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Involvement of direct inhibition of NMDA receptors in the effects of σ-receptor ligands on glutamate neurotoxicity in vitro

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Abstract

This study was performed to examine the roles of the *N*-methyl-D-aspartate (NMDA) receptor/phencyclidine (PCP) channel complex in the protective effects of σ-receptor ligands against glutamate neurotoxicity in cultured cortical neurons derived from fetal rats. A 1-h exposure of cultures to glutamate caused a marked loss of viability, as determined by Trypan blue exclusion. This acute neurotoxicity of glutamate was prevented by NMDA receptor antagonists. Expression of σ_1 receptor mRNA in cortical cultures was confirmed by reverse transcription polymerase chain reaction (RT-PCR). σ Receptor ligands with affinity for NMDA receptor channels including the PCP site, such as (+)-*N*-allylnormetazocine ((+)-SKF10,047), haloperidol, and *R*(−)-*N*-(3-phenyl-1-propyl)-1-phenyl-2-aminopropane ((−)-PPAP), prevented glutamate neurotoxicity in a concentration-dependent manner. In contrast, other σ-receptor ligands without affinity for NMDA receptors, such as carbetapentane and *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine ((+)-3-PPP), did not show neuroprotective effects. Putative endogenous σ receptor ligands such as pregnenolone, progesterone, and dehydroepiandrosterone did not affect glutamate neurotoxicity. The protective effects of (+)-SKF10,047, haloperidol, and (−)-PPAP were not affected by the σ_1 receptor antagonist rimcazole. These results suggested that a direct interaction with NMDA receptors but not with σ receptors plays a crucial role in the neuroprotective effects of σ receptor ligands with affinity for NMDA receptors.

Keywords: Glutamate; Neurotoxicity; σ Receptor; Phencyclidine site; Cortical culture

1. Introduction

Glutamate is an excitatory neurotransmitter in the vertebrate central nervous system. Glutamate excitotoxicity has also been postulated to play an important role in various neurodegenerative diseases including hypoxic–ischemic brain injury (Choi et al., 1987; Bresnick, 1989). In primary cultures of rat cortical neurons, glutamate induces Ca^{2+} influx into the cytoplasm via *N*-methyl-D-aspartate (NMDA) receptors and triggers the formation of reactive oxygen species such as nitric oxide and superoxide, resulting in glutamate-related neuronal death (Dawson et al., 1991, 1993; Akaike et al., 1994a).

σ Receptors are defined as non-opiate and non-phencyclidine (PCP) binding sites that mediate the psychotomimetic actions of certain opioid derivatives (Martin et al., 1976). These receptors are expressed in the brain as well as in several tissues of the endocrine and immune systems (Su et al., 1988; Itzhak and Stein, 1990; Walker et al., 1990; Ferris et al., 1991). The σ receptor can be classified into at least two subtypes termed σ_1 and σ_2 (Walker et al., 1990). Recently, cDNAs of σ_1 receptors were cloned, and subsequently their amino acid sequences were deduced (Hanner et al., 1996; Pan et al., 1998; Prasad et al., 1998; Seth et al., 1998).

σ-receptor ligands have been shown to exhibit a wide variety of actions in the central nervous system and to prevent the neuronal death associated with glutamate cytotoxicity both in vitro (Lysko and Feuerstein, 1990; Lysko et al., 1992; DeCoster et al., 1995; Lockhart et al., 1995; Senda et al., 1998) and in vivo (Contreras et al., 1992; Lysko et al., 1992; O'Neill et al., 1995; Takahashi et al.,

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1995, 1996). Although there is increasing evidence suggesting that the neuroprotective effects of σ-receptor ligands are mediated via σ_1 receptors (DeCoster et al., 1995; Nakazawa et al., 1998; Senda et al., 1998), the mechanism of the neuroprotective effects of these ligands remains to be clarified. Many σ-receptor ligands, including the typical ligand (+)-*N*-allylnormetazocine ((+)-SKF10,047), have affinity for not only σ receptors but also for the PCP site in NMDA receptor channels (Largent et al., 1986). Some groups have suggested that σ receptors play an important role in these neuroprotective effects of σ-receptor ligands, because σ-receptor ligands with negligible affinity for the NMDA receptor channel complex (including PCP site) show neuroprotective effects in ischemia models (Contreras et al., 1992; O'Neill et al., 1995; Takahashi et al., 1995, 1996). In contrast to those findings, other groups have suggested that the neuroprotective effects of σ receptor ligands are related to the NMDA/PCP receptor channel complex, because their potencies are correlated with their affinity for the NMDA/PCP receptor channel complex (Goldberg et al., 1988; Lysko and Feuerstein, 1990; Lysko et al., 1992; Lockhart et al., 1995). Thus, it is still not clear whether inhibition of NMDA receptors is involved in the neuroprotective action of σ-receptor ligands.

In this study, we investigated the effects of several σ-receptor ligands on glutamate neurotoxicity using primary cultures of rat cortical neurons to clarify the involvement of σ receptors in the σ-receptor ligand-induced neuroprotective effect. We used the two types of σ-receptor ligands: those which bind to both σ receptors and the PCP site in NMDA receptor channels, such as (+)-SKF10,047, and those which bind to σ receptors but not NMDA receptor channels, such as carbetapentane. We used an acute glutamate neurotoxicity model in vitro to determine the direct effects of these drugs on NMDA receptor-mediated glutamate neurotoxicity. Our results indicated that only σ-receptor ligands that bind to both the σ receptor and NMDA receptor channel complex prevent acute glutamate neurotoxicity, suggesting that inhibition of the NMDA receptor channel complex is essential for the neuroprotective effects of σ-receptor ligands with affinity for NMDA receptors, such as (+)-SKF10,047.

2. Materials and methods

2.1. Materials

Eagle's minimal essential medium (Eagle's MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Drugs were obtained from the following sources: D-2-amino-5-phosphonovaleric acid (APV; Sigma, St. Louis, MO, USA), carbetapentane citrate (Research Biochemicals International, Natick, MA, USA), dehydroepiandrosterone (Sigma), dehydroepiandrosterone sulfate (Sigma), 6,7-dichloro-2,3-quinoxalinedione (DCQX; RBI), *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy) phenyl]ethylamine (NE-100; a generous gift from Santen Pharmaceutical), dizocilpine maleate (MK-801; RBI), L-glutamic acid monosodium salt (Nacalai Tesque, Kyoto, Japan), haloperidol (Wako, Osaka, Japan), *R*(−)-*N*-(3-phenyl-1-propyl)-1-phenyl-2-aminopropane ((−)-PPAP; RBI), *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine ((+)-3-PPP; RBI), pregnenolone (Sigma), progesterone (Sigma), rimcazole dihydrochloride (RBI) and (+)-SKF-10,047 (RBI).

2.2. 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., 1997a, 2000). Briefly, single cells dissociated from the whole cerebral cortex of fetal rats were plated on plastic coverslips placed in Falcon 60-mm dishes ($5.1–6.0 \times 10^6$ 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 (8–13 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.

2.3. Measurement of neurotoxicity

Neurotoxicity induced by glutamate was quantitatively assessed by examining cultures under Hoffman modulation microscopy according to the methods described in our previous reports (Kume et al., 1997a, 2000). All experiments were performed in Eagle's MEM at 37°C. Cell viability was assessed by Trypan blue exclusion. After drug treatment, cell cultures were immediately stained with 1.5% Trypan blue solution for 10 min at room temperature, fixed with isotonic formalin (pH 7.0, 2–4°C), and rinsed with physiological saline. Cells stained with Trypan blue were regarded as non-viable. The viability of the cultures was calculated as the percentage of unstained cells (viable cells) relative to the total number of cells counted (viable cells plus non-viable cells). To determine cell viability, at least 200 cells were counted on each coverslip. In each experiment, the cells on five coverslips were counted to obtain the means ± S.E.M. of cell viability.

2.4. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultures using ISOGEN as recommended by the manufacturer (Nippon Gene, Tokyo, Japan). Final RNA concentrations were determined

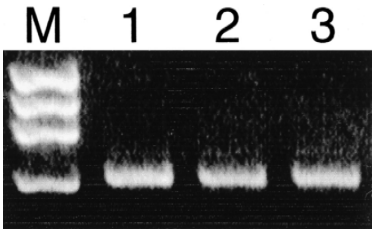

Fig. 1. Expression of σ_1 receptor mRNA in cultured rat cortical neurons. RT-PCR was performed as described in Materials and methods. M: marker, lane 1: cultured rat cortical neurons, lane 2: adult rat cerebral cortex, lane 3: adult rat whole brain.

by measuring optical density at 260 nm. Aliquots of 2 μg of total RNA were reverse transcribed in a total volume of 50 μl using random hexamers and superscript reverse transcriptase (Life Technologies, Rockville, MD, USA) for 2 h at 37°C. Aliquots of 1 μl of RT product were subjected to PCR to amplify rat σ_1 receptor (forward primer, 5′-TCTGACTATTGTGGCGGTGC-3′; reverse primer, 5′-CAAAGAGGTAGGTGGTGAGC-3′) in a thermocycler. The amplification protocol involved denaturation at 94°C for 1 min, primer annealing at 68°C for 1 min and extension at 72°C for 1 min. This cycle was repeated 35 times. PCR products were subjected to 3% agarose gel electrophoresis and visualized with 0.1% ethidium bromide.

2.5. Statistics

Data are expressed as means ± S.E.M. The statistical significance of differences was determined by Dunnett's two-tailed test. Statistical significance was defined as a probability value of less than 5%.

3. Results

3.1. NMDA receptor-mediated glutamate neurotoxicity

Exposure of the cortical cultures to 500 μM of glutamate for 1 h significantly reduced cell viability. We examined the effects of three types of NMDA receptor antagonists on acute glutamate neurotoxicity; MK-801 as a

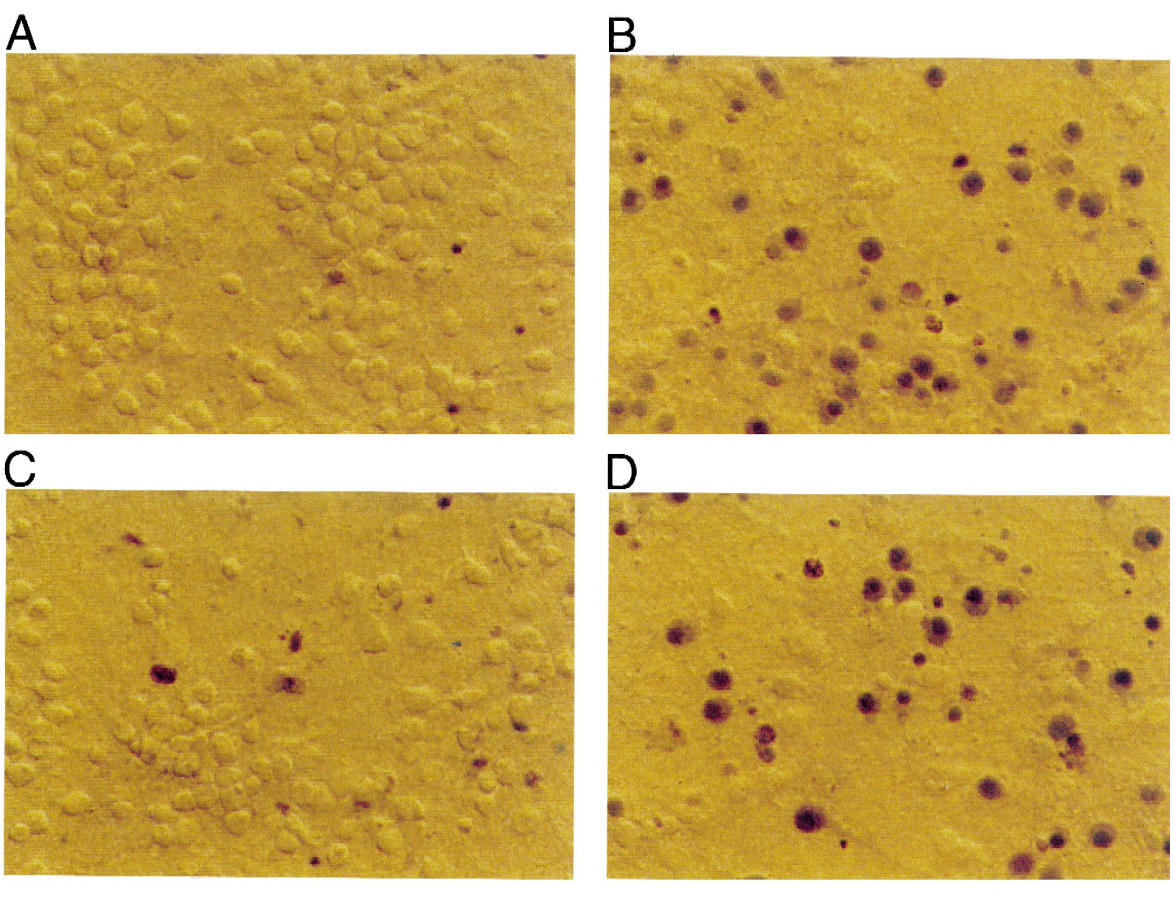

Fig. 2. Hoffman modulation photomicrographs showing effects of (+)-SKF10,047 and carbetapentane on glutamate neurotoxicity. Culture fields were photographed after Trypan blue staining followed by formalin fixation. A–D show control, glutamate-treated, glutamate plus (+)-SKF10,047 (10 μM) and glutamate plus carbetapentane (10 μM), respectively. In C and D, σ receptor ligands were added to glutamate-containing medium for 1 h. Calibration bar = 50 μm.

non-competitive antagonist which binds to the PCP site in the NMDA receptor channel complex, APV as a competitive antagonist, and DCQX as an antagonist of the strychnine-insensitive glycine recognition site (GlyB site). Consistent with the findings of our previous studies (Akaike et al., 1994a,b; Tamura et al., 1994; Kume et al., 1997b), the concomitant application of either MK-801 (1 μM), APV (1 mM) or DCQX (1–10 μM) with glutamate significantly inhibited glutamate neurotoxicity (data not shown). These findings indicate that acute glutamate neurotoxicity is mediated by NMDA receptors.

3.2. Expression of σ_1-receptor mRNA in cultured cortical neurons

Recent studies indicated that there were at least two distinct σ-receptor subtypes termed σ_1 and σ_2 (Walker et al., 1990). However, only cDNA of the σ_1 receptor has been cloned, and the amino acid sequence of the σ_2 receptor has not yet been determined (Hanner et al., 1996; Pan et al., 1998; Prasad et al., 1998; Seth et al., 1998). Therefore, we examined the expression of σ_1-receptor mRNA in cultured cortical neurons using RT-PCR to examine the expression of σ_1 receptors in cortical cultures maintained in our laboratory. σ_1-receptor mRNA was expressed in cultured cortical neurons (Fig. 1). The whole brain and cerebral cortex of adult rats, used as positive controls, also showed σ_1-receptor mRNA expression. In negative controls in which RT was performed without reverse transcriptase, neither band was observed (data not shown).

3.3. Effects of σ_1-receptor ligands on glutamate neurotoxicity

Fig. 2 shows typical photographs of the effects of σ-receptor ligands on glutamate neurotoxicity. Most of the cells in sham-treated cultures were viable (Fig. 2A). Expo-

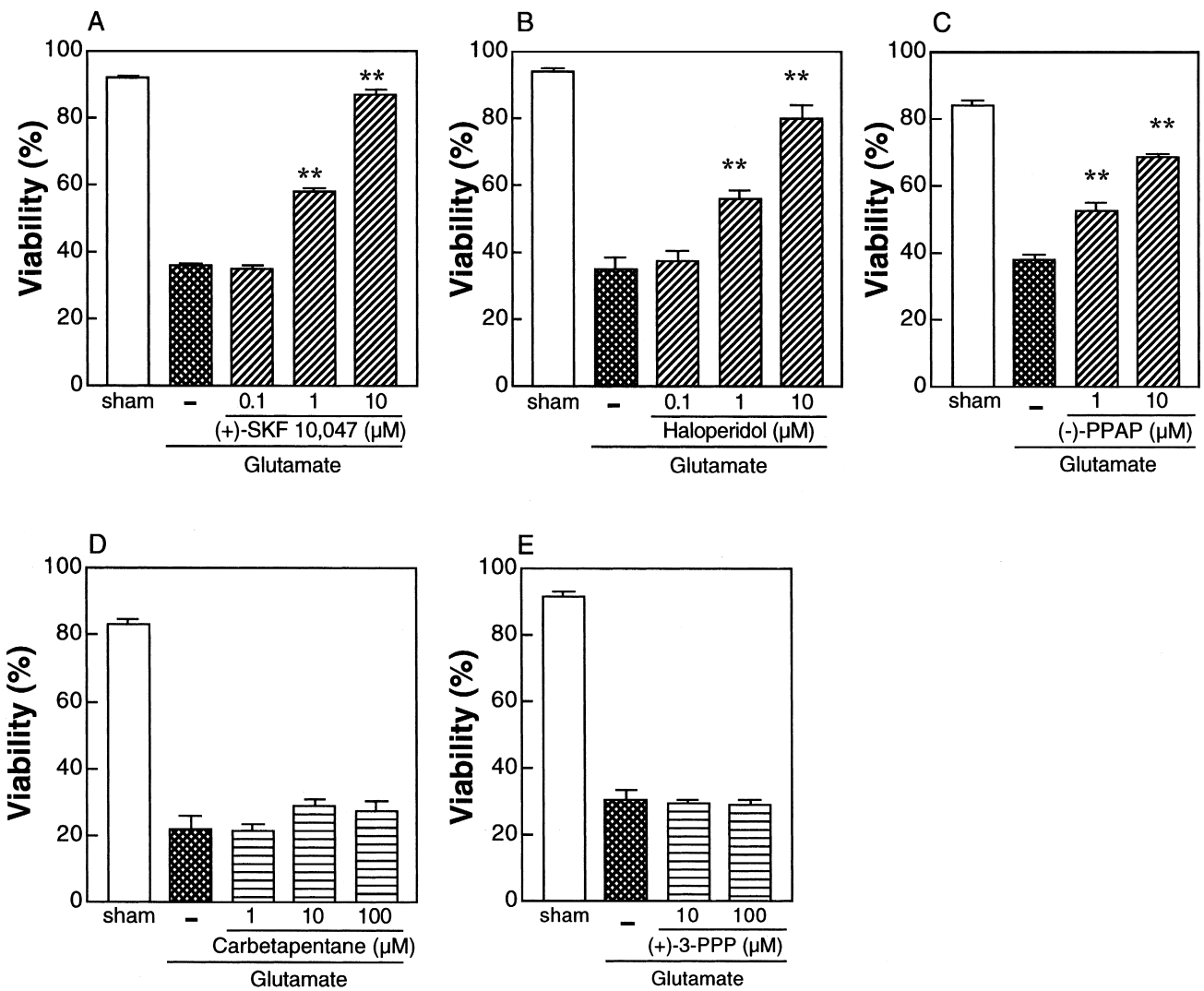

Fig. 3. Effects of σ receptor ligands on glutamate neurotoxicity in cultured rat cortical neurons. Cultures were exposed to glutamate (500 μM) for 1 h. (+)-SKF10,047 (A), haloperidol (B), (−)-PPAP (C), carbetapentane (D) and (+)-3-PPP (E) were added to glutamate-containing medium. $^{**}P < 0.01$, compared with glutamate alone.

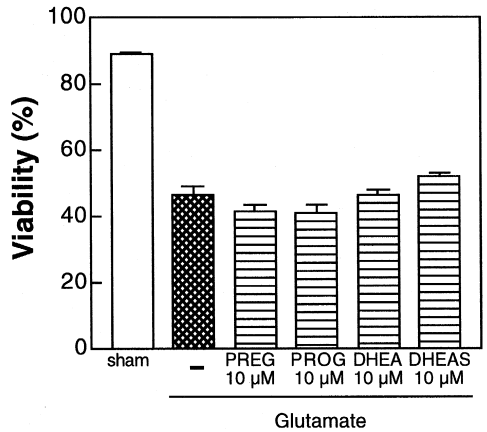

Fig. 4. Effects of steroid hormones on glutamate neurotoxicity in cultured rat cortical neurons. Cultures were exposed to glutamate (500 μM) for 1 h. Steroids were added to glutamate-containing medium. These steroids showed no significant protective effects. PREG: pregnenolone, PROG: progesterone, DHEA: dehydroepiandrosterone, DHEAS: dehydroepiandrosterone sulfate.

sure of cultures to glutamate (500 μM) for 1 h markedly reduced cell viability, as indicated by the increase in number of cells stained by Trypan blue (Fig. 2B). The concomitant application of (+)-SKF10,047 (10 μM), a prototypical σ-receptor ligand which binds to both σ_1 receptors and the PCP site in NMDA receptor channels, with glutamate reduced the number of cells stained by Trypan blue (Fig. 2C), indicating that glutamate neurotoxicity was prevented by the drug. (+)-SKF10,047 (1–10 μM) prevented glutamate neurotoxicity in a concentration-dependent manner (Fig. 3A). Moreover, other σ ligands that possessed affinity for NMDA receptors but which were structurally distinct from (+)-SKF10,047, such as haloperidol (1–10 μM) and (−)-PPAP (1–10 μM), also prevented glutamate neurotoxicity (Fig. 3B–C). Carbetapentane and (+)-3-PPP, σ_1 and σ_1/σ_2-receptor ligands, respectively, had no affinity for the NMDA receptor including the PCP site. Carbetapentane (1–100 μM) and (+)-3-PPP (10–100 μM) did not show neuroprotective effects in contrast to σ-receptor ligands with affinity for NMDA receptors (Fig. 2D, Fig. 3D–E). It has been reported that several steroid hormones, such as pregnenolone, progesterone, dehydroepiandrosterone and dehydroepiandrosterone sulfate, regulate σ receptors and may

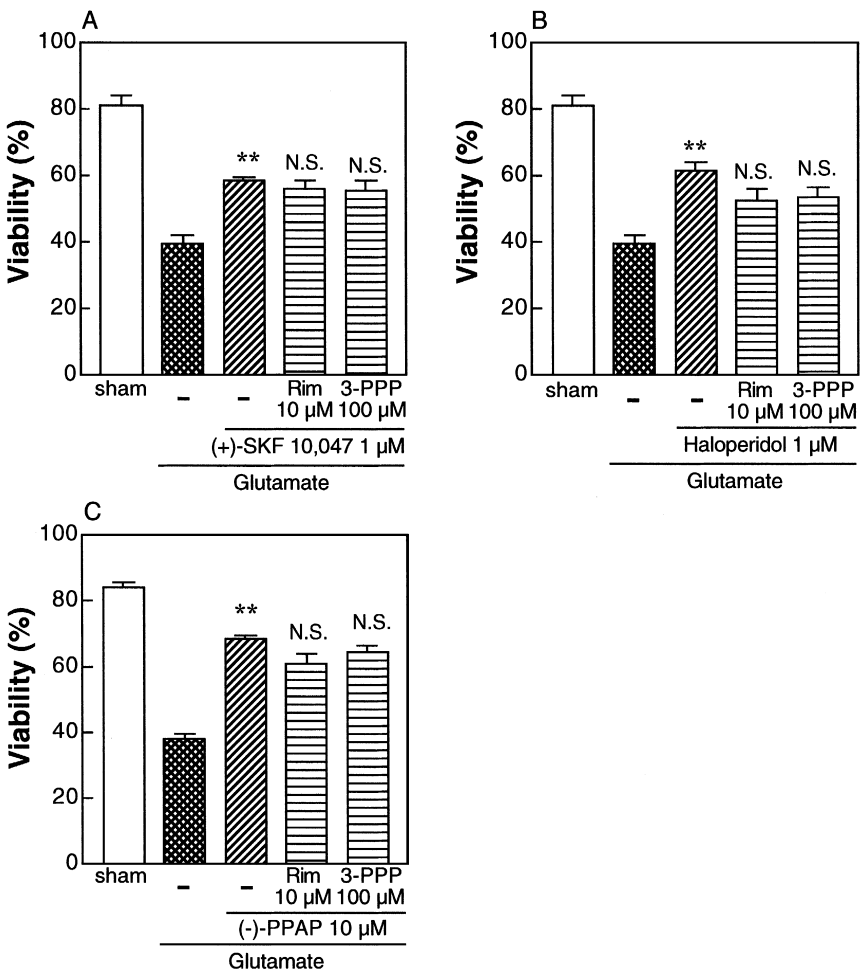

Fig. 5. Effects of σ receptor antagonists on neuroprotection by (+)-SKF10,047, haloperidol, and (−)-PPAP. Cortical cultures were exposed to glutamate (500 μM) for 1 h. All the drugs were added to glutamate-containing medium for 1 h. $^{**}P < 0.01$, compared with glutamate alone. N.S.: not significant, compared with glutamate plus either (+)-SKF10,047, haloperidol or (−)-PPAP. RIM: rimcazole.

act as endogenous σ-receptor ligands (Su et al., 1988; Monnet et al., 1995; Bergeron et al., 1996; Maurice et al., 1998). However, these steroid hormones (10 μM) showed no effect on glutamate neurotoxicity (Fig. 4).

3.4. Effects of σ_1-receptor antagonists on neuroprotective effects of (+)-SKF10,047, haloperidol, and (−)-PPAP

Since only σ-receptor ligands with affinity for NMDA receptors prevented acute glutamate neurotoxicity, we hypothesized that these neuroprotective effects were not mediated by the interaction with σ_1 receptors, but rather by direct interaction with NMDA receptor channel complexes. Therefore, we examined the effects of σ-receptor antagonists on the neuroprotective effects of (+)-SKF10,047, haloperidol, and (−)-PPAP to clarify whether the neuroprotective effects of these ligands on glutamate neurotoxicity were mediated by the activation of σ receptors. Certain σ-receptor ligands are regarded as σ-receptor antagonists, although the distinction between agonist and antagonist for σ-receptor ligands is not absolute. In this study, we used rimcazole, NE-100, and (+)-3-PPP as σ-receptor antagonists according to previous studies.

Rimcazole is considered to be a σ_1-receptor antagonist (Steardo et al., 1995, 1996). In this study, rimcazole (1–10 μM) did not prevent glutamate neurotoxicity and failed to attenuate the protective effects of (+)-SKF10,047 (1 μM), haloperidol (1 μM), and (−)-PPAP (10 μM) (Fig. 5). Similarly, NE-100 (10 μM), which is also a σ_1-receptor antagonist, but structurally distinct from rimcazole (Senda et al., 1996; Tokuyama et al., 1997), did not attenuate the neuroprotective effect of 1 μM of (+)-SKF10,047 (data not shown). Rimcazole at 10 μM might be insufficient to prevent the binding of (+)-SKF10,047 (1 μM), haloperidol (1 μM), and (−)-PPAP (10 μM) to σ_1 receptors. However, we could not use higher concentrations of rimcazole because of the neurotoxicity observed at higher concentrations. Therefore, we decided to use (+)-3-PPP as an antagonist although this drug reportedly binds to both σ_1 and σ_2 receptors. (+)-3-PPP did not show protective effects against glutamate neurotoxicity. (+)-3-PPP is widely used to displace the binding of (+)-SKF10,047 or other σ-receptor ligands from σ receptors (Largent et al., 1986; Matsuno et al., 1996). An amount of (+)-3-PPP (100 μM) sufficient to displace (+)-SKF10,047 (1 μM) from σ receptors did not attenuate the neuroprotective effect of (+)-SKF10,047 (Fig. 5A). (+)-3-PPP also had no effect on the actions of haloperidol and (−)-PPAP. These findings suggest that the neuroprotective effects of (+)-SKF10,047, haloperidol, and (−)-PPAP were not mediated by the activation of σ receptors.

4. Discussion

We examined the effects of σ-receptor ligands on glutamate neurotoxicity using primary cultures of rat cortical neurons. Consistent with our previous studies (Akaike et al., 1994a,b; Tamura et al., 1994; Kume et al., 1997b), exposure of cortical cultures to glutamate for 1 h significantly reduced cell viability. The acute glutamate neurotoxicity was prevented by the NMDA receptor antagonists MK-801, APV, and DCQX. These results indicate that NMDA receptors play a dominant role in the acute glutamate neurotoxicity observed in cortical cultures.

In this study, we used several synthetic σ-receptor ligands and steroid hormones regarded as intrinsic σ-receptor ligands (Su et al., 1988; Monnet et al., 1995; Bergeron et al., 1996; Maurice et al., 1998). (+)-SKF10,047 prevented neuronal death both in vivo and in vitro models (Lysko and Feuerstein, 1990; Lysko et al., 1992; DeCoster et al., 1995; Lockhart et al., 1995). However, (+)-SKF10,047 is also known to bind to σ_1 receptors and the NMDA/PCP receptor channel complex (Largent et al., 1986). The present results showed that (+)-SKF10,047 protected cortical cultures against glutamate neurotoxicity. The neuroprotective effect of (+)-SKF10,047 is probably not mediated by σ_1 receptors because the protective effect of this drug was not attenuated by the σ_1-receptor antagonists rimcazole and NE-100. σ_1-receptor mRNA was expressed in our cortical cultures. Thus, the effect of (+)-SKF10,047 should be antagonized by σ_1-receptor antagonists if its effect is mediated by σ_1 receptors. Similarly, haloperidol, a σ_1 and σ_2-receptor ligand, and (−)-PPAP, a σ_1-receptor ligand, also protected cortical cultures against glutamate neurotoxicity, and the neuroprotective effects of these drugs were not attenuated by σ-receptor antagonists. Previous studies showed that haloperidol inhibited the NMDA-activated membrane current response in *Xenopus* oocytes expressing the NR1a/NR2B subunit of NMDA receptor (Whittemore et al., 1997), that haloperidol interacted with the GlyB site at the NMDA receptor (Fletcher and McDonald, 1993) and that (−)-PPAP weakly interacts with the NMDA/PCP receptor channel complex (Glennon et al., 1990). Therefore, we concluded that haloperidol and (−)-PPAP may have protective effects against glutamate neurotoxicity by interacting with NMDA receptors.

It has been reported that carbetapentane, a σ_1-receptor ligand, and (+)-3-PPP, a σ_1 and σ_2-receptor ligand, do not possess affinity for NMDA receptors (Largent et al., 1986). These σ-receptor ligands did not show neuroprotective effects in the present study. Moreover, steroid hormones, such as pregnenolone, progesterone, dehydroepiandrosterone and dehydroepiandrosterone sulfate, had no effect on glutamate neurotoxicity. These findings suggested that several σ-receptor ligands such as (+)-SKF10,047, haloperidol, and (−)-PPAP protect cortical cultures against glutamate neurotoxicity by antagonizing NMDA receptor channels including the PCP site. In contrast, carbetapentane and (+)-3-PPP, σ-receptor ligands without affinity for NMDA receptors, did not confer protection against acute glutamate neurotoxicity.

Rimcazole at 10 μM did not attenuate the neuroprotective effects of (+)-SKF10,047, haloperidol or (−)-PPAP. Although this concentration of rimcazole might be insufficient to prevent the binding of (+)-SKF10,047, haloperidol, and (−)-PPAP to σ_1 receptors, we could not use this agent at higher concentrations because of its neurotoxicity (data not shown). Therefore, we also used (+)-3-PPP as a σ receptor antagonist. (+)-3-PPP did not show neuroprotective effects against glutamate neurotoxicity. It has been reported that 100 μM (+)-3-PPP is sufficient to prevent the binding of 1 μM (+)-SKF10,047 to σ_1 receptors (Largent et al., 1986). However, the neuroprotective effect of (+)-SKF10,047 was not attenuated by (+)-3-PPP, indicating that the neuroprotection conferred by (+)-SKF10,047 was not mediated by σ_1 receptors. We confirmed that σ_1-receptor mRNA was expressed in the cortical cultures maintained in our laboratory. Therefore, it is unlikely that the lack of neuroprotective action of σ receptor ligands without affinity for NMDA receptors was due to the lack of σ receptors.

In the present study, we added σ-receptor ligands to glutamate-containing medium. Exposure of cortical cultures for 1 h to a high concentration of glutamate induced acute and severe cell death with the characteristics of necrotic cell death. This severe glutamate neurotoxicity was prevented by NMDA receptor antagonists. In this study, only σ-receptor ligands that have been reported to interact with NMDA receptors prevented glutamate neurotoxicity. Nakazawa et al. (1998) reported that SA4503, a selective σ_1-receptor ligand, and (+)-pentazocine had no effect on NMDA-induced neurotoxicity but that these ligands showed neuroprotection via σ_1-receptors against hypoxia/hypoglycemia-induced toxicity. These observations indicated that the activation of σ_1 receptors suppressed excitatory amino acid release from presynaptic sites. Hence, neuroprotection by σ-receptor ligands against hypoxia/hypoglycemia-induced toxicity was not correlated with NMDA receptor antagonism, suggesting that other neuroprotective mechanisms may be involved.

In conclusion, the present study showed that only σ-receptor ligands with affinity for NMDA/PCP receptor channels, such as (+)-SKF10,047, prevented acute glutamate neurotoxicity in cultured rat cortical neurons. It is possible that the direct interaction of the drugs with NMDA/PCP receptor channels contributes to their protective action against glutamate neurotoxicity.

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