treated repeatedly with antipsychotics. Phencyclidine/olanzapine-treated mice showed fear conditioning-induced ERK activation in the amygdalae and hippocampi but not phencyclidine/vehicle-treated mice. Although haloperidol and olanzapine increased the basal phospho-ERK levels in the hippocampi of saline- and phencyclidine-treated mice, haloperidol, unlike olanzapine, failed to recover fear conditioning-induced ERK activation in the amygdalae and hippocampi of phencyclidine-treated mice. Although the mechanism by which antipsychotics increased basal and postconditioning phospho-ERK levels is unknown, an important finding is that the improvement of associative learning on repeated olanzapine treatment accompanied fear conditioning-induced ERK activation in the amygdala and hippocampus. Other signaling pathways might also process associative learning in the amygdalae of phencyclidine/olanzapine-treated mice, because the phospho-ERK level after conditioning in the amygdalae of phencyclidine/olanzapine-treated mice was not significantly different from that in phencyclidine/vehicle-treated mice.

The effect of olanzapine would not be caused by prevention of phencyclidine-induced neurodegeneration, because olanzapine treatment was initiated after the cessation of repeated phencyclidine treatment. It is possible that olanzapine restores normal function in the fear conditioning test as a result of complex changes in the interaction of various neural

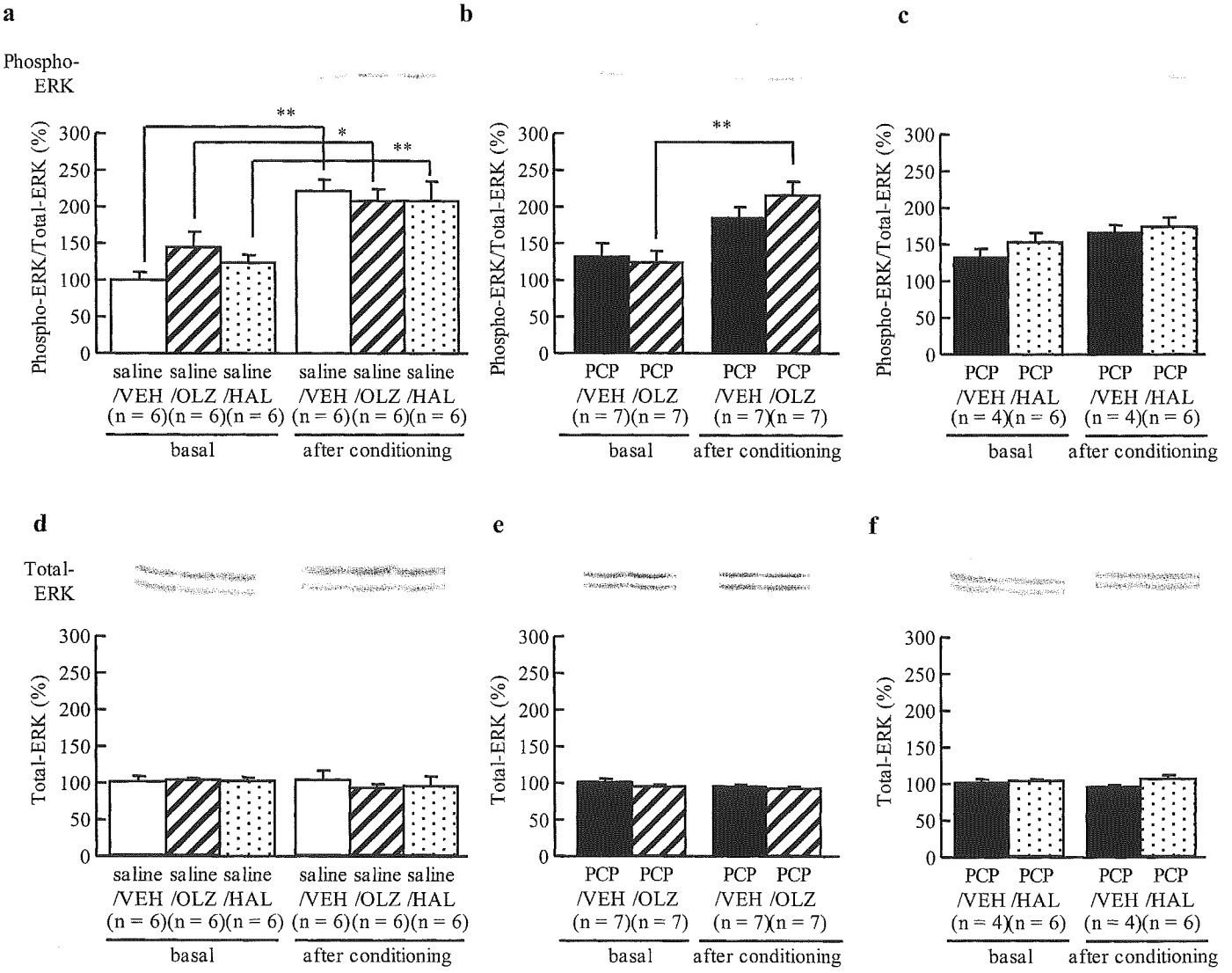

Fig. 6. Effects of repeated antipsychotic treatment on ERK activation in the amygdala after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine-treatment (10 mg/kg/day s.c. for 14 days). Fear conditioning was performed at 1 day after the final antipsychotic treatment. Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae of mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (a), phencyclidine/vehicle, phencyclidine/olanzapine (b), phencyclidine/vehicle, and phencyclidine/haloperidol (c). Representative Western blots and total ERK immunoreactivity in the amygdalae of mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (d), phencyclidine/vehicle, phencyclidine/olanzapine (e), phen-cyclidine/vehicle, and phencyclidine/haloperidol (f). Values correspond to the mean ± S.E.M. Results with two-way ANOVA were: a, treatment, $F_{2,30} = 0.38$ ($p = 0.68$); time, $F_{1,30} = 36.34$ ($p < 0.01$); treatment-by-time interaction $F_{2,30} = 1.33$ ($p = 0.28$); b, treatment, $F_{1,24} = 0.57$ ($p = 0.46$); time, $F_{1,24} = 18.51$ ($p < 0.01$), treatment-by-time interaction, $F_{1,24} = 1.36$ ($p = 0.26$); c, treatment, $F_{1,16} = 1.18$ ($p = 0.29$); time, $F_{1,16} = 4.08$ ($p = 0.06$); treatment-by-time interaction, $F_{1,16} = 0.20$ ($p = 0.66$); d, treatment, $F_{2,30} = 0.16$ ($p = 0.86$); time, $F_{1,30} = 0.65$ ($p = 0.42$); treatment-by-time interaction, $F_{2,30} = 0.28$ ($p = 0.76$); e, treatment, $F_{1,24} = 0.78$ ($p = 0.38$); time, $F_{1,24} = 1.48$ ($p = 0.24$); treatment-by-time interaction, $F_{1,24} = 0.17$ ($p = 0.68$); f, treatment, $F_{1,16} = 2.08$ ($p = 0.17$); time, $F_{1,16} = 0.06$ ($p = 0.82$); treatment-by-time interaction, $F_{1,16} = 0.65$ ($p = 0.43$). *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

circuits that were altered by repeated phencyclidine administration. The inability of haloperidol to reverse phencyclidine-induced impairment of associative learning suggests that the blocking of D_2 receptors alone is insufficient to reverse the impairment of learning in this model. Olanzapine would activate glutamate neurotransmission, because it induces the increase of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor binding in the rat hippocampus (Tascedda et al., 2001). It has been reported that olanzapine, but not haloperidol, enhances brain-derived neurotrophic factor (BDNF) mRNA expression (Bai et al., 2003) and antagonizes the MK-801-induced reduction of BDNF mRNA expression in rat hippocampus (Fumagalli et al., 2003). We have shown previously that BDNF is involved in learning and memory by enhancing the phosphorylation of NMDA receptors (Mizuno et al., 2003). Therefore, it is possible that olanzapine might reverse the phencyclidine-induced impairment of associative learning and ERK activation by enhancing the activities of BDNF and NMDA receptors. These results are compatible with the clinical findings that olanzapine but not haloperidol improves cognitive dysfunction in patients with schizophrenia (Bhana et al., 2001). Because the pharmacological effects of these antipsychotics in this model would reflect their clinical effectiveness, phencyclidine-in-

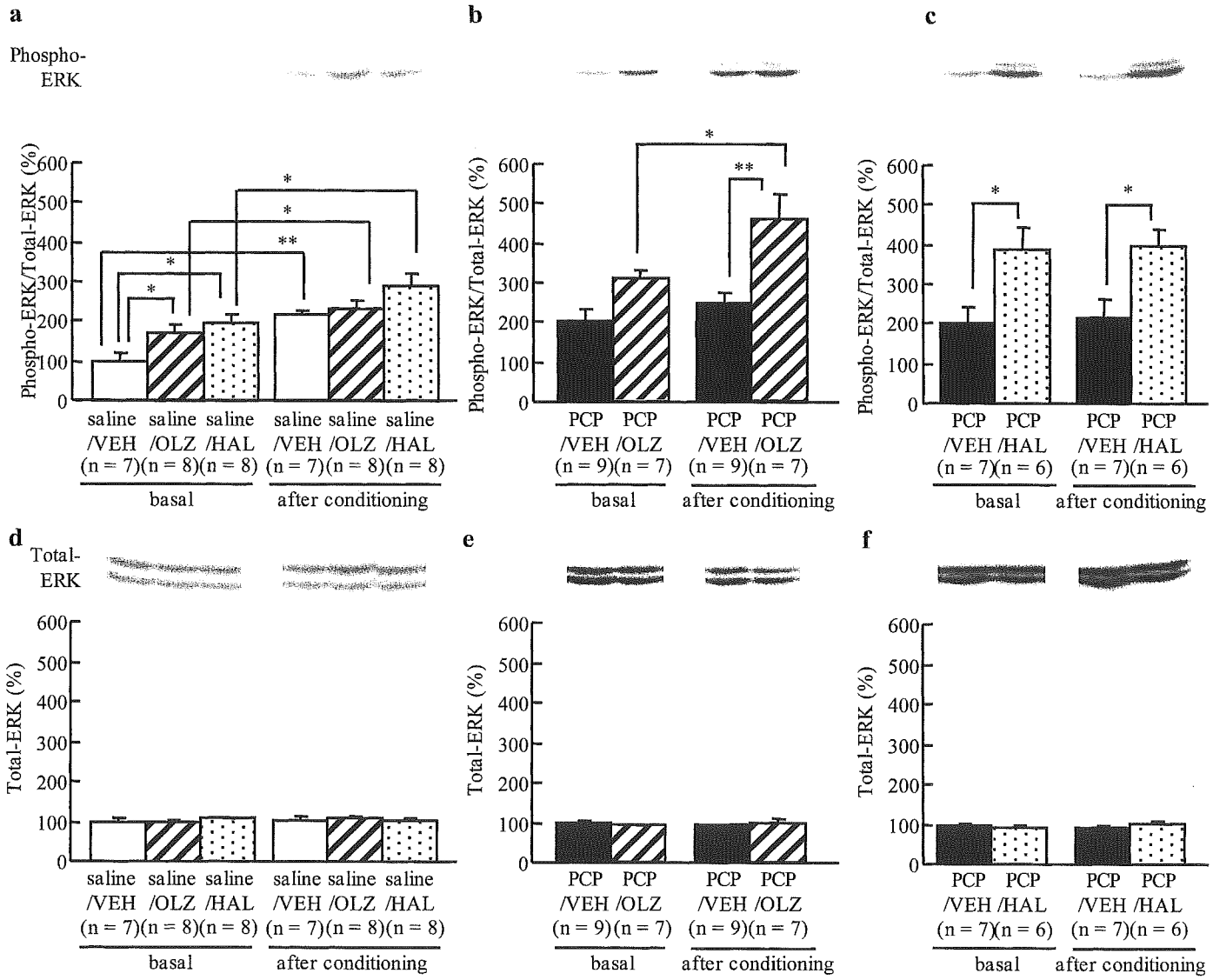

Fig. 7. Effects of repeated antipsychotic treatment on ERK activation in the hippocampus after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Fear conditioning was performed 1 day after the final antipsychotic treatment. Representative Western blots and phospho-ERK/total ERK immunoreactivity in the hippocampi of mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (a), phencyclidine/vehicle, phencyclidine/olanzapine (b), phencyclidine/vehicle, and phencyclidine/haloperidol (c). Representative Western blots and total ERK immunoreactivity in the hippocampi mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (d), phencyclidine/vehicle, phencyclidine/olanzapine (e), phen-cyclidine/vehicle, and phencyclidine/haloperidol-treated mice (f). Values correspond to the mean ± S.E.M. Results with two-way ANOVA were the following: a, treatment, $F_{2,40} = 7.40$ ($p < 0.01$); time, $F_{1,40} = 25.08$ ($p < 0.01$); treatment-by-time interaction, $F_{2,40} = 0.77$ ($p = 0.47$); b, treatment, $F_{1,28} = 18.83$ ($p < 0.01$); time, $F_{1,28} = 6.88$ ($p < 0.05$); treatment-by-time interaction, $F_{1,28} = 2.21$ ($p = 0.15$); c, treatment, $F_{1,22} = 16.32$ ($p < 0.01$); time, $F_{1,22} = 0.10$ ($p = 0.76$); treatment-by-time interaction, $F_{1,22} = 0.0009$ ($p = 0.98$); d: treatment, $F_{2,40} = 0.18$ ($p = 0.84$); time, $F_{1,40} = 0.95$ ($p = 0.34$); treatment-by-time interaction, $F_{2,40} = 1.20$ ($p = 0.31$); e, treatment, $F_{1,28} = 0.21$ ($p = 0.65$); time, $F_{1,28} = 0.18$ ($p = 0.68$); treatment-by-time interaction, $F_{1,28} = 2.02$ ($p = 0.17$); f, treatment, $F_{1,22} = 0.53$ ($p = 0.47$); time, $F_{1,22} = 0.49$ ($p = 0.49$); treatment-by-time interaction, $F_{1,22} = 0.90$ ($p = 0.35$). *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

duced impairments of associative learning would be a useful model of cognitive dysfunction in schizophrenia.

Repeated phencyclidine treatment produces a long-lasting impairment of associative learning in mice. This impairment is accompanied by a dysfunction of NMDA-ERK signaling. This finding is the first step to understanding the mechanism of cognitive dysfunction in schizophrenic and/or phencyclidine psychoses. Furthermore, this animal model would provide a useful system for studying the effect of antipsychotics on the impairment of associative learning in schizophrenia, because the impairment was reversed by olanzapine but not by haloperidol.

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Research Report

Protective effects of nicergoline against neuronal cell death induced by activated microglia and astrocytes

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Abstract

We examined the neuroprotective role of nicergoline in neuron–microglia or neuron–astrocytes co-cultures. Nicergoline, an ergoline derivative, significantly suppressed the neuronal cell death induced by co-culture with activated microglia or astrocytes stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ. To elucidate the mechanism by which nicergoline exerts a neuroprotective effect, we examined the production of inflammatory mediators and neurotrophic factors in activated microglia and astrocytes following nicergoline treatment. In microglia stimulated with LPS and IFN-γ, nicergoline suppressed the production of superoxide anions, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in a dose-dependent manner. In astrocytes, nicergoline also suppressed the production of proinflammatory cytokines and enhanced brain-derived neurotrophic factor (BDNF). Thus, nicergoline-mediated neuroprotection resulted primarily from the inhibition of inflammatory mediators and the upregulation of neurotrophic factors by glial cells.

Theme: Disorders of the nervous system
Topic: Neurotoxicity

Keywords: Nicergoline; Microglia; Astrocyte; BDNF; Neurotoxicity

1. Introduction

Microglia and astrocytes are the main effectors of innate immune responses in the central nervous system (CNS). They are activated in pathological conditions, such as demyelinating and neurodegenerative diseases. The release of proinflammatory mediators, including interleukin (IL)-1 [13], IL-6 [11], tumor necrosis factor (TNF)-α [28,39], nitric oxide (NO) [20], and reactive oxygen species (ROS) [35], by microglia and astrocytes function to exacerbate demyelinating diseases, such as multiple sclerosis and the cognate animal model experimental allergic encephalomyelitis (EAE). These inflammatory substances act to destroy myelin or oligodendrocytes, producing demyelinating lesions [29,30]. Proinflammatory cytokines can also cause neuronal cell death, both directly and indirectly via the induction of NO and free radicals [16]. Peroxynitrite, formed by NO and ROS-mediated reactions, are reported to play a role in amyloid beta neurotoxicity [41].

Astrocytes provide structural, metabolic, and trophic support for neurons. Activated astrocytes, however, can secrete these inflammatory mediators to exert strong neurotoxic effects [2,7]. Cytosine arabinofuranoside-stimulated astrocytes increase neuronal susceptibility to glutamate [1], suggesting that agents that suppress glial cell activation may be useful for the future treatment of neuroinflammatory or neurodegenerative disorders.

Nicergoline is an ergoline derivative used to treat various symptoms, such as cognitive deficits and dizziness related to cerebrovascular disease [4,10]. In rats, this compound increases regional cerebral blood flow and improves mono-

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amine turnover and cholinergic function by reversing the acetylcholine deficits in aged rats [6]. A protective effect of nicergoline against anoxic or ischemic brain damage has been reported in ischemic brain models [31,34]. In aged rats, repeated injections of nicergoline induced significant increases in the NGF levels within the frontal region of the brain [23].

In this study, we investigated the protective effect of nicergoline against neuronal cell death induced by activated microglia or astrocytes. To elucidate the mechanism of nicergoline neuroprotection, we examined the effects of nicergoline on the production of toxic and trophic mediators by activated microglia and astrocytes.

2. Materials and method

2.1. Reagent

Nicergoline was the kind gift of Tanabe Pharmaceuticals (Osaka, Japan). LPS was purchased from Sigma (St. Louis, MO, USA). IFN-γ was obtained from Genzyme/Techne (Minneapolis, MN, USA).

2.2. Cell cultures

Microglia were isolated from primary mixed glial cell cultures from newborn C57/BL6 mice on the 14th day using the "shaking off" method described previously [32]. Cultures were 97 to 100% pure as determined by Fc receptor-specific immunostaining. Cultures were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 5 μg/ml bovine insulin, and 0.2% glucose. Microglial cells (5×10^5) were cultured for 48 h with or without 1 μg/ml LPS in the presence of graded concentrations of nicergoline. Culture supernatants were collected for cytokine ELISA assays; the remaining cells were used for RNA extraction.

Astrocytes were purified from the primary mixed glial cell cultures by three to four repetitions of trypsinization and replating. The purity of astrocytes was greater than 95% when determined by indirect immunofluorescence staining with an anti-GFAP antibody. Astrocytes were grown to confluency and cultured in serum-free medium for 3 days. Cells were then cultured for an additional 48 h with or without 10 μg/ml LPS in the presence of graded concentrations of nicergoline. The supernatants were collected for cytokine ELISA assays; the cells at 12, 24, and 48 h were used for RNA extraction.

Neuronal cultures were prepared from C57/BL6 mice at embryonic day 17. Briefly, cortices were dissected and freed of meninges. Cortical fragments were dissociated into single cells using dissociation solution (Sumitomo Bakelite), then resuspended in Neuron Medium (Sumitomo Bakelite). Primary neuronal cells were plated on 12 mm poly-ethyleneimine (PEI)-coated cover glass in 24-well multidishes at a density of 5×10^4 cells per well. Neuron–microglia co-cultures were prepared as follows: 2.5×10^4 microglial cells in 10 μl neuron medium were added to neuronal cultures on day 10 (5×10^4 neuronal cells). Six hours later, cultures were stimulated with or without 1 μg/ml LPS and 100 ng/ml IFN-γ for 24 h in the presence of graded concentrations of nicergoline. For neuron–astrocytes co-cultures, confluent monolayer astrocytes were grown on 12 mm poly-ethyleneimine (PEI)-coated cover glass in 24-well multidishes. Neuronal cells (2.5×10^4 per well) in 10 μl neuron medium were added to astrocyte cultures (5×10^4 cells). After 7 days, cultures were stimulated with or without 10 μg/ml LPS and 100 ng/ml IFN-γ for 24 h in the presence of graded concentrations of nicergoline.

2.3. Immunocytochemistry and quantification of survival neuron

Neuron–microglia or neuron–astrocyte co-cultures were fixed in 4% paraformaldehyde for 30 min. After treatment with blocking reagent (0.3% Triton-X-100, 1% goat serum, and 1% BSA) for 1 h at room temperature, neuronal cells were stained with anti-microtubule-associated protein (MAP)-2 antibody (1:500, Chemicon), while astrocytes were stained with an anti-glial fibrillary acidic protein (GFAP) antibody (1:500, Sigma). After washing three times, appropriate secondary antibodies and Hoechst 33342 (0.1 mg/ml, Molecular Probes) were added for an additional hour. Microglia were stained with an R-phycoerythrin (PE)-conjugated anti-CD11b antibody (1:1000, BD) prior to fixation. Neuronal survival was calculated as follows: the number of intact neurons with treatment/those without treatment × 100. Number of MAP-2 positive neurons with intact nuclei by Hoechst staining was counted. The viability of untreated neuronal cells was arbitrarily normalized to 100%. MAP-2 staining has been useful to assess neurotoxicity in mixed neuron–glial cultures. Cytotoxicity assessed by cytoskeleton change is more sensitive than that by common cell viability assay such as calcein/AM fluorescence [19].

2.4. Measurement of cytokines and superoxide anion

Supernatants from microglial or astrocytes cultures were assessed for cytokine levels (IL-1β, IL-6, and TNF-α) using commercial ELISA kits (Pharmingen). Superoxide anion formation was measured by an NBT reduction assay as described [33]. Microglia and astrocytes at 2×10^4 cells/100 μl were respectively seeded in a 96-well test plate and cultured for 48 h with LPS and IFN-γ in the presence of nicergoline as above. Then 100 μl of 0.15% NBT solution containing 0.2 μg/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added and incubated at 37 °C for 30 min. Supernatant was discarded, and the reaction was stopped with 100 μl of 1 N HCl in PBS. The absorbance was read at 630 nm in a microtiter plate reader.

2.5. Measurement of neurotrophic factors

RNA was extracted from the cells at 12, 24, and 48 h to examine mRNA expression levels of neurotrophic factors (NGF and BDNF). Total RNA was extracted from microglia using the guanidinium thiocyanate method (RNeasy Mini Kit; QIAGEN). cDNAs encoding mouse NGF and BDNF were generated by reverse transcription-PCR (RT-PCR) using Super Script II (Invitrogen) and Ampli Taq DNA polymerase (Applied Biosystems) with the following primers:

NGF sense, 5′-CATAGCGTAATGTCCATGTTGTTCT
NGF antisense, 5′-CTTCTCATCTGTTGTCAACGC
BDNF sense, 5′-GAAAGTCCCGGTATCCAAAG
BDNF antisense, 5′-CCAGCCAATTCTCTTTTT
GAPDH forward, 5′-ACTCACGGCAAATTCAACG
GAPDH reverse, 5′-CCCTGTTGCTGTAGCCGTA

NGF and BDNF content was measured by an enzyme immunoassay (EIA) method [18,24]. In short, multiwell plates (Falcon 3910; Becton Dickinson and Co., Franklin Lakes, NJ) were incubated with 5 μl of anti-BDNF antibody in 0.1 M Tris–HCl buffer (pH 9.0) (10 μg/ml) per well for 12 h, washed with washing buffer (0.1 M Tris–HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.02% Na_3N 0.1% BSA, and 1 mM $MgCl_2$), and then blocked with washing buffer containing 1% (w/v) skimmed milk. Tissue extract or BDNF standard (30 μl) in washing buffer was then added to each antibody-coated well; and incubation was carried out for 5 h at 25 °C. After three washes with washing buffer, 30 μl of biotinylated anti-BDNF-antibody (10 ng/ml) in washing buffer was added to each well; and the plate was incubated for 12 to 18 h at 4 °C. The biotinylated secondary antibodies were reacted with avidin-conjugated β-galactosidase (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h.

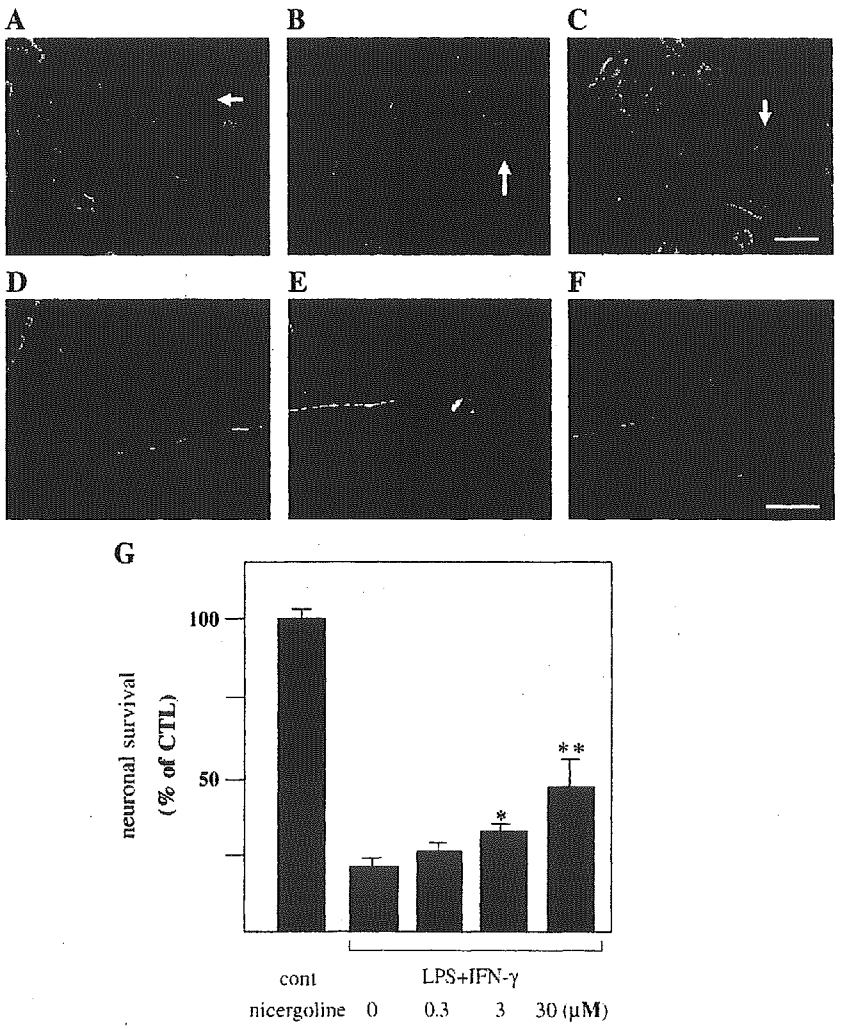

Fig. 1. Neuronal cell death by activated microglia and neuroprotection by nicergoline. In neuron–microglial co-cultures, microglia (white arrows) were stimulated by LPS (1 μg/ml) and IFN-γ (100 ng/ml) for 48 h in the presence or absence of nicergoline. Panels A–C display phase-contrast images; neuronal cells are indicated by black arrows. Panels D–F exhibit MAP-2-positive neuronal cells. Cultures were either (A) (D) left unstimulated, (B) (E) stimulated with LPS (1 μg/ml) and IFN-γ (100 ng/ml), or (C) (F) treated with nicergoline (30 μM) following the activation of microglia with LPS and IFN-γ. (G) Neuronal survival rates following nicergoline treatment are shown after normalizing values such that the viability of untreated cells was set to 100%. $*P < 0.05$, $**P < 0.01$ versus LPS and IFN-γ alone. Data represent the mean ± SD of five independent experiments. Scale bars: 20 μm.

Then following thorough washing with washing buffer, enzyme activity retained in each well was measured by incubation with fluorogenic substrate; 4-methylumbelliferyl-β-D-galactoside (100 μM) in the washing buffer. The intensity of fluorescence was monitored with 360-nm excitation and 448-nm emission. The detection limit of the EIA was as low as 5 pg/ml. The recovery of BDNF (61.8 pg/ml) exogenously added into the homogenizing buffer following disruption of the rat hippocampus was 80.5%. The value of BDNF content thus obtained was expressed without correction.

Each experiment, measured in triplicate, was repeated at least three times. The results are presented as mean ± SD. Statistical significance was assessed using a one-way ANOVA, followed by post hoc Tukey's test.

3. Results

3.1. Nicergoline attenuates activated microglia-induced neuronal cell death

Unstimulated microglia did not exert any toxic effects on neuronal cells in neuron–microglial co-cultures (Fig. 1A). Neurons in these cultures, stained with an anti-MAP2 antibody, did not exhibit any abnormalities, showing intact cell bodies and dendrites (Fig. 1D). When microglia were activated by LPS (1 μg/ml) and IFN-γ (100 ng/ml), they were transformed into the ameboid form. These activated microglia induced severe neuronal cell death. Damaged neuronal cells in neuron–microglial co-cultures exhibited many fragmented neurites and swollen cell bodies (Fig. 1B), accompanied with dendritic beading and reduced numbers of MAP-2-positive neurons (Fig. 1E). Bead formation is considered to represent dendritic toxicity [14]. The addition of 30 μM nicergoline inhibited the activation of microglia and subsequent neuronal cell death (Fig. 1C), significantly reducing dendritic beading (Fig. 1F). In neuron–microglial co-cultures stimulated with LPS and IFN-γ, neuronal survival rate was reduced to approximately 20% in 24 h. The addition of 3 or 30 μM nicergoline, however, significantly increased the neuronal survival rate in a dose-dependent manner to 33 and 44%, respectively (Fig. 1G). Nicergoline had no effect on the microglial population. The addition of nicergoline to unstimulated microglia did not affect neuronal survival. LPS and IFN-γ treatment alone did not affect neuronal survival (data not shown).

3.2. Nicergoline suppresses the production of proinflammatory cytokines and superoxide anion by activated microglia

To investigate the effect of nicergoline on proinflammatory cytokine production by activated microglia, we tested the levels of IL-1β, IL-6, and TNF-α in the supernatants of LPS-stimulated microglial cultures using cytokine-specific ELISA kits. Nicergoline significantly suppressed both TNF-α and IL-1β production at 30 μM and IL-6 production at 3 and 30 μM (Figs. 2A–C). Same results were obtained at LPS and IFN-γ stimulation. To examine the effect of nicergoline on free radical formation by microglia, we examined the production of superoxide anions by microglia stimulated with LPS (1 μg/ml) and IFN-γ (100 ng/ml). Nicergoline significantly inhibited the production of superoxide anions by activated microglia at 3 and 30 μM (Fig. 2D).

3.3. Nicergoline attenuates the neuronal cell death in neuron–astrocyte co-cultures

Unstimulated astrocytes did not exert any toxic effects on neuronal cells in neuron–astrocyte co-cultures (Fig. 3A). MAP-2-positive neurons had normal cell bodies and dendrites (Fig. 3D). However, astrocytes also produce inflammatory cytokines at higher LPS concentration (10 μg/ml). When co-cultures were stimulated with LPS (10 μg/ml) and IFN-γ (100 ng/ml), the neurons exhibited fragmented neurites and shrunken cell bodies (Fig. 3B). The number of MAP-2-positive neurons decreased and exhibited disrupted dendrite formation (Fig. 3E). The stimulation of neuron–

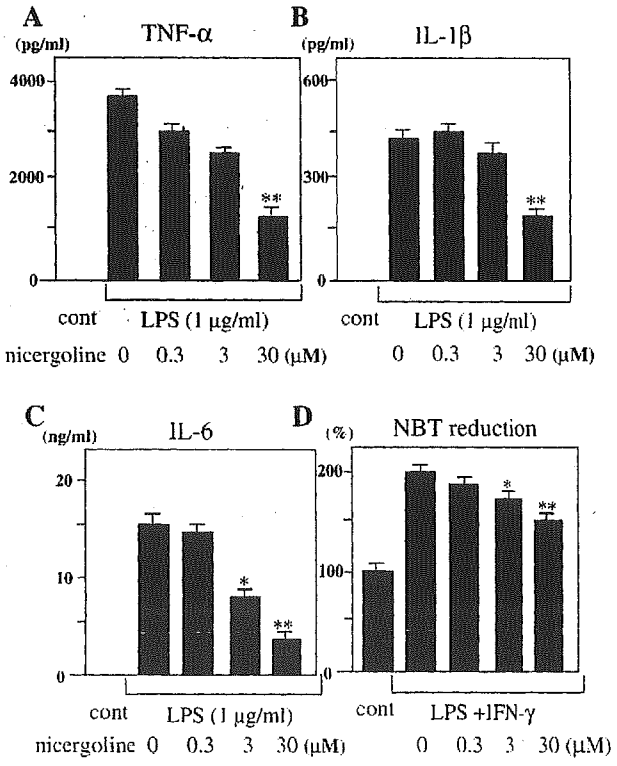

Fig. 2. The effect of nicergoline on proinflammatory cytokine production and superoxide anion by activated microglia. Microglia were treated with LPS (1 μg/ml) for 48 h. The production of the proinflammatory cytokines (A) TNF-α, (B) IL-1β, and (C) IL-6 were evaluated by EIA. $*P < 0.05$, $**P < 0.01$ versus LPS alone. (D) Superoxide anion formation was detected by NBT reduction assay. Microglia treated with various concentrations of nicergoline were stimulated with LPS (1 μg/ml) and IFN-γ (100 ng/ml) for 48 h. $*P < 0.05$, $**P < 0.01$ versus LPS and IFN-γ. Data represent the mean ± SD of five independent experiments.

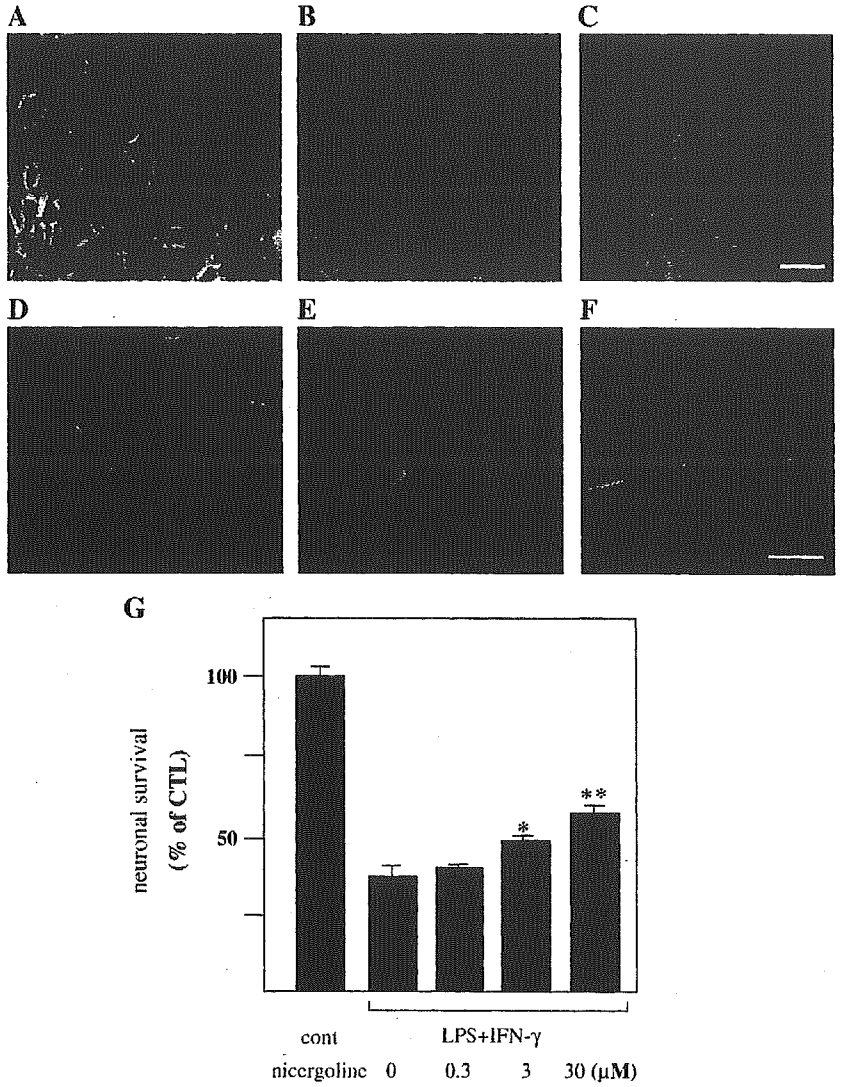

Fig. 3. Neuronal cell death by activated astrocytes and neuroprotection by nicergoline. In neuron–astrocyte co-cultures, astrocytes were stimulated with LPS (10 μg/ml) and IFN-γ (100 ng/ml) for 48 h in the presence or absence of nicergoline. Panels A–C display phase-contrast images in which neuronal cells are indicated with black arrows. Panels D–F exhibit MAP-2-positive neuronal cells. Cultures were either (A) (D) left unstimulated, (B) (E) stimulated with LPS (10 μg/ml) and IFN-γ (100 ng/ml), or (C) (F) given nicergoline (30 μM) following the activation of astrocytes with LPS and IFN-γ. (G) Neuronal survival rates following nicergoline treatment were normalized such that the viability of untreated cells was set to 100%. $*P < 0.05$, $**P < 0.01$ versus LPS and IFN-γ alone. Data represent the mean ± SD of five independent experiments. Scale bars: 20 μm.

astrocyte co-cultures with LPS and IFN-γ reduced the neuronal survival rate to 35% in 24 h. The addition of 30 μM nicergoline inhibited neuronal cell death (Fig. 3C), preserving intact cell bodies and dendrites (Fig. 3F). The addition of 3 or 30 μM nicergoline significantly increased the neuronal survival rate to 48 and 56%, respectively (Fig. 3G).

3.4. Nicergoline suppresses the production of proinflammatory cytokines and upregulates the generation of BDNF by activated astrocytes

At 30 μM, nicergoline significantly suppressed TNF-α, IL-1β, and IL-6 production by activated astrocytes, as in microglia (Figs. 4A–C). Same results were obtained at LPS and IFN-γ stimulation. The production of superoxide anions by activated astrocytes treated with LPS and IFN-γ, however, was not inhibited by nicergoline (Fig. 4D).

As astrocytes also produce neurotrophic factors, we investigated the upregulation of NGF and BDNF production following treatment with nicergoline. Nicergoline did not change the mRNA expression and protein levels of NGF in LPS-stimulated astrocytes (Fig. 5A). However, both mRNA expression and protein levels of BDNF were dose dependently increased in LPS-stimulated astrocytes (Fig. 5B).

4. Discussion

In this study, we demonstrate that nicergoline suppressed neuronal cell death induced by activated microglia or

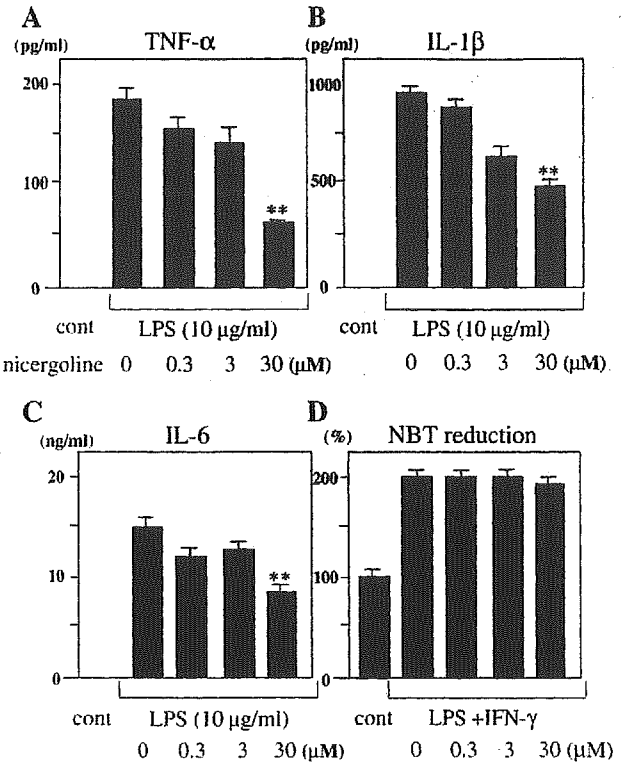

Fig. 4. The effect of nicergoline on proinflammatory cytokine production and superoxide anion by activated astrocytes. Astrocytes were treated with LPS (10 μg/ml) for 48 h. The production of the proinflammatory cytokines (A) TNF-α, (B) IL-1β, and (C) IL-6 were evaluated by EIA. $^{**}P < 0.01$ versus LPS alone. (D) Superoxide anion formation was detected by NBT reduction assay. Astrocytes treated with various concentrations of nicergoline were stimulated with LPS (1 μg/ml) and IFN-γ (100 ng/ml) for 48 h. Data represent the mean ± SD of five independent experiments.

astrocytes. Upon activation, both types of glial cells release a variety of proinflammatory cytokines, NO, ROS, and peroxynitrite, all of which are toxic to neurons. Although the beneficial and deleterious effects of microglial activation remain highly debated [40], microglia may have a pathological role in both demyelinating and neurodegenerative disorders. Alzheimer's disease (AD) is characterized by an accumulation of β-amyloid in neuritic plaques surrounded by activated microglia and astrocytes [17]. Increased levels of TNF-α, inducible nitric oxide synthase, and the peroxynitrite marker, nitrotyrosine, have been observed in the brains of patients with AD [15,36]. As TNF-α is the major neurotoxic agent secreted by β-amyloid-stimulated microglia [8], and cross-talk between the TNF-α and mitogen-activated protein kinase/c-Jun N-terminal kinase (MAPK/JNK) signaling pathways potentially provides a mechanism for neuronal cell death in the setting of chronic AD [9]. IL-1β is also neurotoxic, promoting ischemic injury in rat retinal ganglion cells [42]. Although IL-6 promotes the in vitro survival, differentiation, and growth of neurons, hippocampal neurogenesis is reduced by IL-6 [38]. In culture, IL-6 treatment reduces the number of granule neurons and enhances neurotoxicity mediated through NMDA receptors [27]. Peroxynitrite, which is formed by the action of NO and ROS, may also play a significant role in neurotoxicity [41]. In this study, nicergoline suppressed proinflammatory cytokine production by both microglia and astrocytes. While superoxide anion generation by microglia was suppressed by nicergoline, as observed in previous reports [43], superoxide anion formation by astrocytes was not inhibited by nicergoline treatment. The reason for this discrepancy remains unclear. Although we did not observe an inhibition of NO production by either microglia or astrocytes by nicergoline in this study (data not shown), the production of peroxynitrite may be suppressed by reducing the generation of superoxide anions in microglia. It has been shown that nicergoline has antioxidant activity, preventing not only GSH depletion but also lipid peroxidation [37]. Thus, suppression of proinflammatory cytokine and superoxide anion production may contribute to the favorable effects of nicergoline on neuronal cell survival. The neuroprotective effect observed in these co-culture system may also include a direct effect on neurons. There are some reports that nicergoline can exert a direct protective effect on neurons. Nicergoline has a neuroprotective role to reduce the extracellular glutamate concentration through its effect on glutamate transporters [22], and nicergoline protects cultured neurons against β-amyloid toxicity by inducing transforming growth factor-β and glial-derived neurotrophic factor [5]. Moreover, the degeneration of cholinergic neurons induced by nerve growth factor deprivation is inhibited by nicergoline [12].

Phosphodiesterase inhibitors (PDEI) increase cytoplasmic cAMP, resulting in the downregulation of nuclear factor κB (NF-κB) and the activation of cAMP responsive element-binding protein (CREB) [25] via protein kinase A (PKA)-mediated phosphorylation. Suppression of either the activation and/or translocation of NF-κB results in the depression of proinflammatory cytokine and NO produc-

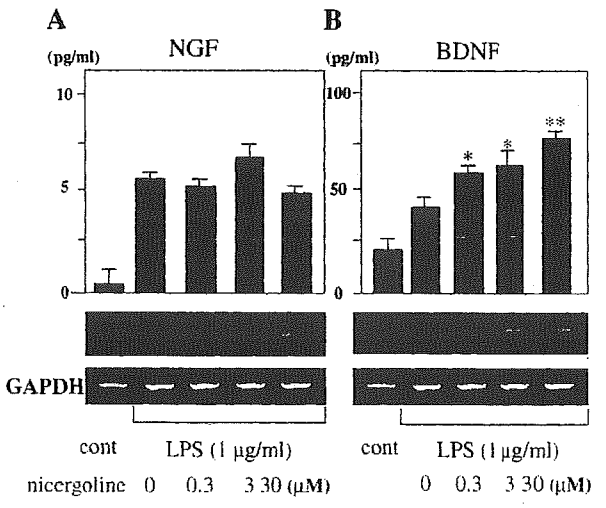

Fig. 5. The effect of nicergoline on NGF and BDNF production by activated astrocytes. Data indicate (A) NGF and (B) BDNF mRNA expression and protein production at 48 h. $^{*}P < 0.05$, $^{**}P < 0.01$ versus LPS alone.

tion. We previously showed that the PDEI ibudilast inhibited the generation of proinflammatory cytokines, NO, and ROS and suppressed neuronal cell death induced by activated microglia [21]. Nicergoline reportedly also leads cAMP elevation [26]. The inhibition of proinflammatory cytokine production may be due to a similar mechanism.

Neurotrophic factors are critical in the regulation of neuronal cell survival. NGF and BDNF promote neuronal survival via the ligation of surface tyrosine kinase receptors [3]. The nicergoline-mediated upregulation of such BDNF may also support neuronal cell survival. The reduction of extracellular glutamate concentrations by nicergoline through an effect on glutamate transporters [22] may also be involved in neuroprotection.

Nicergoline is now widely and safely used to treat patients with stroke, and this compound is known to cross the blood–brain barrier. The inhibitory effect on activated microglia and astrocytes supports the potential use of nicergoline in the treatment of inflammatory and neurodegenerative disorders.

Acknowledgments

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Research report

Relapse of methamphetamine-seeking behavior in C57BL/6J mice demonstrated by a reinstatement procedure involving intravenous self-administration

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Abstract

There is an urgent need to develop a reliable mouse model of relapse to address the genetic factors involved in susceptibility to relapse of drug-seeking behavior by using mutant mice. This paper presents a feasible way to reinstate extinguished methamphetamine (METH)-seeking behavior. Male C57BL/6J mice acquired stable nose-poking responses for taking METH after approximately 10 daily 3-h sessions of METH (0.1 mg/kg/infusion) self-administration under a fixed ratio 1 or 2 (FR1/2) schedule. During the self-administration, cue- and hole-lamps indicated the availability of METH and were inactivated simultaneously with each infusion for 5 s. The mice were exposed to extinction training in the absence of METH-paired stimuli (cue- and hole-lamps) and METH infusion, until they met the extinction criterion (less than 25 active responses or 30% of active responses in the stable self-administration phase on 2 consecutive days). METH-paired stimuli (cue- and hole-lamps) during METH self-administration reliably triggered a relapse of METH-seeking behavior in the absence of METH infusion. A combination of non-contingent intravenous (i.v.) priming and self-injected METH also increased the reinstatement of METH-seeking behavior in the absence of METH-paired stimuli (cue- and hole-lamps) and without METH infusion posterior to the self-injection. These results suggest that the mouse model of relapse in our study might provide a new stage for the exploration of genetic factors involved in relapse of drug dependence and of the underlying mechanisms of drugs of abuse.

Keywords: Mouse model of relapse; Methamphetamine; C57BL/6J mice; Extinction; Cue-induced reinstatement; Drug-primed reinstatement; Intravenous self-administration

1. Introduction

A major clinical problem in treating drug abusers or addicts is relapse of drug dependence even after long-term abstinence. To address this issue, an experimental reinstatement procedure has been established in monkeys and rats [37], which reliably represents relapse of drug-seeking behavior in addicts. This is based on findings of similarities in the development of drug dependence between humans and animals, including compulsive drug taking [9,13,24,44,45], cue-induced relapse [14,15,20,30,38], drug-primed relapse [21,26,39] and stress-triggered relapse [38,39] of drug-seeking behavior. In the typical reinstatement paradigm, the ability of acute exposure to drugs or non-drug conditioned cues to reinstate operant conditioning responses is determined following drug self-administration and subsequent extinction of the drug-reinforced behavior [7,37]. Using this paradigm, potential anatomical neuronal substrates and transmitter systems have been identified [18,19,36,39,46], and clinical data support these findings [3,22,23,25,27].

Clinical therapeutic candidates have also been tested using the reinstatement procedure in experimental animals [37,42]. However, no direct evidence has been obtained of genetic factors contributing to susceptibility to relapse although there is evidence of considerable individual variability in the propensity for relapse to drug seeking and taking in human addicts or animals [2,10,12,13,17,42,45]. Since most genetically modified model animal strains are inbred mice, there is an impetus to develop self-administration and reinstatement procedures for

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mice [6,12,20,26,31,32,37,42]. In the last decade, mouse models of i.v. self-administration have been well developed and used to identify several specific genes involved in the acquisition and maintenance of drug dependence, including serotonin 1B receptor [35], acetylcholine receptor containing the beta2 [32], dopamine transporter [34], mu-opioid receptor [33], cannabinoid receptor 1 [11,40], metabotropic glutamate receptor 5 [8], dopamine D2 receptor [5,10,16], Kir3 potassium channel subunit, Homer 2 [28], Homer 2 [41], activator of G protein signaling 3 [4], etc. In contrast, a model of relapse has only just been established in 129 × 1/SvJ and C57BL/6 mice, respectively, by two groups of researchers [20,26]. Both strains exhibited a conditioned stimulus-induced, but not intraperitoneal (i.p.) cocaine-primed, reinstatement of extinguished cocaine-seeking behavior, although 129 × 1/SvJ mice demonstrated a modest i.v. cocaine-primed reinstatement. However, it seems to be difficult to extend the reinstatement procedures presented in those two reports to transgenic mice since no further study has been published to date. Thus it largely remains to be determined which genes are specifically involved in the propensity for relapse.

Over the past several years, abuse of METH has spread worldwide, resulting in serious health and social issues [2,8,29,43]. Similar to other drugs of abuse, a major clinical concern in the treatment of METH addiction is relapse even after long-term abstinence [2,9,43]. However, no research has been conducted to develop reliable procedures to reinstate METH-seeking behavior in mice. In the present study, a newly developed reinstatement procedure was used to characterize the cue-induced and METH-primed reinstatement of extinguished METH-seeking behavior in C57BL/6J mice.

2. Materials and methods

2.1. Subjects and drugs

Male C57BL/6J mice (8 weeks old; SLC, Japan) weighing 20–25 g were used in this study. All mice were kept in a regulated environment (23 ± 0.5 °C; 50 ± 0.5% humidity) with a 12-h reverse light/dark cycle (lights on between 9:00 a.m. and 9:00 p.m.). Water and food were available ad libitum throughout the experiment unless otherwise noted. All experiments were performed in accordance with Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. METH hydrochloride (Dainippon Pharmaceutical, Osaka, Japan) was dissolved in sterile saline and self-administered at a dose of 0.1 mg/kg/infusion over 5 s (infusion volume, 2.1 μl). The unit dose for METH self-administration is based on previous reports [1,6] and our pilot observations.

2.2. Apparatus for nose-poking task and self-administration

Nose-poking task and the subsequent self-administration were conducted in standard mouse operant conditioning chambers (ENV-307A, Med Associates, Georgia, Vermont, USA) located within ventilated sound attenuation cubicles.

For nose-poking task, the chamber was equipped with two nose-poke sensors (ENV-313M, Med Associates) in two holes, two cue-lamps in and above each hole, and a food pellet dispenser (ENV-203-20, Med Associates) connected with a rectangular opening (2.25 cm × 2.25 cm) between the two holes. The bottom of the opening was 5 mm above the chamber floor and was equidistant from the holes. A house light was located at the top of the chamber opposite the holes. During the training period, nose-poking responses in the active hole resulted in the delivery of a pellet to the opening by the dispenser (ENV302M, Med Associates). Nose-poking responses in the inactive hole had no programmed consequences but were recorded by the software MED-PC for Windows (Med Associates).

For self-administration, the operant conditioning chambers were equipped with nose-poke sensors (ENV-313M, Med Associates) in two holes located on one side of the chamber 1.0 cm above the floor, cue- and hole-lamps located, respectively, above and in each hole, and a red house light located on the top of the chamber opposite the holes. Nose-poking responses in one hole (the active hole) activated an infusion pump (PHM-100, Med Associates) and led to the inactivation of METH-associated cues (cue- and hole-lamps). Nose-poking responses in the other hole (the inactive hole) had no programmed consequences but were recorded by the software MED-PC for Windows. The components of the infusion line were connected to each other from the injector to the exit port of the mouse's catheter by joint FEP tubing (inner diameter, 0.25 mm; outer diameter, 0.55 mm; Eicom Co., Ltd., Japan), which was encased in steel spring leashes (Instech, Plymouth Meeting, PA, USA). Swivels were suspended above the operant conditioning chamber. One pump/syringe set was used for each self-administration chamber located inside of the cubicle.

2.3. Nose-poking training under food-deprived conditions

Nose-poking training was conducted in the standard operant chamber mentioned above. Mice were deprived of food for at least 12 h and then trained to make nose-poking responses to food pellet (dustless precision pellets 20 mg, A Holton Industries Co., Frenchtown, NJ, USA) reinforcement under a fixed ratio 1 (FR1) schedule for 2–8 h. After the nose-poking response training, mice were returned to home cages for at least 24 h before surgery.

2.4. Catheter implantation

At least 24 h following the nose-poking training, mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Indwelling catheters were constructed using a micro-silicone tube (inner diameter, 0.50 mm; outer diameter, 0.7 mm; IMG, Imamura Co., Ltd., Japan) and a polyethylene tube (inner diameter, 0.50 mm; outer diameter, 0.8 mm). Incisions were made on the skin of the head and ventral neck, and the right jugular vein was externalized. The end of the catheter was inserted into the jugular vein via a small incision and was secured to the vein and surrounding tissue with silk sutures. The exit port of the catheter passed subcutaneously to the top of the skull where it was attached to a modified 24-gauge cannula, which was secured to the mouse's skull with quick self-curing acrylic resin (Shofu Inc., Japan). To extend their patency, the catheters were flushed immediately after surgery, and in the morning and evening of the days that followed, with 0.03 ml of an antibiotic solution of cefmetazole sodium (20.0 mg/ml; Sankyo Co., Ltd., Japan) dissolved in heparinized saline (70 U/ml; Leo Pharmaceutical Products, Ltd., Japan). Patency was usually confirmed by taking blood back from the catheter. The intravenous injection of 0.15 ml of pentobarbital sodium solution was also used for such a purpose in a few cases (e.g. responses of mice were much different from the previous session in the same phase).

2.5. Saline or METH self-administration

After recovery from surgery, saline or METH self-administration was conducted for 10 consecutive daily 3-h sessions during the light cycle under an FR1 (day 1–4) to FR2 (day 5–10) schedule of saline or METH reinforcement. METH was self-administered at a dose of 0.1 mg/kg/infusion over 5 s (infusion volume, 2.1 μl) for the METH self-administration group of mice ($n = 50$). Saline was self-administered at 2.1 μl/infusion over 5 s for the saline self-administration group of mice ($n = 5$). The house light was illuminated throughout the session. Nose-poking responses in the active hole resulted in a 5-s simultaneous activation of the infusion pump and inactivation of cue- and hole-lamps. During the remaining timeout period, responses in the active hole had no consequences. Throughout the session, responses in the inactive hole had no programmed consequences but were recorded by the software MED-PC for Windows. In order to facilitate METH self-administration, food was not available in the chambers during the daily sessions. METH self-administration was continued until the deviations

were less than 15% of the mean of active responses on 3 consecutive training days. At the end of the METH self-administration training phase, 32 mice of the METH self-administration group demonstrated a clear ability to discriminate active from inactive responses and then were subjected to extinction training. The other 18 mice were excluded from the analysis of METH self-administration data since the mice could not acquire stable METH self-administration because of a failure of catheter patency.

2.6. Extinction training

The extinction training conditions were the same for all groups of mice ($n=32$) in both cue-induced and drug-primed reinstatement of METH-seeking behavior. During the extinction phase, the syringe was removed from the infusion pump and METH was unavailable. The cue- and hole-lamps were not presented during these sessions. Accordingly, the nose-poking responses in the previous active hole were disassociated from METH infusion. The house light was on throughout extinction sessions. Nose-poking responses in the previous active hole were included in the number of active responses. Nose-poking responses in the previous inactive hole were included in the number of inactive responses. Extinction sessions were conducted daily (6–10 days) until the criterion was met (less than 25 active responses or 30% of active responses in the stable self-administration phase on 2 consecutive days). Accordingly, the mice could not discriminate the active from inactive nose-poking responses when the criteria were achieved in the late phase of extinction training. Two mice could not meet the extinction criterion and were excluded from all the data in this study.

2.7. Cue-induced reinstatement testing

Tests for cue-induced reinstatement were conducted under the conditions used in the extinction sessions but with METH-paired cue- and hole-lamps. Following the last extinction session, mice ($n=16$) were separated into two groups. One group of mice (no relapse (No-RLP) group, $n=8$) was subjected to an additional 3-h extinction session (without METH and METH-paired cue- and hole-lamps), whereas the other group of mice (cue-induced relapse (Cue-RLP) group, $n=8$) was subjected to a 3-h cue-induced reinstatement testing session. During the cue-induced reinstatement session, cue- and hole-lamps were on, which indicated the availability of METH during the self-administration phase. However, METH was unavailable after mice made active nose-poking responses under the same schedule of reinforcement as that in the self-administration phase. Nose-poking responses in previously active holes and inactive holes were recorded by the software MED-PC for Windows, respectively. Throughout the cue-induced reinstatement session, the house light was on.

2.8. METH-primed reinstatement testing

Following the last extinction session, mice ($n=14$) were separated into three groups. Two groups of mice ($n=4$ for each group) were subjected to i.p. METH-primed reinstatement testing at doses of 0.5 mg/kg and 1.0 mg/kg METH. Another group of mice ($n=6$) was subjected to both i.v. METH injection (0.2 mg/kg) and self-injected METH (0.5 mg/kg) primed reinstatement testing. For i.p. METH-primed reinstatement testing, mice were placed into the operant chamber and subjected to a 3-h test session immediately after 0.5 mg/kg or 1.0 mg/kg of METH was administered intraperitoneally. During the test session, conditions in the test chamber were the same as those in extinction sessions. For reinstatement induced by both i.v. injection of METH and self-injected METH primed reinstatement, mice were first administered 0.2 mg/kg of METH intravenously via the catheter, then immediately placed into the operant conditioning chamber for METH self-administration in the absence of cue- and hole-lamps (house light was on) until they self-administered 0.5 mg/kg of METH (total METH intake, including passive and active intake, 0.7 mg/kg) within 1 h. Subsequently, mice were subjected to a 3-h test session, in which the drug-primed reinstatement of METH-seeking behavior was controlled by the computer program used in the extinction phase in the absence of both METH infusion and predictive cue- and hole-lamps but with the house light on throughout the test session.

2.9. Data analysis

All data were expressed as the mean ± S.E. For statistical analyses, a repeated two-way measure of variance (ANOVA) was performed for the nose-poking responses during the self-administration and the subsequent extinction training phase, followed post hoc by the Bonferroni/Dunn test. The other data in our study were examined with the one-way ANOVA, followed post hoc by the Bonferroni/Dunn test. In all cases, a significant difference was set at $P<0.05$.

3. Results

3.1. Saline or METH self-administration and extinction training

After the same number of sessions of self-administration under an FR1/FR2 schedule of reinforcement (FR1 on day 1–4 and FR2 on day 5–10), mice in the saline group (Fig. 1A) could not discriminate active from inactive nose-poking responses. By contrast, mice in the METH self-administration group could gradually discriminate active from inactive nose-poking responses (Fig. 1B, a repeated measure two-way ANOVA, $F_{(2,599)}=222.35$, $P<0.001$), suggesting that the increase in active nose-poking responses in the METH self-administration group of mice depended on the METH reinforcement. Then, mice exhibited stable active nose-poking response rates during the late phase of METH self-administration with a within-subject variability of less than 15% in daily active nose-poking responses for at least 3 consecutive days. The mean number of active nose-poking responses to METH reinforcement during the stable phases of self-administration was 58.7 ± 2.9 in a daily 3-h session. The average amount of METH taken by mice of the METH self-administration group ($n=30$) during a daily 3-h session was 1.89 ± 0.12 mg/kg.

Following the stable responses to METH self-administration, the mice were then subjected to daily 3-h sessions of extinction training. The data in Fig. 1C demonstrate the operant conditioning behavior in the first 2 days and the last 4 days during the extinction training phase. The subsequent daily 3-h sessions of extinction training resulted in a gradual decrease in active nose-poking responses. After 6–10 daily 3-h sessions of extinction training, almost all the mice met the extinction criterion (less than 25 active responses or 30% of active responses in the stable self-administration phase during a daily 3-h session on 2 consecutive days). The data for two mice obtained during the self-administration, extinction and reinstatement phases were excluded since the mice could not meet the extinction criterion after 10 sessions of extinction training. On the last day of the extinction phase, the average number of active nose-poking responses (16.1 ± 3.2) was significantly small as compared with that (80.4 ± 4.9) on the first day of the extinction phase (Fig. 1C, a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test, $P<0.001$) and that (59.4 ± 3.0) on the last day of self-administration (Fig. 1B and C, a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test, $P<0.001$). During the last two sessions of the extinction, the mice could not discriminate the active (e.g. 16.1 ± 3.0 for the last session) from the inactive (13.8 ± 5.6 for the last session) nose-poking responses, whereas the mice could discriminate the

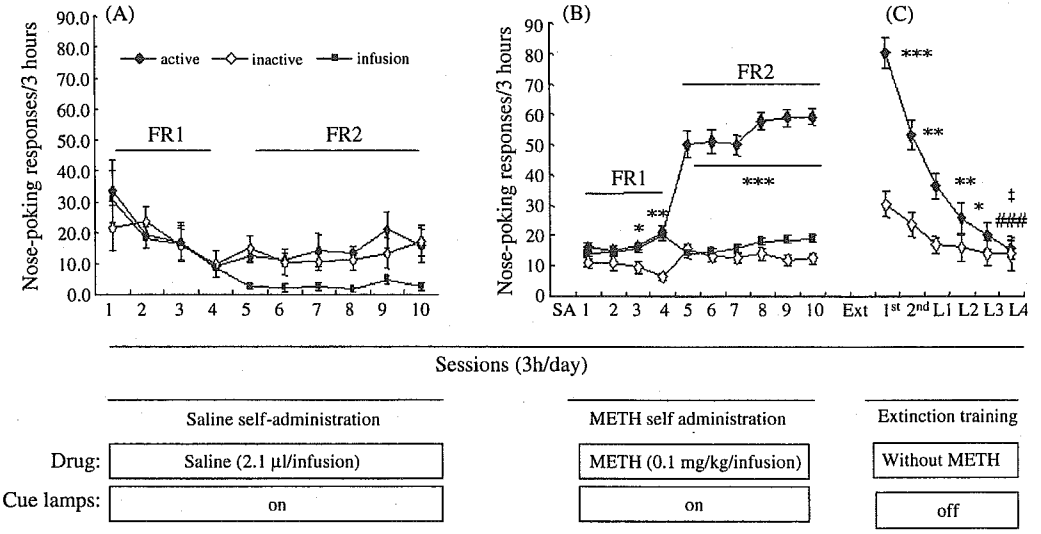

Fig. 1. Nose-poking responses during saline self-administration in the saline group ($n=5$), and METH self-administration and the extinction training phase in the reinstatement group ($n=30$). (A) Active, inactive, nose-poking responses and the number of infusions during 10 daily 3-h sessions of saline self-administration in the saline group. (B) Active, inactive, nose-poking responses and the number of infusion during METH self-administration in the reinstatement group. (C) Active and inactive nose-poking responses during extinction training in the reinstatement group. The data were from the first 2 daily 3-h sessions (indicated by 1st and 2nd) and the last 4 daily 3-h sessions (indicated by L1, L2, L3 and L4, respectively) during 6–10 extinction training sessions. Data are presented as the mean ± S.E. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ active vs. inactive nose-poking responses during METH self-administration and extinction (a repeated two-way measure of variance (ANOVA), followed post hoc by the Bonferroni/Dunn test). $^{\#\#\#}P<0.001$ vs. active nose-poking responses on the last day of the self-administration phase. $^{\ddagger}P<0.001$ vs. active nose-poking responses on the first day of extinction training (a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test).

active (80.4 ± 4.9) from the inactive (30.6 ± 4.5) nose-poking responses in the first session of extinction training. These data suggested that the METH-paired active nose-poking responses were extinguished after 6–10 sessions of extinction training.

3.2. Cue-induced reinstatement of METH-seeking behavior

After meeting the extinction criterion, mice ($n=16$) were separated into two groups. The No-RLP group of mice ($n=8$) were subjected to an additional 3-h session of extinction training on the following day. As shown in Fig. 2, the mice in this group showed similar nose-poking responses to those in the last day of the extinction training phase, which were significantly lower than those in the last day of the METH self-administration phase (a one-way ANOVA followed post hoc by the Bonferroni/Dunn test, $P<0.001$). On the following day, after exposure to both cue- and hole-lamps as in the METH self-administration phase, the Cue-RLP group of mice ($n=8$) made a significantly larger number of active nose-poking responses (a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test, $P<0.001$) than the No-RLP group of mice although METH was not delivered. However, there was no difference in inactive nose-poking responses between the Cue-RLP and the No-RLP group, suggesting that contingent cues could reliably reinstate METH-seeking behavior in the Cue-RLP group of mice. During the reinstatement period, the Cue-RLP group of mice could also discriminate the active responses, previously associated with METH infusion, from the inactive nose-poking responses (a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test, $P<0.001$).

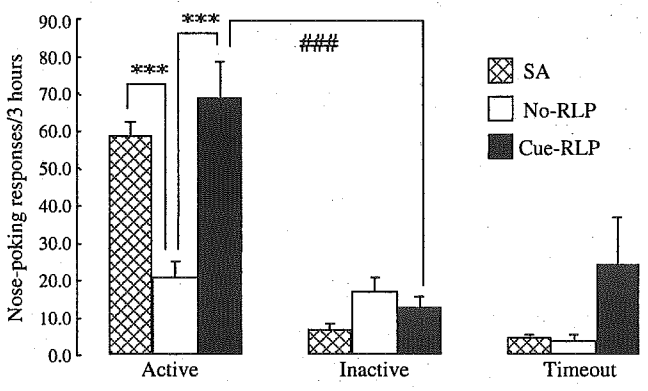

Fig. 2. Nose-poking responses in all groups of mice during METH self-administration (SA, $n=16$), in the control group of mice (No-RLP, without cue- and hole-lamps and METH infusion, $n=8$) and in the cue-induced reinstatement group (Cue-RLP, $n=8$). Data are presented as the mean ± S.E. $^{***}P<0.001$ vs. active nose-poking in the No-RLP group. $^{\#\#\#}P<0.001$ vs. inactive nose-poking responses in the Cue-RLP group. Statistical analysis was conducted with a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test. RLP: relapse.

3.3. METH-primed reinstatement of drug-seeking behavior

After meeting the extinction criterion, mice ($n=14$) were separated into three groups. Two groups of mice ($n=4$ for each group) were subjected to an additional 3-h session of i.p. priming-induced reinstatement testing immediately after the i.p. priming injection of METH (0.5 mg/kg or 1.0 mg/kg) on the following day. Compared with active nose-poking responses on the last day of the extinction training phase (extinction group, $n=14$), two i.p. priming groups of mice (Fig. 3) failed to show reinstatement of METH-paired active nose-poking responses, respectively, at 0.5 mg/kg or 1.0 mg/kg of METH. Another group of mice ($n=6$), on the following day, were firstly administered 0.2 mg/kg of METH via the catheter, then immediately placed

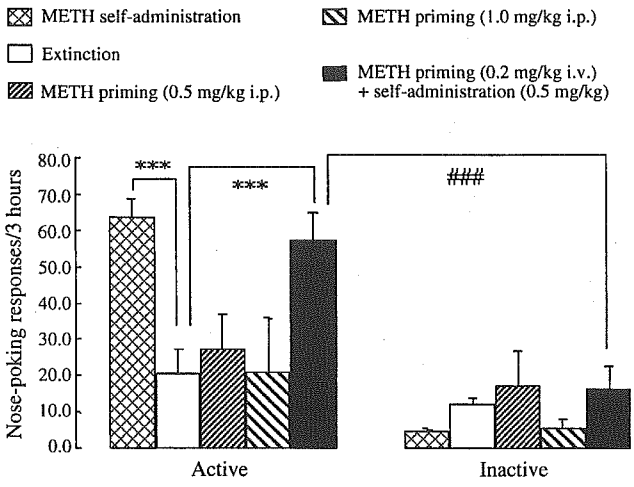

Fig. 3. Nose-poking responses in all groups of mice during METH self-administration (SA, $n = 14$), on the last day of extinction training (extinction group, $n = 14$) and in different subgroups during the METH-primed reinstatement test. Data are presented as the mean ± S.E. $^{***}P < 0.001$ vs. active nose-poking responses in the last session of extinction. $^{###}P < 0.001$ vs. inactive nose-poking responses during the METH-primed reinstatement test. Statistical analysis was conducted with a one-way ANOVA, followed post hoc by the Bonferroni/Dunn test.

into the chamber for self-administration (without METH-paired cue- and hole-lamps) until 0.5 mg/kg of METH was earned within 1 h. Subsequently, the reinstatement was measured in the mice for an additional 3-h session, in which METH was unavailable and without METH-paired cue- and hole-lamps. The mice in this group demonstrated more active nose-poking responses (Fig. 3, a one-way ANOVA followed post hoc by the Bonferroni/Dunn test, $P < 0.001$) compared with active nose-poking responses on the last day of the extinction training phase (extinction group). The mice could also discriminate active from inactive nose-poking responses during reinstatement testing (Fig. 3, a one-way ANOVA followed post hoc by the Bonferroni/Dunn test, $P < 0.001$).

4. Discussion

A new procedure for reinstating extinguished METH-seeking behavior in mice was established in the present study. Two groups of researchers have already reported similar procedures using cocaine in mice [20,26]. However, no further investigation based on their reinstatement procedures has been published to date. Compared with the previous procedures, the present procedure has the following advantages. First, a nose-poking response task was introduced instead of the more difficult lever-pressing response task. Lever-pressing is hard by mice, since the task is complicated for them and their muscle is not strong enough to press the lever in relation to reinforcement. Body weight of mice for experiments (8-week-old) usually is 20–30 g. Therefore, it seems to take longer time for mice to acquire lever-pressing responses. On the contrary, nose-poking task is very easy for mice, since the mice have an inherent ability to do nose-poking responses. The introduction of the nose-poking task might be useful to facilitate the acquisition of METH self-administration. Second, instead of a single fixed ratio 1 (FR1) schedule of cocaine self-administration, an FR1/FR2 schedule of METH reinforcement was taken into the late phase of self-administration. Thus, it seemed to be easier for researchers to examine whether the mice depended on METH-taking during self-administration and whether METH-paired active nose-poking responses were extinguished after extinction training. Mice would exhibit many more active than inactive nose-poking responses if they had already depended on METH-taking. In contrast, mice would emit similar numbers of active and inactive nose-poking responses if they have already been extinguished of METH-seeking behavior. Third, the present reinstatement procedure under an FR1/FR2 schedule seemed to be useful to shorten the duration (6–10 days in our study) of the extinction training phase. In the present experiment, only two mice were excluded since they could not meet the extinction criterion (less than 25 active responses or 30% of active responses at the stable self-administration phase during a daily 3-h session on 2 consecutive days) after 10 sessions of extinction training. In contrast, Fuchs et al. have shown that mice are considerably resistant to extinction (18.3 ± 2.7 days) to reach a similar criterion (less than 25 active responses in a 2-h test session), although it could not be excluded that the different extinction duration between cocaine-conditioned lever-press responses and METH-conditioned nose-poking responses in mice results from different mechanisms between cocaine- and METH-conditioning effects. Together, the present procedure for the METH self-administration, extinction and cue-induced reinstatement is a feasible, even useful, mouse model of drug-seeking behavior with which to identify target genes involved in the relapse of drug dependence, since there is only one limitation for developing intravenous self-administration and the subsequent reinstatement of drug seeking in mice, which is the relatively short duration of jugular catheter patency. However, it remained unclear in the present study whether the new reinstatement procedure could be extended to other drugs of abuse such as nicotine or other inbred strains of mice such as 129 × 1/SvJ mice.

Consistent with previous studies using cocaine in 129 × 1/SvJ and C57BL/6 mice [20,26], exposure to METH-paired cues, in the present study, reliably triggered a relapse of extinguished METH-seeking behavior in C57BL/6J mice, and an i.p. priming injection of METH at the doses examined in this study failed to reinstate drug-seeking behavior in C57BL/6J mice although similar doses of i.p. METH priming reliably provoked the reinstatement of extinguished METH-seeking behavior in rats [1]. These findings suggested that the reinstatement procedure in determining extinguished cocaine-seeking behavior in C57BL/6J and 129 × 1/SvJ mice could be extended to other drugs of abuse such as METH in the present study. However, failure of the i.p. priming injection of cocaine to reinstate drug-seeking behavior has been demonstrated once again in the present study by using METH in C57BL/6J mice. Although it was postulated that C57BL/6J would demonstrate similar or lower sensitivity to the priming effect of cocaine than 129 × v1/SvJ mice [26], Fuchs et al. demonstrated clearly that wide range of cocaine doses (1–40 mg/kg, i.p.) did not reinstate cocaine-seeking behavior in C57BL/6 mice whereas Highfield et al. showed that 6 mg/kg of i.v. cocaine priming modestly rein-

stated cocaine-seeking behavior in 129 × 1/SvJ mice. Together with previous studies in rats, these findings indicate at least two possibilities. First, mice may be much more sensitive than rats to differences in the subjective effects of i.v. versus i.p. cocaine during self-administration training and reinstatement testing, thus leading to a failure of i.p. cocaine primed reinstatement in mice. Second, the reinstatement procedure itself (e.g. the short duration of extinction training, and the same day for the extinction training and the following reinstatement testing in the report from Highfield et al.) may play an important role in drug-primed reinstatement of extinguished drug-seeking behavior in mice. In our pilot studies, however, an i.v. primed injection of METH (0.2 mg/kg or 0.8 mg/kg) via the catheter also failed to reinstate METH-seeking behavior (data not showed). Therefore, we further tried to develop new procedure for METH-primed reinstatement by using a combination of i.v. injection and self-injected METH under the same self-administration, extinction schedule as METH-paired cue-induced reinstatement test. Such a combination successfully increased the reinstatement of METH-seeking behavior. Some may make the following points regarding this combination-primed reinstatement procedure. First, such a combination of priming could not exactly distinguish the reinstatement induced by passive METH-priming (i.p. priming injection) from that induced by active METH-priming (self-injection of METH). Second, the METH primed reinstatement might reflect an acute extinction behavior immediately after the cessation of the 1-h METH self-injection experience. Third, such METH-primed reinstatement might just demonstrate re-self-administration behavior after 6–10 sessions of extinction training. However, any possibility mentioned above, to some extent, might reflect relapse behavior in humans [17,29,42,43,45]. In some sense, such a combination-primed reinstatement procedure may be one way to investigate specific gene functions in drug-primed relapse until a more optimal drug-primed reinstatement procedure is established in mice.

In conclusion, the present findings suggested that the cue-induced and drug-primed reinstatement of extinguished METH-seeking behavior in the new procedure was a feasible way to identify genes involved in the relapse of drug-seeking behavior in genetically modified mouse strains and the mechanism of action of drugs of abuse. The introduction of a natural nose-poking response system and an FR1/FR2 schedule of reinforcement might shorten the duration of self-administration and extinction training phase, thus ameliorating the effectiveness of the procedure to reinstate extinguished drug-seeking behavior in mice. However, a more practical way to achieve drug-primed reinstatement in mice remains to be established.

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