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Antagonism of NMDA receptors by σ receptor ligands attenuates chemical ischemia-induced neuronal death in vitro

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Abstract

We investigated the effects of σ receptor ligands on neuronal death induced by chemical ischemia using primary cultures of rat cerebral cortical neurons. The induction of chemical ischemia by sodium azide and 2-deoxy-D-glucose led to delayed neuronal death in a time- and concentration-dependent manner, as determined by trypan blue exclusion. The neurotoxicity was inhibited by *N*-methyl-D-aspartate (NMDA) receptor antagonists, indicating the involvement of glutamate. The σ receptor ligands (+)-*N*-allylnormetazocine ((+)-SKF10,047) and haloperidol, but not carbetapentane and *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine ((+)-3-PPP), prevented chemical ischemia-induced neurotoxicity in a concentration-dependent manner. The protective effects of (+)-SKF10,047 and haloperidol were not affected by the σ receptor antagonists. (+)-SKF10,047 and haloperidol, but not carbetapentane and (+)-3PPP, inhibited the glutamate-induced increase in intracellular Ca^{2+}, and the inhibitory effects were not attenuated by σ receptor antagonists. These results suggest that direct interaction with NMDA receptors but not σ receptors is crucial to the neuroprotective effects of σ receptor ligands with affinity for NMDA receptors.

Keywords: Chemical ischemia; Glutamate; Neurotoxicity; σ Receptor ligand; Cortical culture

1. Introduction

The physiological functions of the brain are dependent on supplies of oxygen and glucose from the blood. Since neurons are very vulnerable to hypoxia/hypoglycemia, they sustain lethal damage even if supplies of oxygen and glucose fail for just a few minutes. Glutamate excitotoxicity has been postulated to play an important role in ischemic brain injury (Choi et al., 1987; Bresnick, 1989; Kristian et al., 1998; Li et al., 2000; Manzerra et al., 2001; Yu et al., 2001). To mimic certain aspects of ischemic brain injury, a number of in vitro models have been proposed. In this study we used both sodium azide, an inhibitor of oxidative phosphorylation, and 2-deoxy-D-glucose, an inhibitor of glycolysis, to induce cortical cultures to undergo hypoxia and hypoglycemia (Imura et al., 1999). Since in vivo cerebral ischemia often consists of both reversible ischemia and blood flow reperfusion, we incubated cortical neurons in normal medium following the induction of chemical hypoxia/hypoglycemia to mimic the in vivo blood flow reperfusion period. Thus, we first determined whether or not this chemical ischemic model involves glutamate excitotoxicity-related delayed neuronal death as shown in previous reports (Choi et al., 1987; Dawson et al., 1991, 1993; Akaike et al., 1994; Kume et al., 2000).

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). σ Receptors can be classified into at least two subtypes termed σ_1 and σ_2 (Walker et al., 1990; Qurion et al., 1992). 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

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prevent the neuronal death associated with glutamate excitotoxicity 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., 1996). Recently, evidence is mounting 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). However, we have recently reported that a direct interaction with *N*-methyl-D-aspartate (NMDA) receptors but not with σ receptors is crucial to the neuroprotective effects of σ receptor ligands including the typical ligand (+)-*N*-allylnormetazocine ((+)-SKF10,047) on acute glutamate neurotoxicity (Nishikawa et al., 2000).

In this study, we investigated the effects of several σ receptor ligands on chemical ischemia-induced neurotoxicity using primary cultures of rat cortical neurons to clarify the involvement of σ receptors in the σ receptor ligand-induced neuroprotective effect. Only σ receptor ligands that bind to both the σ receptors and NMDA receptor-ion channel complex prevented chemical ischemia-induced neurotoxicity. These results suggest that inhibition of the NMDA receptor-ion channel complex, but not activation of σ receptors, is essential for the protective effects of σ receptor ligands on ischemic injury mediated by glutamate excitotoxicity.

2. Materials and methods

2.1. Materials

Drugs were obtained from the following sources: Eagle's minimal essential medium (Eagle's MEM; Nissui Pharmaceutical, Tokyo, Japan), D-2-amino-5-phosphonovaleric acid (APV; Sigma, St. Louis, MO), carbetapentane citrate (Research Biochemicals International (RBI), Natick, MA), 2-deoxy-D-glucose (Nacalai Tesque, Kyoto, Japan), *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy) phenyl]-ethylamine (NE-100; a gift from Santen Pharmaceutical, Japan), dizocilpine maleate (MK-801; RBI), Fura-2 acetoxymethyl ester (Wako, Osaka, Japan), L-glutamic acid monosodium salt (Nacalai Tesque), haloperidol (Wako), hexadecyltrimethylammonium bromide (Nacalai Tesque), *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine ((+)-3-PPP; RBI), rimcazole dihydrochloride (RBI), (+)-SKF-10,047 (RBI) and sodium azide (Nacalai Tesque).

2.2. Cell culture

Primary cultures were obtained from the cerebral cortex of fetal Wistar rats (17–19 days of gestation) according to procedures described previously (Kume et al., 1997; Nishikawa et al., 2000). Briefly, single cells dissociated from the whole cerebral cortex of fetal rats were plated on plastic coverslips placed in Falcon 60-mm dishes (5.1×10^6 cells per dish) or on 24-well plates (1.0×10^6 cells per well). 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, nonneuronal cells were removed by adding cytosine arabinoside (10 μM). Only mature cultures (10–13 days in vitro) were used for experiments. Determination by immunochemical labeling with anti-MAP2 antibody and anti-glial fibrillary acidic protein antibody revealed that cells cultured for 12 days consisted of 91.4% neurons and 8.6% astrocytes in 60-mm dishes and 88.5% neurons and 11.5% astrocytes in 24-well plates. The animals were treated in accordance with the guidelines of Kyoto University animal experimentation committee, and the guidelines of the Japanese Pharmacological Society.

2.3. Induction of chemical ischemia and drug application

Sodium azide, an inhibitor of oxidative phosphorylation, and 2-deoxy-D-glucose, an inhibitor of glycolysis, were used to induce chemical hypoxia and hypoglycemia, respectively. The chemical ischemia was induced to expose cortical neurons to both metabolic inhibitors according to procedures described previously (Imura et al., 1999). Cortical neurons cultured on plastic coverslips were moved into either chemical ischemic HEPES-buffered saline (CI-HBS: NaCl 120 mM, KCl 5.4 mM, $MgSO_4$ 0.62 mM, 1.8 $CaCl_2$ 1.8 mM, HEPES 10 mM, sodium azide 3 mM and 2-deoxy-D-glucose 10 mM) or normal HEPES-buffered saline (sham treatment, HBS: NaCl 120 mM, KCl 5.4 mM, $MgSO_4$ 0.62 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM and D-glucose 10 mM) for 15 min (or as indicated). Then, coverslips were placed into 10% heat-inactivated horse serum-containing Eagle's MEM and incubated for 24 h.

Drugs were delivered as follows: D-Glucose, NMDA receptor antagonists and σ receptor ligands were added to the incubation buffer or medium during both chemical ischemia and the post-incubation period. Nitric oxide synthase (NOS) inhibitors were added from 30 min prior to the induction of chemical ischemia to the end of the post-incubation period. The extracellular calcium ion was removed so as to incubate cortical cultures in Ca^{2+}-free 1 mM EGTA-containing CI-HBS and this was followed by post-incubation in normal medium containing Ca^{2+}.

2.4. Measurement of neurotoxicity

The neurotoxicity induced by chemical ischemia was quantitatively assessed by Hoffman modulation microscopy as described in our previous reports (Kume et al., 1997, 2000). All experiments were performed in Eagle's MEM at 37 °C. Cell viability was assessed by trypan blue exclusion. After post-incubation, cell cultures were immediately stained with a 1.5% trypan blue solution for 10 min at room

temperature, fixed with isotonic formalin (pH 7.0, 4 °C), and rinsed with physiological saline. Cells stained with trypan blue were regarded as nonviable. 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 nonviable 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 mean ± S.E.M. of cell viability.

2.5. Glutamate release analysis

Cortical neurons were incubated in CI-HBS for 0 or 15 min, and then the supernatant of the culture medium was collected for glutamate release analysis. After the supernatants were removed, we performed a protein assay by the Bradford method to calibrate glutamate contents among the samples. The analysis of glutamate content was performed by high-performance liquid chromatography (HPLC) with electric chemical detection (EICOM 300 series system; LC pump EP-300 and electric chemical detector ECD-300 (platinum electrode)) on a pre-separation column (EICOMPAK E-GEL, 4.6 × 150 mm) and glutamate oxidase-fixing enzyme column (EICOMPAK E-Enzympak, 4.0 × 5 mm). Each sample solution was separated isocratically on the pre-separation column E-GEL using a mobile phase consisting of phosphate buffer (15 mM, pH 7.4) and hexadecyltrimethylammonium bromide (250 mg/l). The sample (10 μl) was manually injected 1 min prior to the automatic injection of an external glutamate standard solution by an auto injector (EICOM EAS-20). The peak area of each sample was determined with the analysis system EICOM EPC-300 (Power Chrom) and compared with the peak area of the external standard to calculate the glutamate content.

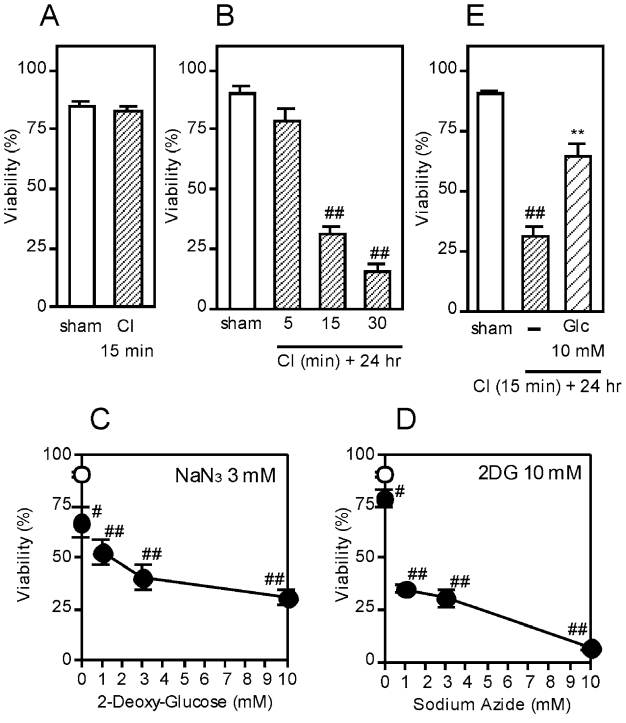

Fig. 1. Delayed neuronal death induced by chemical ischemia (CI) in rat cortical cultures. (A) Cortical cultures were incubated in CI HEPES-buffered saline (CI-HBS) which contained sodium azide (3 mM) and 2-deoxy-D-glucose (10 mM) for 15 min. (B) Cortical cultures were incubated in CI-HBS containing sodium azide (3 mM) and 2-deoxy-D-glucose (10 mM) for the period indicated, followed by post-incubation in normal medium for 24 h. (C and D) CI-induced neurotoxicity was dependent on the concentration of both sodium azide (C) and 2-deoxy-D-glucose (D). Cortical cultures were incubated in CI-HBS containing the indicated concentrations of sodium azide and 2-deoxy-D-glucose for 15 min, followed by post-incubation in normal medium for 24 h. Open circles, sham-operated cultures; closed circles, sodium azide and/or 2-deoxy-D-glucose-treated cultures. (E) Effects of D-glucose on chemical ischemia-induced neurotoxicity. $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with sham (one-way ANOVA followed by Dunnett's two-tailed test). $^{**}P<0.01$, compared with chemical ischemia (one-way ANOVA followed by Dunnett's two-tailed test).

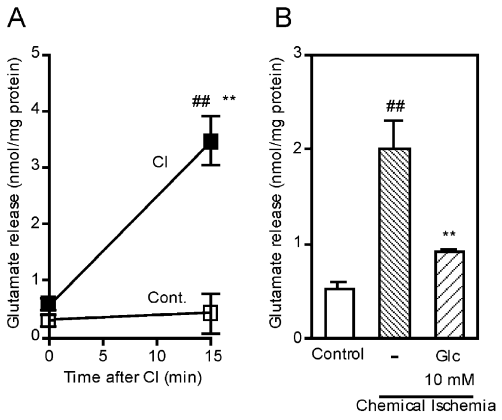

Fig. 2. Chemical ischemia (CI)-induced increase in extracellular glutamate content. (A) Glutamate levels in incubation buffer 0 or 15 min after induction of CI. $^{\#\#}P<0.01$, compared with CI at 0 min (Student *t*-test). $^{**}P<0.01$, compared with control (Cont.) at 15 min (Student *t*-test). (B) Effect of D-glucose on glutamate release triggered by chemical ischemia. D-Glucose (10 mM) was added to CI-HBS for 15 min. $^{\#\#}P<0.01$, compared with control (Student *t*-test). $^{**}P<0.01$, compared with chemical ischemia (Student *t*-test).

Table 1
Involvement of NO-mediated glutamate neurotoxicity in chemical ischemia (CI)-induced neuronal death

Cell viability (percent of sham)		
Drugs	CI	CI + drug
NMDA receptor antagonists		
MK-801 (1 μM)	36.8 ± 4.6	85.2 ± 2.6[b]
APV (1 mM)	47.0 ± 2.6	98.2 ± 1.7[b]
Ca^{2+} removal	36.8 ± 4.6	54.7 ± 5.0[b]
NO synthase inhibitors		
L-NAME (300 μM)	31.2 ± 2.8	39.4 ± 2.3[a]
L-NAME (1 mM)	31.2 ± 2.8	46.0 ± 1.6[b]

Data are percentages of sham-treated cultures and are expressed as the mean ± S.E.M. Cortical cultures were incubated in CI-HBS for 15 min followed by post-incubation in normal medium for 24 h. The drugs were added as described in Materials and methods. $^{a}P<0.05$, $^{b}P<0.01$, compared with CI alone (one-way ANOVA followed by Dunnet's two-tailed test).

2.6. Intracellular Ca^{2+} imaging

The intracellular Ca^{2+} concentration was measured with the fluorescent Ca^{2+} chelator Fura-2 acetoxymethyl ester (Fura-2 AM). Cortical cultures on glass coverslips were incubated in Fura-2 AM (5 μM)-containing HEPES buffered saline for 30 min, and then rinsed with the buffer. Each coverslip was mounted on a recording chamber placed under a fluorescent microscope. The cells were alternatively excited with 340 and 380 nm of light at an interval of 2 s, and the emission was measured at 500 nm by the ARGUS-HiSCA imaging system (HAMAMATSU Photonics K.K., Shizuoka, Japan). Fluorescent imaging was performed for 10 min at room temperature (≈ 25 °C). Drugs were applied to the recording chamber. As an index of the level of Ca^{2+} influx induced by glutamate, the area under the curve (AUC) from glutamate addition to the end of the analysis was calculated.

2.7. Statistics

Data are expressed as means ± S.E.M. The statistical significance of differences was determined by Student's *t*-test or one-way ANOVA followed by Dunnett's two-tailed test or Tukey's test using the InStat (GraphPad Software, San Diego, USA) program. Statistical significance was defined as a probability value of less than 5%.

3. Results

3.1. Delayed neuronal death induced by chemical ischemia

The incubation of cortical cultures in CI-HBS containing 3 mM of sodium azide and 10 mM of 2-deoxy-D-glucose for 15 min without post-incubation had no effect on cell

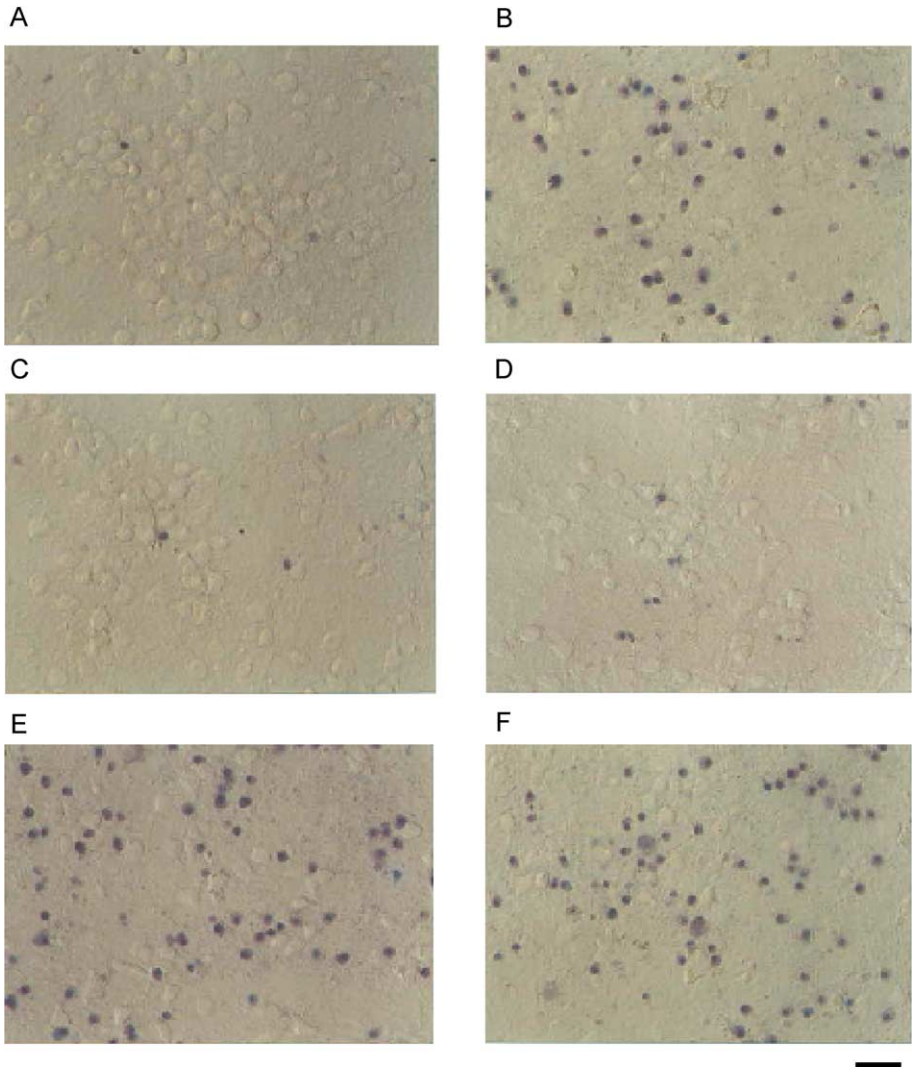

Fig. 3. Hoffman modulation photomicrographs showing effects of σ receptor ligands on chemical ischemia-induced neuronal death. (A)–(F) shows control, chemical ischemia (CI), CI plus (+)-SKF10,047 (10 μM), CI plus haloperidol (10 μM), CI plus carbetapentane (10 μM) and CI plus (+)-3-PPP (100 μM)-treated cultures, respectively. Calibration bar = 50 μm.

viability, as compared with sham-operated cultures (Fig. 1A). On the other hand, when cortical cultures were incubated in CI-HBS for 15 min, followed by post-incubation in normal medium for 24 h, cell viability was significantly reduced. The reduction of cell viability was dependent on induction time of chemical ischemia (Fig. 1B). These results suggest that a brief exposure to chemical ischemia leads to delayed neuronal death in cortical cultures. Accordingly, we assessed the chemical ischemia-induced cytotoxicity by this procedure in the present study. Under the conditions, the chemical ischemia-induced neurotoxicity was dependent on the concentration of sodium azide or 2-deoxy-D-glucose (Fig. 1C–D).

The application of D-glucose (10 mM), during both chemical ischemia and the post-incubation (total of 21 mM because Eagle's MEM contained 11 mM D-glucose) period, prevented the neurotoxicity (Fig. 1E). These results indicate that chemical ischemia-induced neurotoxicity is dependent on the metabolic inhibition of both oxygen consumption and glycolysis.

3.2. Involvement of glutamate neurotoxicity in chemical ischemia-induced neuronal death

We measured the amount of glutamate released from cultures into the incubation medium using the EICOM 300 series analysis system. Fifteen minutes after the induction of chemical ischemia, the glutamate content of the supernatant of the medium was significantly increased, as compared with control cultures or cultures at 0 min after the induction of chemical ischemia (Fig. 2A). The increase in glutamate was prevented by the application of D-glucose (10 mM) (Fig. 2B). These results suggest that metabolic inhibition caused by chemical ischemia leads to the release of glutamate from cortical cultures, and that glutamate is involved in chemical ischemia-induced neuronal death.

It has been reported that glutamate receptors, especially NMDA receptors, play a crucial role in neuronal death during cerebral ischemia and in other neurodegenerative disorders. Therefore, to investigate the contribution of glutamate neurotoxicity, we examined the effects of NMDA receptor antagonists on chemical ischemia-induced neurotoxicity. As shown Table 1, the application of MK-801 (1 μM), a noncompetitive NMDA receptor antagonist, and APV (0.3–1 mM), a competitive NMDA receptor antagonist, during both chemical ischemia and the post-incubation period significantly prevented chemical ischemia-induced neurotoxicity. These results indicate that glutamate neurotoxicity via NMDA receptors contributes to the neuronal death induced by chemical ischemia. Moreover, the removal of extracellular Ca^{2+} during the period of chemical ischemia prevented the neuronal death though the recovery was partial. Nitric oxide (NO) is regarded as a mediator of glutamate neurotoxicity. Therefore, we investigated the involvement of NO in chemical ischemia-induced neuronal death. N^{ω}-nitro-L-arginine methyl ester (L-NAME; 0.3–1 mM), an inhibitor of NOS, had only minor effects to the neuroprotection (Table 1). A similar result was obtained on the application of another NOS inhibitor, N^{ω}-nitro-L-arginine (*N*-Arg) (data not shown). These findings suggest that NO plays a minor role in chemical ischemia-induced neuronal death.

3.3. Effects of σ receptor ligands on chemical ischemia-induced neurotoxicity

Fig. 3 shows typical photographs of the effects of σ receptor ligands on chemical ischemia-induced neuronal death. Most of the cells in the control culture were viable (Fig. 3A). The induction of chemical ischemia markedly reduced cell viability, as indicated by the increase in the number of cells stained by trypan blue (Fig. 3B). The application of (+)-SKF10,047 (10 μM), a prototypical σ receptor ligand which binds to both σ_1 receptors and the phencyclidine (PCP) site in NMDA receptor channels, or haloperidol (10 μM), a σ_1/σ_2 receptor ligand structurally distinct from (+)-SKF10,047, reduced the number of cells stained by trypan blue (Fig. 3C and D), indicating that chemical ischemia-induced neuronal death was prevented by these drugs. (+)-SKF10,047 (1–10 μM) and haloperidol (1–10 μM) prevented chemical ischemia-induced neurotoxicity in a concentration-dependent manner (Fig. 4A and B). On the other hand, carbetapentane (10 μM) and (+)-3-PPP (100 μM), σ_1 and σ_1/σ_2 receptor ligands, respectively,

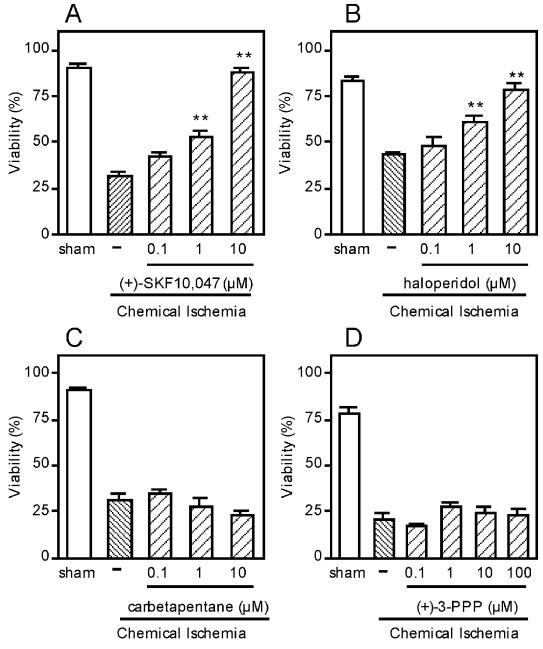

Fig. 4. Effects of σ receptor ligands on chemical ischemia-induced neurotoxicity. Cortical cultures were incubated in CI-HBS for 15 min followed by post-incubation in normal MEM. (+)-SKF10,047 (A), haloperidol (B), carbetapentane (C) and (+)-3-PPP (D) were added during both the induction of chemical ischemia and the post-incubation period. $^{**}P<0.01$, compared with chemical ischemia (one-way ANOVA followed by Dunnett's two-tailed test).

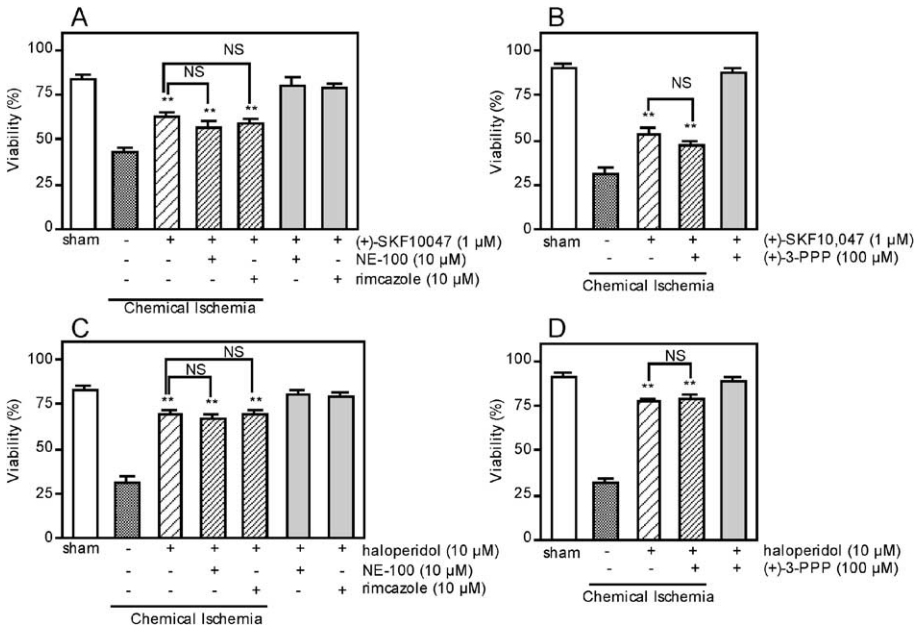

Fig. 5. Effects of σ receptor antagonists on neuroprotection by (+)-SKF10,047 (A, B) and haloperidol (C, D). Cortical cultures were incubated in CI-HBS for 15 min followed by post-incubation in normal medium for 24 h. Sigma receptor ligands were added during both the induction of chemical ischemia and the post-incubation period. $^{**}P < 0.01$, compared with chemical ischemia (one-way ANOVA followed by Dunnett's two-tailed test). NS, not significant.

had no effect on chemical ischemia-induced neuronal death (Figs. 3E–F and 4C–D).

To clarify whether the protective effects of (+)-SKF10,047 and haloperidol on chemical ischemia-induced neurotoxicity were mediated via σ receptors, we examined the influence of σ receptor antagonists on the neuroprotection. Certain σ receptor ligands are regarded as σ receptor antagonists, although the distinction between agonists and antagonists for σ receptor ligands is not absolute. In this study, we used rimcazole and NE-100 as σ_1 receptor antagonists according

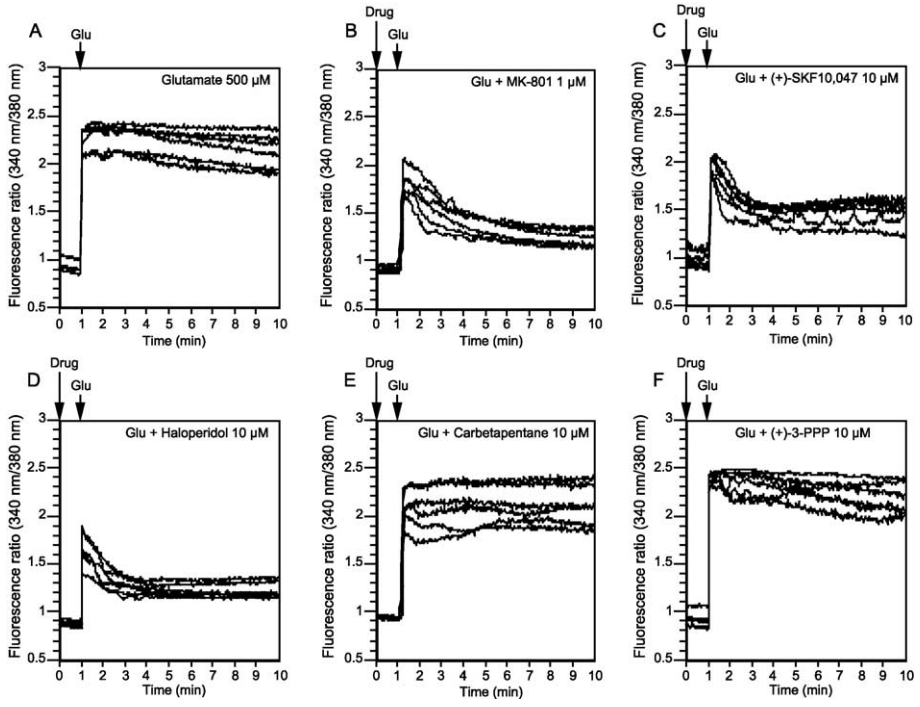

Fig. 6. Typical traces of effects of σ receptor ligands on the glutamate-induced increase in intracellular Ca^{2+}. Intracellular Ca^{2+} levels were measured with Fura-2, as described in Materials and methods. (A)–(F) show typical traces of glutamate (500 μM) alone, glutamate plus MK-801 (1 μM), glutamate plus (+)-SKF10,047 (10 μM), glutamate plus haloperidol (10 μM), glutamate plus carbetapentane (100 μM) and glutamate plus (+)-3-PPP (100 μM), respectively. Arrows indicate the application of drugs or glutamate.

to previous studies (Balster, 1986; Senda et al., 1996; Steardo et al., 1996; Nakazawa et al., 1998) and (+)-3-PPP as a σ_1/σ_2 receptor antagonist since it had no effect on chemical ischemia-induced neurotoxicity. Rimcazole (10 μM) and NE-100 (10 μM) had no effect on chemical ischemia-induced neurotoxicity. We did not use these agents at higher concentrations (>10 μM) because of their neurotoxicity (data not shown).

Rimcazole is considered to be a σ_1 receptor antagonist (Steardo et al., 1996). Co-application of rimcazole (10 μM) failed to attenuate the protective effects of (+)-SKF10,047 (1 μM) and haloperidol (10 μM). Similarly, NE-100 (10 μM), which is also a σ_1 receptor antagonist (Senda et al., 1996; Tokuyama et al., 1997) but structurally distinct from rimcazole, did not attenuate the neuroprotective actions of (+)-SKF10,047 (1 μM) and haloperidol (10 μM) (Fig. 5A and C). These results suggest that the σ_1 receptor is not involved in the neuroprotective effects of (+)-SKF10,047 and haloperidol. Since haloperidol is a σ_1/σ_2 receptor ligand, the effect of the drug might be mediated via σ_2 receptors. Therefore, to investigate the involvement of σ_2 receptors in the effect of haloperidol, we examined the influence of (+)-3-PPP on the neuroprotection provided by haloperidol. (+)-3-PPP, a σ_1/σ_2 receptor ligand, is widely used to displace σ receptor ligands from σ receptors (Largent et al., 1986; Matsuno et al., 1996). (+)-3-PPP (100 μM) also did not attenuate the neuroprotective effects of (+)-SKF10,047 (1 μM) and haloperidol (10 μM) (Fig. 5B and D), suggesting that neither σ_1 nor σ_2 receptors were involved in the mechanism of the neuroprotective actions of (+)-SKF10,047 and haloperidol. These findings indicate that the neuroprotective activities of (+)-SKF10,047 and haloperidol are not mediated by the activation of σ receptors.

3.4. Effects of σ receptor ligands on glutamate-induced increase in intracellular Ca^{2+}

Since it was suggested that the neuroprotective effects of (+)-SKF10,047 and haloperidol were not mediated by the activation of σ receptors, we hypothesized that these effects were mediated by direct interaction with the NMDA receptor-ion channel complex. In the present study using a chemical ischemic model, we obtained results similar to our previous findings (Nishikawa et al., 2000), which showed that the exposure of cortical cultures to glutamate (500 μM) for 1 h significantly reduced cell viability, and that this acute glutamate neurotoxicity was prevented by (+)-SKF10,047 and haloperidol, but not by carbetapentane and (+)-3-PPP. To investigate the involvement of the direct interaction with NMDA receptors in the protective effects of σ receptor ligands, we examined the effects of σ receptor ligands on the increase in intracellular Ca^{2+} induced by exposure to glutamate.

Glutamate (500 μM) significantly evoked an increase in intracellular Ca^{2+}, and the response was persistent during the recording period. In the presence of MK-801, the glutamate

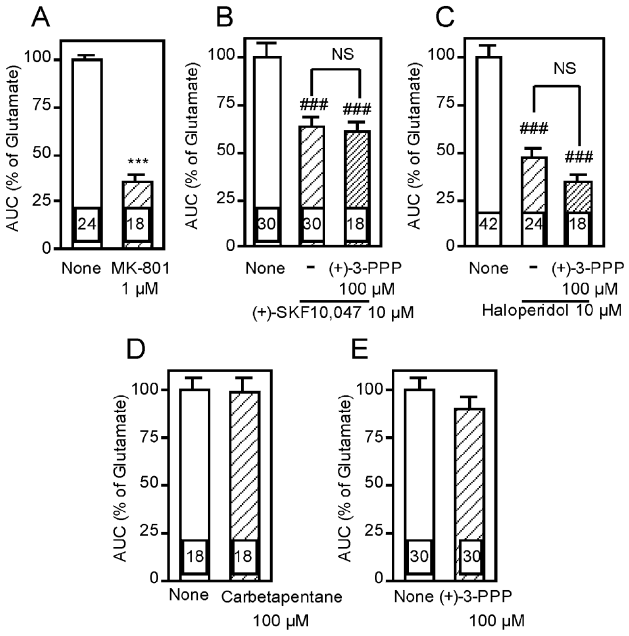

Fig. 7. Effects of σ receptor ligands on the glutamate-induced increase in intracellular Ca^{2+}. Intracellular Ca^{2+} levels were measured with Fura-2, as described in Materials and methods. As an index of Ca^{2+} influx induced by glutamate, the area under the curve (AUC) from glutamate addition to the end of the analysis was calculated. (A)–(E) show the results for MK-801 (1 μM), (+)-SKF10,047 (10 μM), haloperidol (10 μM), carbetapentane (100 μM) and (+)-3-PPP (100 μM), respectively. In (B) and (C), (+)-3-PPP (100 μM) was applied with (+)-SKF10,047 (10 μM) or haloperidol (10 μM). The number in the columns indicates that of neurons examined. $^{***}P<0.001$, compared with None. (Glutamate alone, Student *t*-test). $^{###}P<0.001$, compared with None (glutamate alone, one-way ANOVA followed by Tukey's test). NS, not significant.

induced-increase in intracellular Ca^{2+} was markedly reduced, with a transient increase followed by a significant decrease to a plateau. These results indicate that the glutamate-induced Ca^{2+} influx was mainly mediated via NMDA receptors. Similar to the effect of MK-801, (+)-SKF10,047 (10 μM) and haloperidol (10 μM) also attenuated the glutamate-induced Ca^{2+} influx, but not carbetapentane (100 μM) or (+)-3-PPP (100 μM) (Figs. 6 and 7). Moreover, the inhibition of glutamate-induced Ca^{2+} influx by (+)-SKF10,047 (10 μM) and haloperidol (10 μM) was not antagonized by (+)-3-PPP (100 μM) co-applied as a σ_1/σ_2 receptor antagonist (Fig. 7B and C). These results suggest that (+)-SKF10,047 and haloperidol inhibit the glutamate-induced Ca^{2+} influx via direct interaction with the NMDA receptor-ion channel complex, and not by the activation of σ receptors.

4. Discussion

We performed an in vitro study to examine the effects of several σ receptor ligands on ischemia-induced neuronal death. To induce hypoxia/hypoglycemia in rat cortical cultures, sodium azide and 2-deoxy-D-glucose were used. Consistent with a previous study (Varming et al., 1996),

sodium azide and 2-deoxy-D-glucose both induced neuronal death in a concentration-dependent manner. The induction of chemical ischemia followed by post-incubation for 24 h led cortical cultures to sustain lethal damage. The delayed neuronal death observed in this study has similar properties to the glutamate neurotoxicity observed in previous studies (Choi et al., 1987; Dawson et al., 1991; Kume et al., 2000). A significant increase in glutamate in the supernatant of the incubation buffer was observed after the induction of chemical ischemia. Moreover, the chemical ischemia-induced neurotoxicity was prevented by NMDA receptor antagonists, MK-801 and APV. These results indicate that glutamate neurotoxicity via NMDA receptors plays a dominant role in chemical ischemia-induced neurotoxicity. Moreover, NOS inhibitors partially inhibited the chemical ischemia-induced neurotoxicity, suggesting the involvement of NO in the neuronal death.

We have previously reported that direct interaction with NMDA receptors but not σ receptors is crucial to the neuroprotective effects of σ receptor ligands, including (+)-SKF10,047, on acute glutamate neurotoxicity (Nishikawa et al., 2000). In the present study, we examined the effects of several σ receptor ligands on chemical ischemia-induced neurotoxicity in rat cortical cultures. Consistent with our previous report, (+)-SKF10,047 and haloperidol, but not carbetapentane or (+)-3-PPP, prevented chemical ischemia-induced neurotoxicity. (+)-SKF10,047 prevented neuronal death both in vivo and in vitro (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 PCP sites in the NMDA receptor channel (Largent et al., 1986). The present results showed that (+)-SKF10,047 protected cortical cultures against chemical ischemia-induced neurotoxicity. It is unlikely that the neuroprotective effect of (+)-SKF10,047 is mediated by σ_1 receptors because it was not attenuated by the σ_1 receptor antagonists, rimcazole and NE-100, or the σ_1/σ_2 receptor ligand, (+)-3-PPP, which had no effect on chemical ischemia-induced neurotoxicity. (+)-3-PPP has been widely used to displace (+)-SKF10,047 and other ligands from σ_1 receptors (Largent et al., 1986; Matsuno et al., 1996). Moreover, (+)-SKF10,047 inhibited the glutamate-induced increase in intracellular Ca^{2+} in a similar manner to MK-801, a PCP site blocker. Although Yamamoto et al. (1995) have suggested that σ receptor ligands indirectly modulate the NMDA receptor–ion channel complex, the inhibitory effect on Ca^{2+} influx was not attenuated by (+)-3-PPP. Therefore, it is plausible that the inhibitory effect of (+)-SKF10,047 on the glutamate-induced increase in intracellular Ca^{2+} is not mediated via σ_1 receptors. These results support the idea that direct inhibition of the NMDA receptor-ion channel complex by (+)-SKF10,047 is essential to protect cortical cultures against glutamate exicitoxicity-related neuronal death.

Haloperidol, a σ_1/σ_2 receptor ligand, also protected cortical cultures against chemical ischemia-induced neurotoxicity, and the neuroprotective effects of the drug were not attenuated by σ_1 receptor antagonists or a σ_1/σ_2 receptor ligand (+)-3-PPP. These results suggest that the neuroprotective effect of haloperidol was not mediated by either σ_1 or σ_2 receptors. Previous studies showed that haloperidol inhibited the NMDA-activated membrane current response in *Xenopus* oocytes expressing the NR1A/NR2B subunit of the NMDA receptor (Ilyin et al., 1996; Whittemore et al., 1997). Haloperidol may interact directly with the strychnine-insensitive glycine site (Fletcher and McDonald, 1993) or a site distinct from the polyamine or ifenprodil sites on the NMDA receptor (Lynch and Gallagher, 1996). Consistent with these reports, haloperidol prevented the glutamate-induced increase in intracellular Ca^{2+}, and the effect was not attenuated by (+)-3-PPP. Therefore, it is concluded that haloperidol provides protection against chemical ischemia-induced neurotoxicity by interacting with NMDA receptors. Moreover, our preliminary studies showed that (+)-SKF10,047 and haloperidol also inhibited the NMDA-induced increase in intracellular Ca^{2+} as determined by Fura-2 Ca^{2+} imaging (data not shown). These results support our conclusion that (+)-SKF10,047 and haloperidol directly regulate NMDA receptor channels.

Carbetapentane, a σ_1 receptor ligand, and (+)-3-PPP, a σ_1/σ_2 receptor ligand, did not have a neuroprotective effect on chemical ischemia-induced neuronal death. These results were consistent with our previous study examining the effects of these ligands on acute glutamate neurotoxicity (Nishikawa et al., 2000). It has been reported that carbetapentane and (+)-3-PPP do not possess affinity for NMDA receptors (Largent et al., 1986; Whittemore et al., 1997). Since we confirmed the expression of σ_1 receptor mRNA in the cortical cultures (Nishikawa et al., 2000), it is unlikely that the lack of neuroprotective action by σ receptor ligands without affinity for NMDA receptors was due to a lack of σ receptors. Moreover, carbetapentane and (+)-3-PPP had no effect on glutamate-induced Ca^{2+} influx. These findings indicate that the inhibition of Ca^{2+} influx evoked by glutamate is crucial to the protective effects of σ receptor ligands on both the acute glutamate neurotoxicity and the chemical ischemia-induced neurotoxicity. This idea is supported by the finding that σ receptor ligands without affinity for NMDA receptors had no effect on chemical ischemia-induced neurotoxicity, while those with affinity for NMDA receptors including PCP sites had a neuroprotective effect.

Recent reports offer conflicting explanations as to the mechanism by which σ site ligands inhibit NMDA receptors. On one hand, binding and Ca^{2+} imaging studies suggest that inhibition is mediated indirectly via σ sites (Hayashi et al., 1995; Yamamoto et al., 1995). On the other hand, electrophysiological studies indicate that inhibition is due to direct effects on NMDA receptors (Fletcher et al., 1995). It is also reported that the σ receptor ligands with the affinity for NMDA receptors act as use-dependent blockers of NMDA receptors (Whittemore et al., 1997). In the present study, only the σ receptor ligands with the affinity

for NMDA receptors showed the protective effect on glutamate neurotoxicity and these protective effects were not attenuated by the σ receptor antagonists. Therefore, we concluded that direct inhibition of NMDA receptors plays a crucial role in the protective effect of σ receptor ligands with the affinity for NMDA receptors. However, we cannot exclude the possibility that σ receptor ligands prevented glutamate neurotoxicity because of use-dependent block of NMDA receptors. To reveal detailed mechanisms, further experiments might be necessary.

In conclusion, the present study showed that only σ receptor ligands with affinity for NMDA/PCP receptor channels, such as (+)-SKF10,047, prevented chemical ischemia-induced neuronal death in cultured rat cortical neurons. These results indicate that the direct interaction of the drugs with NMDA/PCP receptor channels contributes to their protective action against neuronal death caused by exicitotoxicity.

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