptor activity.

In conclusion, we have demonstrated that several compounds with σ receptor affinity provide neuroprotection against NMDA cytotoxicity on midbrain dopaminergic neurons. As excitotoxicity may participate in the pathogenesis of Parkinson's disease (Blandini et al., 1996), σ receptor ligands are possible candidates for neuroprotective therapy for Parkinson's disease.

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