

# Amphetamine Inhibits the *N*-Methyl-D-Aspartate Receptor-Mediated Responses by Directly Interacting with The Receptor/Channel Complex

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Received August 21, 2001; accepted November 21, 2001 This article is available online at <http://jpet.aspetjournals.org>

## ABSTRACT

Amphetamine (AMPH) induces behavioral sensitization and neurotoxicity primarily by enhancing the dopamine-mediated neurotransmission. However, the involvement of the *N*-methyl-D-aspartate (NMDA) receptor in AMPH-induced neuropathology is also known. Recent investigation has found that high concentration of dopamine could inhibit NMDA receptor-mediated responses by blocking the NMDA receptor channel. By virtue of the structure similarity between dopamine and AMPH, we determined whether *d*-AMPH and its analogs, *l*-AMPH and methamphetamine (MAMH), could affect the NMDA receptor-mediated [<sup>3</sup>H]*N*-[1-(2-thienyl)cyclohexyl] piperidine ([<sup>3</sup>H]TCP) binding in rat cortical membrane preparations and intracellular <sup>45</sup>Ca<sup>2+</sup> accumulation and cell death in the rat primary cortical cell cultures. AMPH concentration-dependently inhibited NMDA- and glycine-stimulated [<sup>3</sup>H]TCP binding and intracellu-

lar <sup>45</sup>Ca<sup>2+</sup> accumulation with two distinct potencies; a minor inhibition with high potency and a major inhibition with low potency. [<sup>3</sup>H]TCP binding suggested that the high-potency inhibition was produced by decreasing agonist-induced activation of the NMDA receptor channel. On the other hand, the low-potency inhibition was produced by competing with [<sup>3</sup>H]TCP binding in the NMDA receptor channel, like the action of noncompetitive antagonist of the NMDA receptor. However, AMPH analogs were less potent in inhibiting NMDA- and glycine-induced cultured cell death. Thus, this result indicates that AMPH could antagonize the NMDA receptor-mediated responses in vitro by two different mechanisms, probably, through directly interacting with two distinct sites on this receptor/channel complex.

Amphetamine (AMPH), a commonly abused psychostimulant, induces behavioral sensitization or neurotoxicity primarily by enhancing the activities of dopaminergic systems (Robinson and Becker, 1986; Kalivas and Stewart, 1991; Segal and Kuczenski, 1992; Robinson and Berridge, 1993). However, available evidences have indicated the involvement of the *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptors. Administration of NMDA receptor antagonists effectively prevented or attenuated a variety of AMPH-induced responses such as behavioral sensitization, neuronal degeneration, and gene expression, indicating that activation of the NMDA receptor is required for the development and expression of AMPH-induced neuropathological responses (Karler et al., 1989; Lowy, 1990; Hemrick-

Luecke et al., 1992; Bristow et al., 1994; Wang et al., 1994; Konradi et al., 1996). The recruitment of the NMDA receptor-mediated neurotransmission in AMPH-mediated response has been thought to be as a result of an increase in glutamate release (Nash and Yamamoto, 1992, 1993) and an increase in the sensitivity to glutamate agonist (White et al., 1995) of the dopaminergic neurons after repeatedly being exposed to AMPH. Recent investigation had shown that dopamine at concentrations higher than 100  $\mu$ M was able to inhibit the NMDA receptor-mediated electrophysiological responses in a concentration-dependent manner by directly blocking the NMDA receptor channel (Castro et al., 1999). Given that AMPH shares a structural similarity with dopamine (Fig. 1) and that the dopamine transporter could potentially uptake both compounds, we proposed that AMPH might be able to regulate directly the NMDA receptor-mediated activities. To test this possibility, we determined whether AMPH analogs could affect the NMDA receptor-mediated responses in vitro, including NMDA- and glycine-stimulated [<sup>3</sup>H]TCP binding in

This work is supported by National Science Council of Taiwan Grants 85-2331-B-038-027-M27 and 86-2314-B-038-006.

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; AMPH, amphetamine; MAMH, methamphetamine; [<sup>3</sup>H]TCP, [<sup>3</sup>H]*N*-[1-(2-thienyl)cyclohexyl] piperidine; LDH, lactose dehydrogenase; MEM, modified Eagle's medium; D-APV, 2-amino-5-phosphopentanoic acid; 7-CKA, 7-cholorkyneurenic acid; PCP, phencyclidine; ANOVA, analysis of variance; MAMPH, *d*-methamphetamine.

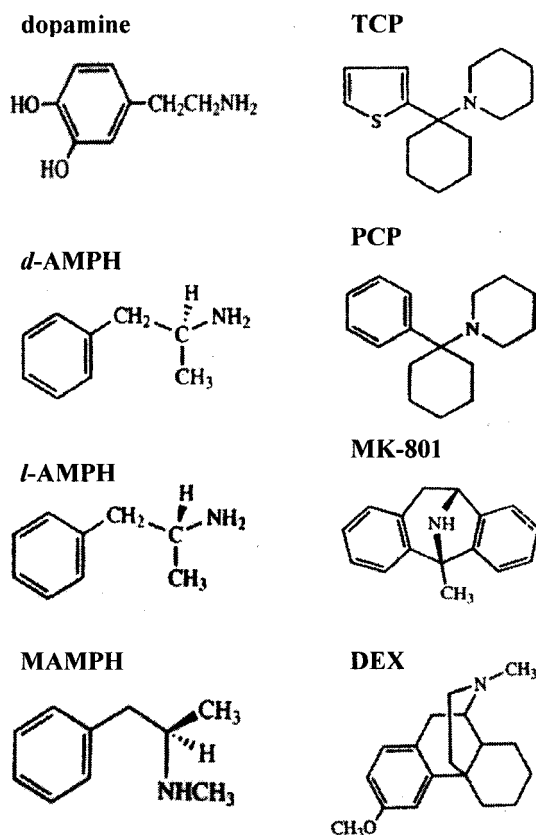


Fig. 1. The chemical structures of dopamine, *d*-AMPH, *l*-AMPH, MAMPH, TCP, PCP, MK-801, and dextromethorphan (DEX).

membrane preparation of rat brain tissues and NMDA- and glycine-elicited intracellular  $^{45}\text{Ca}^{2+}$  accumulation and cell death in rat primary cortical cell cultures. The result of this study provided evidences that AMPH could directly inhibit the NMDA receptor-mediated responses by interacting with two distinct sites on this receptor/channel complex.

## Experimental Procedures

**Animals.** Male adult Sprague-Dawley rats (200–250 g, Animals Center of National Science Council, Taiwan) or pregnant female rats were maintained a 12-h light/dark cycle with free access to food and water. All procedures were provided by the Taipei Medical University Animals Center.

**Materials.** Materials were obtained from the following companies. *S*(+)-D-Amphetamine sulfate, *R*(-)-L-amphetamine sulfate, and *R*(-)-D-methamphetamine, MK-801, TCP, NMDA, glycine, dextromethorphan, nifipidine, 2-amino-5-phosphopentanoic acid (D-APV), dopamine from RBI (Natick, MA); 7-cholorkyneurenic acid (7-CKA) from Tocris Cookson (St. Louis, MO); MEM, fetal bovine serum, calf serum, DNase I, trypsin, *l*-glutamine from Invitrogen (Carlsbad, CA); [ $^3\text{H}$ ]TCP (45–51 Ci/mmol), [ $^3\text{H}$ ]glutamate (48 Ci/mmol), [ $^3\text{H}$ ]glycine (47 Ci/mmol), scintillating cocktail from DuPont-NEN (Boston, MA);  $^{45}\text{Ca}^{2+}$ , (5–50 mCi/mg) from Amersham (Arlington Heights, IL); all other materials were purchased from Sigma (St. Louis, MO).

**Crude Membrane Preparation.** Animals were decapitated under ether anesthesia, and the cortex, striatum, hippocampus, and cerebellum were grossly dissected. These brain tissues were subjected to intensive washing with eight cycles of homogenization, centrifugation, and resuspension in cold tris-acetate buffer (pH 7.4). The first three cycles of washing were performed in 50 mM tris-

acetate buffer containing 1 mM EDTA (pH 7.4, 4°C). The last five cycles of washing were performed in 5 mM tris-acetate buffer (pH 7.4, 4°C). Membranes were rapidly frozen in a methanol/dry ice bath, slowly thawed at room temperature before the third cycle of washing, and stored in  $-70^\circ\text{C}$  for at least 24 h before the fourth cycle of washing.

**[ $^3\text{H}$ ]TCP Ligand Binding Assay.** [ $^3\text{H}$ ]TCP is a ligand that binds to the phencyclidine (PCP) binding site inside the NMDA receptor channel. It has been widely used to identify the presence of the NMDA receptor as well as to examine the status of activation of the NMDA receptor-coupled ion channel (Bonhaus and McNamara, 1988, 1989; Kloog et al., 1988; McDonald et al., 1989; Stirling et al., 1989; Yeh et al., 1989). The binding of [ $^3\text{H}$ ]TCP in the NMDA channel requires opening of the NMDA receptor-coupled ion channel, which is controlled by the agonist binding on the NMDA binding site and strychnine-insensitive glycine binding sites (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Bonhaus et al., 1989). Consistent with these findings, the binding of [ $^3\text{H}$ ]TCP in the cortical membrane preparation was stimulated by the presence of NMDA and glycine in the incubating mixture. According to previous studies in brain membrane preparation, the binding of [ $^3\text{H}$ ]TCP is mainly affected by three different factors: the affinity of [ $^3\text{H}$ ]TCP binding site, the number of [ $^3\text{H}$ ]TCP binding sites, and the accessibility of [ $^3\text{H}$ ]TCP to its binding site (presumably located within the channel itself) (Bonhaus and McNamara, 1988, 1989; Kloog et al., 1988). The accessibility is mainly controlled by agonist occupancy of the NMDA and glycine binding site on the receptor. All three factors (affinity, number, and access) influence [ $^3\text{H}$ ]TCP binding under nonequilibrium conditions; under equilibrium conditions, only affinity and number regulate the PCP binding sites. To determine whether AMPH modified the [ $^3\text{H}$ ]TCP binding site itself (affinity or number of sites) or modified the channel-gated access of [ $^3\text{H}$ ]TCP to its site (without modifying the interaction of the ligand with its recognition site per se), [ $^3\text{H}$ ]TCP binding was measured under both nonequilibrium and equilibrium conditions. To determine the effects of AMPH and other compounds on the [ $^3\text{H}$ ]TCP binding under nonequilibrium conditions, crude membranes were incubated with 100  $\mu\text{M}$  NMDA, 10  $\mu\text{M}$  glycine, 2.5 nM [ $^3\text{H}$ ]TCP, and various concentrations of examined compounds at 25°C for 15 min. The incubations were terminated by vacuum filtration with a Skatron cell harvester (Molecular Devices Corp., Sunnyvale, CA) and Skatron Filtermat (catalog 11734; Molecular Devices Corp.). The radioactivities were measured by a liquid scintillating counter. Nonspecific bindings were the bindings in the presence of 2.5  $\mu\text{M}$  nonradiolabeled TCP. To determine the effects of AMPH and other compounds on the equilibrated [ $^3\text{H}$ ]TCP binding, crude membranes were incubated with 100  $\mu\text{M}$  NMDA, 10  $\mu\text{M}$  glycine, and 2.5 nM [ $^3\text{H}$ ]TCP at 25°C for 400 min. Various concentrations of examined compounds were added after this 400-min incubation and incubations were terminated 60 min thereafter. To determine the effects of *d*-AMPH and other compounds on the density ( $B_{\text{max}}$ ) and  $K_d$  of [ $^3\text{H}$ ]TCP binding, membranes were incubated with 100  $\mu\text{M}$  NMDA, 10  $\mu\text{M}$  glycine, and various concentrations of [ $^3\text{H}$ ]TCP (1–60 nM) at 25°C for 400 min before various concentrations of examined compounds were added. The reactions were terminated 60 min after the addition of these compounds. To determine the effects of *d*-AMPH and D-APV on the association of [ $^3\text{H}$ ]TCP, membranes were incubated with 100  $\mu\text{M}$  NMDA, 10  $\mu\text{M}$  glycine, and 2.5 nM [ $^3\text{H}$ ]TCP in the presence of various concentrations of *d*-AMPH or D-APV. Incubations were terminated at different time points between 2 and 30 min. To determine the effects of *d*-AMPH and D-APV on the dissociation of [ $^3\text{H}$ ]TCP, membranes were incubated with 100  $\mu\text{M}$  NMDA, 10  $\mu\text{M}$  glycine, and 2.5 nM [ $^3\text{H}$ ]TCP at 25°C for 400 min before addition of 10  $\mu\text{M}$  nonradiolabeled TCP with *d*-AMPH or D-APV. The incubations were terminated at different time points after the addition of TCP and AMPH or D-APV. Values for [ $^3\text{H}$ ]TCP association and dissociation rates are rate (picomoles per milligram per minute), but not rate constant. These were calculated by dividing the specific binding

[<sup>3</sup>H]TCP (picomoles per milligram) derived from a 30-min incubation by 30 min.

**[<sup>3</sup>H]Glycine Binding Assay.** To determine the effect of AMPH on the binding of [<sup>3</sup>H]glycine, membranes were incubated with 40 nM [<sup>3</sup>H]glycine and various concentrations of AMPH at 25°C for 40 min at room temperature. The incubations were terminated by centrifugation. The resulting pellets were washed three times with cold, fresh tris-acetate buffer and then digested by tissue digester (DuPont). Radioactivities were measured by a liquid scintillating counter. Nonspecific bindings were the bindings in the presence of 100 μM glycine.

**NMDA-Displaced [<sup>3</sup>H]Glutamate Binding Assay.** To determine the effect of AMPH on the NMDA-displaced [<sup>3</sup>H]glutamate binding, membranes were incubated with 20 nM [<sup>3</sup>H]glutamate and various concentrations of AMPH at 25°C for 10 min. Incubations were terminated by centrifugation. Nonspecific bindings were the bindings in the presence of 100 μM NMDA. The resulting pellets were washed three times with cold, fresh tris-acetate buffer, and were digested by tissue digester (DuPont). Radioactivities were measured by a liquid scintillating counter.

**Primary Rat Cortical Cell Cultures.** Rat embryos of gestational days 15 to 17 were removed under ether anesthesia, and the cortexes were dissected and freed from the meninges. The tissues were then incubated with 0.25% trypsin in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free modified Eagle's medium (MEM) at 37°C for 60 min. The enzyme solution was removed by aspiration, and tissues were washed with MEM containing 0.02% DNase I. Cells were mechanically dissociated by repetitive pipetting with a fire-polished glass pipette. After low-speed centrifugation, the resulting cell-containing pellet was re-suspended in MEM containing 5% fetal calf serum and 5% horse serum. The cells were plated in D-poly-lysine coated 6-well culture plates (9.62 cm<sup>2</sup>) at a final cell density of 3–5 × 10<sup>5</sup> cells/ml, and maintained for 12 to 15 days in a humidified CO<sub>2</sub> incubator. In the preliminary studies, some cultures were stained with immunocytochemical assay using anti-neuron-specific enolase (Chemicon International, Temecula, CA) and anti-gial fibrillary acidic protein (Chemicon) antibodies to determine the proportion of neuron and glial cell.

**Intracellular <sup>45</sup>Ca<sup>2+</sup> Accumulation Assay.** Primary cortical cell cultures at 12 to 15 days in vitro were incubated with 0.1 Ci/ml <sup>45</sup>Ca<sup>2+</sup>, 100 μM NMDA, and 10 μM glycine in the absence or presence of AMPH or other compounds for 2 min. The cells were washed with fresh MEM buffer three times and disrupted by 0.5 ml of 1% SDS. Radioactivities of cell solutions were counted by a liquid scintillating counter. The nonspecific <sup>45</sup>Ca<sup>2+</sup> accumulation was the <sup>45</sup>Ca<sup>2+</sup> accumulation in the cell culture incubated with 100 μM NMDA, 10 μM glycine, and 10 μM MK-801, which was about 10 to 20% of total calcium accumulation in the presence of NMDA and glycine, and was subtracted from each collected value. The 2-min incubation time was chosen according to our preliminary study, which showed that the maximal inhibition produced by low concentrations of AMPH (from 0.1 μM to 3 μM) on the NMDA- and glycine-elicited <sup>45</sup>Ca<sup>2+</sup> accumulation gradually decreased once the incubation time of AMPH and NMDA exceeded 5 min, and no significant inhibition could be observed when the incubation time exceeded 20 min. Similar phenomenon also found on the effect of NMDA receptor antagonists, like D-APV and MK-801. Their inhibitory efficacy gradually decreased, as incubation time exceeded 10 min. No inhibition was found when incubation time was exceeded 30 min. Thus, this phenomenon is likely due to the continuous accumulation of <sup>45</sup>Ca<sup>2+</sup> inside the cells through the unblocked NMDA receptor channel as time passed, which would finally saturated all the neuronal cells similar to that without the presence of antagonist. Therefore, a 2-min incubation was optimal to display the inhibitory effect of low concentrations of AMPH.

**Lactate Dehydrogenase (LDH) Assay in Primary Cortical Cell Cultures.** Cultured cells with 12 to 15 days in vitro were exposed to 100 μM NMDA and 10 μM glycine in the presence or absence of additional compounds for 30 min. A 0.2-ml medium was

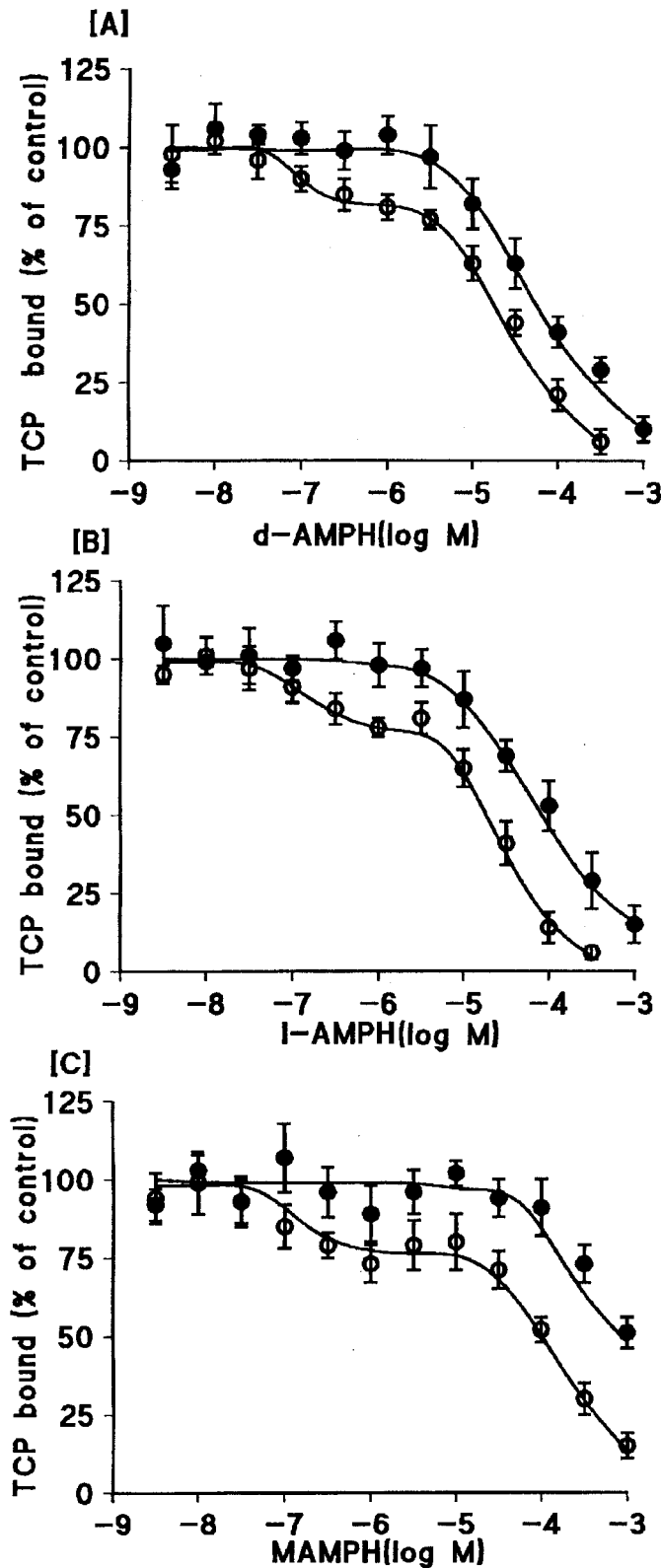
sampled 24 h after NMDA and glycine was washed away with fresh MEM. LDH was measured using an LDH-detecting kit (Sigma). Nonspecific LDH values were the values of cultures incubated with 100 μM NMDA, 10 μM glycine, and 10 μM MK-801 for 30 min and were subtracted from each collected value. Pictures of some cultured cells were taken 24 h after addition of drugs under a phase microscope (20 × 20).

**Statistics.** The EC<sub>50</sub> or IC<sub>50</sub> values of compounds were determined using a nonlinear computerized curve fitting program (GraphPad Prism software, San Diego, CA), and one- and two-site analyses were compared with determine the best fit. The K<sub>d</sub> and B<sub>max</sub> of [<sup>3</sup>H]TCP were determined using a nonlinear computerized curve fitting program (EBDA). Other data were analyzed with either Student's *t* test or with one-way ANOVA followed by a post hoc test, as appropriate. Results were considered significant when the calculated *p* value was <0.05. All data are reported as mean ± S.E.M.

## Results

**AMPH Inhibited the Nonequilibrium and Equilibrated [<sup>3</sup>H]TCP Binding.** The effect of *d*-AMPH on [<sup>3</sup>H]TCP binding was examined under both nonequilibrium and equilibrium condition. Under nonequilibrium conditions, *d*-AMPH inhibited [<sup>3</sup>H]TCP binding in a concentration-dependent manner with two apparent potencies: low concentrations (ranging from 0.01 μM to 3 μM) maximally inhibited 22 ± 2.3% of specific [<sup>3</sup>H]TCP binding with IC<sub>50</sub> of 0.15 ± 0.05 μM, and high concentrations (ranging from 10 μM to 1000 μM) inhibited the remaining specific [<sup>3</sup>H]TCP binding with IC<sub>50</sub> of 53 ± 6 μM (Fig. 2A). However, once [<sup>3</sup>H]TCP was equilibrated, addition of *d*-AMPH inhibited [<sup>3</sup>H]TCP binding with a single potency; the IC<sub>50</sub> was 77 ± 9 μM. Low concentrations of AMPH did not affect the [<sup>3</sup>H]TCP binding under this condition. We also determined the effects of *l*-AMPH and *d*-methamphetamine (MAMPH). These two compounds also inhibited the nonequilibrium [<sup>3</sup>H]TCP binding with two distinct inhibitory phases, but inhibited equilibrated [<sup>3</sup>H]TCP binding with a single phase (Fig. 2B, 2C). The maximal inhibition percentage of the high-potency effect of *l*-AMPH and MAMPH on nonequilibrium [<sup>3</sup>H]TCP binding was 21 ± 3% and 23 ± 3%, respectively, and the IC<sub>50</sub> was 0.15 ± 0.04 and 0.16 ± 0.05 μM, respectively. No difference in the IC<sub>50</sub> and maximal inhibition percentage was found between these two AMPH analogs and *d*-AMPH. On the other hand, the IC<sub>50</sub> of low-potency effect of MAMPH on nonequilibrium [<sup>3</sup>H]TCP binding was 215 ± 31 μM, which was significantly higher than that of *d*-AMPH (53 ± 7 μM) and *l*-AMPH (57 ± 8 μM) (*p* < 0.05, one-way ANOVA with Newman-Keuls test). *l*-AMPH inhibited equilibrated [<sup>3</sup>H]TCP binding with IC<sub>50</sub> of 92 ± 11 μM. Because 1 mM MAMPH could only inhibit about 50% of equilibrated [<sup>3</sup>H]TCP binding, the IC<sub>50</sub> of MAMPH was roughly close to 1 mM. We also examined the effect of competitive antagonist, D-APV and 7-CKA, and noncompetitive antagonist, MK-801, TCP, and dextromethorphan, on both nonequilibrium and equilibrated [<sup>3</sup>H]TCP binding. Under nonequilibrium conditions, D-APV and 7-CKA concentration-dependently inhibited [<sup>3</sup>H]TCP binding with single inhibitory phase. The calculated IC<sub>50</sub> for D-APV and 7CKA was 4.8 ± 0.9 μM (*n* = 3) and 0.12 ± 0.03 μM (*n* = 3), respectively (data not shown). However, neither of these two antagonists had an effect on the equilibrated [<sup>3</sup>H]TCP binding. On the other hand, MK-801, TCP, and dextromethorphan concentration-dependently inhibited nonequilibrium and equilibrated





**Fig. 2.** The effects of *d*-AMPH (A), *l*-AMPH (B), and methamphetamine (MAMPH) (C) on the nonequilibrium (○) and equilibrated (●)  $[^3\text{H}]\text{TCP}$  binding. Cortical membranes were incubated with 2.5 nM  $[^3\text{H}]\text{TCP}$ , 100  $\mu\text{M}$  NMDA, 10  $\mu\text{M}$  glycine, and various concentrations of AMPH analogs for 15 min. Values are mean  $\pm$  S.E.M. of specific  $[^3\text{H}]\text{TCP}$  binding for four experiments. The control values were the binding in the absence of AMPH, and the binding densities was  $0.22 \pm 0.03$  pmol/mg.

$[^3\text{H}]\text{TCP}$  binding with single inhibitory phase. The  $\text{IC}_{50}$  for MK-801, TCP, and dextromethorphan in inhibiting the non-equilibrium  $[^3\text{H}]\text{TCP}$  binding was  $7 \pm 3$  nM,  $11 \pm 2$  nM, and  $0.97 \pm 0.2$   $\mu\text{M}$ , respectively. The  $\text{IC}_{50}$  for MK-801, TCP, and dextromethorphan to inhibit the equilibrated  $[^3\text{H}]\text{TCP}$  binding was  $18 \pm 4$  nM,  $33 \pm 5$  nM, and  $2.1 \pm 0.3$   $\mu\text{M}$ . It is noted that the  $\text{IC}_{50}$  for these compounds to inhibit equilibrated  $[^3\text{H}]\text{TCP}$  binding were significantly higher than their  $\text{IC}_{50}$  for inhibiting nonequilibrium binding ( $p < 0.05$ , paired  $t$  test). To determine whether the inhibitory effect of AMPH has region specificity in the brain, we further examined the effects of *d*-AMPH on  $[^3\text{H}]\text{TCP}$  binding in membranes prepared from three major brain regions, namely the hippocampus, striatum, and cerebellum. In these examined brain regions, *d*-AMPH inhibited  $[^3\text{H}]\text{TCP}$  binding in a manner similar to that observed on the cortex. No significant difference in the maximal inhibition percentage or  $\text{IC}_{50}$  was found between these examined brain regions (data not shown).

**AMPH Decreased the Affinity of  $[^3\text{H}]\text{TCP}$ .** The finding that *d*-AMPH, at high concentrations ( $>10$   $\mu\text{M}$ ), could decrease both nonequilibrium and equilibrated  $[^3\text{H}]\text{TCP}$  binding suggests that AMPH may inhibit  $[^3\text{H}]\text{TCP}$  binding by decreasing the affinity or the number (or density) of the  $[^3\text{H}]\text{TCP}$  binding site. To test this possibility, we determined the effect of *d*-AMPH on the  $K_d$  and  $B_{\text{max}}$  of  $[^3\text{H}]\text{TCP}$  binding. High concentrations of AMPH (30 or 100  $\mu\text{M}$ ) increased the  $K_d$  of  $[^3\text{H}]\text{TCP}$  binding without affecting the  $B_{\text{max}}$  of the binding (Table 1). However, 1 and 10  $\mu\text{M}$  *d*-AMPH did not have any significant effect on either parameter of  $[^3\text{H}]\text{TCP}$  binding.

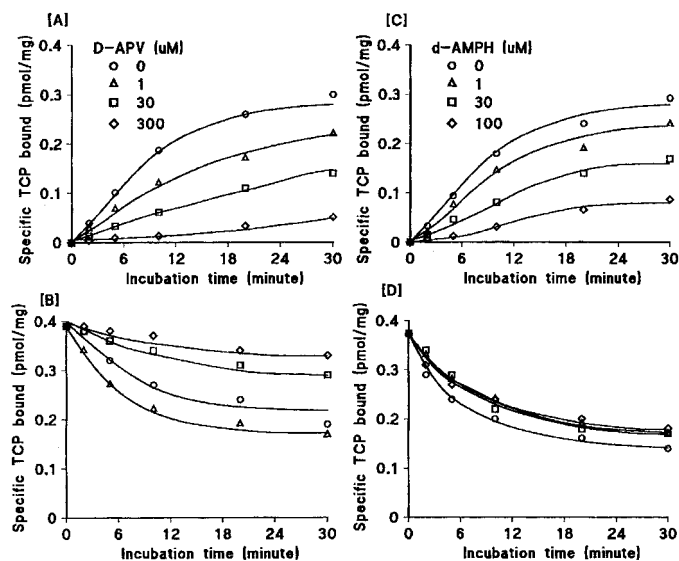
**AMPH Decreased Association Rate and Dissociation Rate of  $[^3\text{H}]\text{TCP}$ .** The finding that lower concentrations of AMPH ( $\leq 3$   $\mu\text{M}$ ) partially decreased the nonequilibrium  $[^3\text{H}]\text{TCP}$  binding without affecting the equilibrated  $[^3\text{H}]\text{TCP}$  binding suggests that AMPH, at lower concentration range, may inhibit the  $[^3\text{H}]\text{TCP}$  binding by decreasing the accessibility of  $[^3\text{H}]\text{TCP}$  to the NMDA receptor-coupled ion channel. Based upon previous studies of  $[^3\text{H}]\text{TCP}$  binding, change in the accessibility could be reflected by a paralleled change in the rate of  $[^3\text{H}]\text{TCP}$  association and dissociation in a membrane preparation (Bonhaus and McNamara, 1988, 1989; Bonhaus et al., 1989; Yeh et al., 1990). NMDA receptor agonist, like NMDA or glycine, will increase both the association rate and dissociation rate of  $[^3\text{H}]\text{TCP}$ . On the contrary, antagonist of NMDA receptor, like D-APV, will decrease both parameters. Consistent with this idea, the addition of D-APV concentration-dependently decreased both the association rate and dissociation rate in a parallel manner (Fig. 3, A and B). The association rate of  $[^3\text{H}]\text{TCP}$  calculated from 30-min incubation in the presence of 0  $\mu\text{M}$ , 1  $\mu\text{M}$ , 30  $\mu\text{M}$ , and 300  $\mu\text{M}$  D-APV was  $9.2 \pm 0.6$  fmol/mg/min ( $n = 4$ ),  $7.4 \pm 0.4$  fmol/mg/min ( $n = 4$ ),  $4.2 \pm 0.6$  fmol/mg/min ( $n = 4$ ), and  $1.7 \pm 0.4$  fmol/mg/min ( $n = 4$ ), respectively. All the association rates were significantly different to each other ( $p < 0.05$ , one-way ANOVA with Newman-Keuls test). Likewise, the dissociation rate of  $[^3\text{H}]\text{TCP}$  in the presence of D-APV was  $6.3 \pm 0.4$  fmol/mg/min ( $n = 4$ ),  $4.4 \pm 0.3$  fmol/mg/min ( $n = 4$ ),  $2.3 \pm 0.3$  fmol/mg/min ( $n = 4$ ), and  $1.2 \pm 0.4$  fmol/mg/min ( $n = 4$ ), respectively. The addition of *d*-AMPH could also decrease both the association rate and dissociation rate of  $[^3\text{H}]\text{TCP}$  but in a different manner (Fig. 3C, 3D). *d*-AMPH concentration-dependently decreased the association of  $[^3\text{H}]\text{TCP}$ . The asso-

TABLE 1

The effects of *d*-AMPH on the  $K_d$  and  $B_{max}$  of [ $^3$ H]TCP binding in the cortical membrane preparation. Data are mean  $\pm$  S.E.M. of four experiments. The  $K_d$  and  $B_{max}$  were determined by saturation binding assay.

	AMPH				
	0	1	10	30	100
$K_d$ (nM)	12 $\pm$ 2	11 $\pm$ 2.4	14 $\pm$ 3	18.4 $\pm$ 3*	24.5 $\pm$ 3.2*
$B_{max}$ (pmol/mg)	2.2 $\pm$ 0.3	1.9 $\pm$ 0.2	2.1 $\pm$ 0.4	2.1 $\pm$ 0.3	2.2 $\pm$ 0.3

\* Significantly different than that of 0  $\mu$ M AMPH (one-way ANOVA with post hoc Newman-Keuls test,  $p < 0.05$ ).



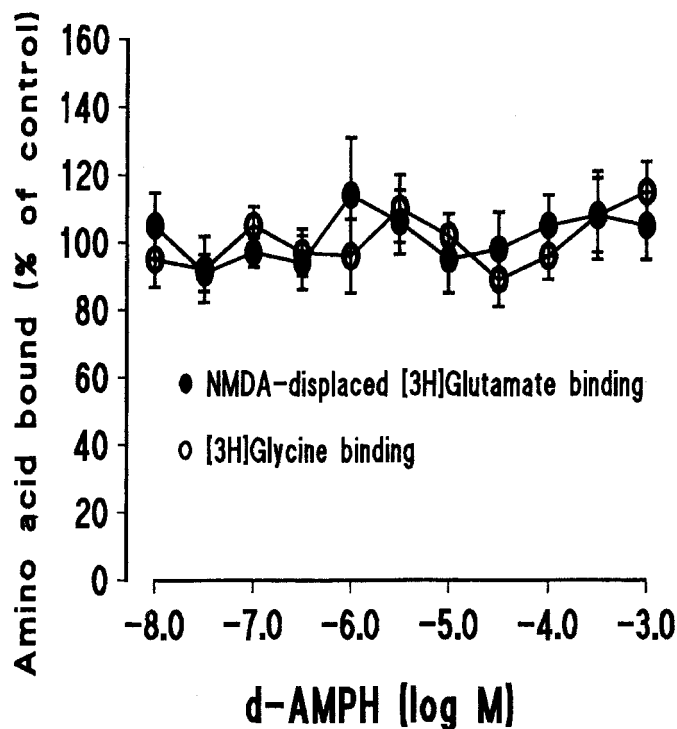
**Fig. 3.** Effects of D-APV on the association (A) and dissociation (B) of [ $^3$ H]TCP, and effects of *d*-AMPH on the association (C) and dissociation (D) of [ $^3$ H]TCP binding in the cortical membrane preparation. Values are one representative experiment of four experiments. Membrane were incubated with 2.5 nM [ $^3$ H]TCP, 100  $\mu$ M NMDA, 10  $\mu$ M glycine, and various concentrations of *d*-AMPH or D-APV following the method described under *Experimental Procedures*.

ciation rate of [ $^3$ H]TCP in the presence of 0, 1, 30, and 100  $\mu$ M *d*-AMPH was  $8.7 \pm 0.6$  fmol/mg/min ( $n = 4$ ),  $7.2 \pm 0.5$  fmol/mg/min ( $n = 4$ ),  $4.7 \pm 0.4$  fmol/mg/min ( $n = 4$ ), and  $2.6 \pm 0.3$  fmol/mg/min ( $n = 4$ ), respectively. All the association rates were significantly different to each other ( $p < 0.05$ , one way ANOVA with Newman-Keuls test). *d*-AMPH, however, inhibited the dissociation rate of [ $^3$ H]TCP to a lesser extent than that for the association rate, and the maximal inhibition was reached with 1  $\mu$ M *d*-AMPH. The dissociation rate of [ $^3$ H]TCP in the presence of 0, 1, 30, and 100  $\mu$ M *d*-AMPH was  $7.1 \pm 0.5$  fmol/mg/min ( $n = 4$ ),  $6.2 \pm 0.4$  fmol/mg/min ( $n = 4$ ),  $6.1 \pm 0.5$  fmol/mg/min ( $n = 4$ ), and  $5.8 \pm 0.4$  fmol/mg/min ( $n = 4$ ), respectively. The dissociation rate in the presence of 1  $\mu$ M *d*-AMPH was significantly lower than that in the absence of *d*-AMPH ( $p < 0.05$ , one way ANOVA with Newman-Keuls test) but was not significantly different to the rates in the presence of 30 or 100  $\mu$ M *d*-AMPH. This result supports the notion that *d*-AMPH could partially inhibit the accessibility of [ $^3$ H]TCP with potency less than 1  $\mu$ M.

**AMPH Did Not Affect the NMDA-Displaced [ $^3$ H]Glutamate Binding and [ $^3$ H]Glycine Binding.** We subsequently determined whether *d*-AMPH could decrease the accessibility of [ $^3$ H]TCP by interfering with the agonist binding on the receptor. The effects of *d*-AMPH on the NMDA-displaced [ $^3$ H]glutamate binding and [ $^3$ H]glycine binding

were examined on the cortical membrane preparation. It is worth noting that the [ $^3$ H]glycine binding in this membrane preparation was not sensitive to the addition of 10  $\mu$ M strychnine, which is consistent with the binding properties of strychnine-insensitive glycine binding sites on the NMDA receptor (data not shown). D-APV inhibited NMDA-displaced [ $^3$ H]glutamate binding with  $IC_{50}$  of  $0.79 \pm 0.3$   $\mu$ M ( $n = 3$ ), whereas 7-CKA inhibited [ $^3$ H]glycine binding with  $IC_{50}$  of  $47 \pm 9$  nM ( $n = 3$ ) (data not shown). However, *d*-AMPH (from 0.1 to 100  $\mu$ M) did not significantly affect these two ligand bindings (Fig. 4).

**AMPH Inhibited the NMDA-and Glycine-Elicited Intracellular  $^{45}Ca^{2+}$  Accumulation in Primary Rat Cortical Cell Cultures.** To further verify the antagonism of AMPH analogs on the NMDA receptor, as revealed by the ligand binding study, we examined the effects of AMPH analogs on the NMDA-and glycine-elicited intracellular  $^{45}Ca^{2+}$  accumulation and cell death in the primary rat cortical cell cultures. In the preliminary immunocytochemical studies on this cell culture, we found that this primary cor-



**Fig. 4.** Effect of *d*-AMPH on the ( $\circ$ ) [ $^3$ H]glycine binding and ( $\bullet$ ) NMDA-displaced [ $^3$ H]glutamate binding in cortical membrane preparation. Values are mean  $\pm$  S.E.M. of four experiments. Control values are the values in the absence of *d*-AMPH. The control value of NMDA-displaced [ $^3$ H]glutamate binding and [ $^3$ H]glycine binding was  $0.47 \pm 0.09$  pmol/mg and  $0.37 \pm 0.08$  pmol/mg, respectively.

tical cell culture is a mixed-type cell culture containing 18 to 25% glial cells and 75 to 82% neuronal cells on days in vitro 12 to 15 (data not shown). After a 2-min incubation, NMDA concentration-dependently increased the  $^{45}\text{Ca}^{2+}$  accumulation with  $\text{EC}_{50}$  of  $22 \pm 7 \mu\text{M}$ . Addition of  $10 \mu\text{M}$  glycine could potentiate the effect of NMDA. The effect of  $100 \mu\text{M}$  NMDA could be abolished by  $10 \mu\text{M}$  MK-801,  $10 \mu\text{M}$  TCP, and  $500 \mu\text{M}$  D-APV, but was not affected by  $10 \mu\text{M}$  nifedipine and  $10 \mu\text{M}$  veratridine, two voltage-dependent calcium channel blockers (data not shown). High concentrations of KCl (50 and  $100 \text{ mM}$ ) could also elicit  $^{45}\text{Ca}^{2+}$  accumulation, but the magnitudes of increase, by subtracting the accumulation in the absence of KCl, were only about 20 to 25% of that induced by  $100 \mu\text{M}$  NMDA, and the increase was not inhibited by MK-801. The addition of MK-801, magnesium, dextromethorphan TCP, D-APV, and 7-CKA concentration-dependently inhibited the NMDA- and glycine-elicited  $^{45}\text{Ca}^{2+}$  accumulation with single inhibitory phase. The  $\text{IC}_{50}$  was  $17 \pm 3 \text{ nM}$ ,  $0.6 \pm 0.1 \text{ mM}$ ,  $1.8 \pm 0.3 \mu\text{M}$ ,  $28 \pm 4 \text{ nM}$ ,  $12 \pm 3 \mu\text{M}$ , and  $0.21 \pm 0.03 \mu\text{M}$ , respectively (data not shown). *d*-AMPH itself did not induce  $^{45}\text{Ca}^{2+}$  accumulation but concentration-dependently inhibited NMDA- and glycine-elicited  $^{45}\text{Ca}^{2+}$  accumulation with two distinct inhibitory phases; low concentrations of *d*-AMPH ( $\leq 3 \mu\text{M}$ ) inhibited  $21 \pm 3\%$  of specific  $^{45}\text{Ca}^{2+}$  accumulation with an  $\text{IC}_{50}$  of  $0.32 \pm 0.09 \mu\text{M}$ , and high concentrations of *d*-AMPH ( $\geq 10 \mu\text{M}$ ) inhibited the remaining  $^{45}\text{Ca}^{2+}$  accumulation with an  $\text{IC}_{50}$  of  $67 \pm 8 \mu\text{M}$  (Fig. 5). *l*-AMPH and MAMPH also inhibited the  $^{45}\text{Ca}^{2+}$  accumulation in a similar way. The  $\text{IC}_{50}$  for high-potency effect of MAMPH and *l*-AMPH was  $0.19 \pm 0.07$  and  $0.22 \pm$

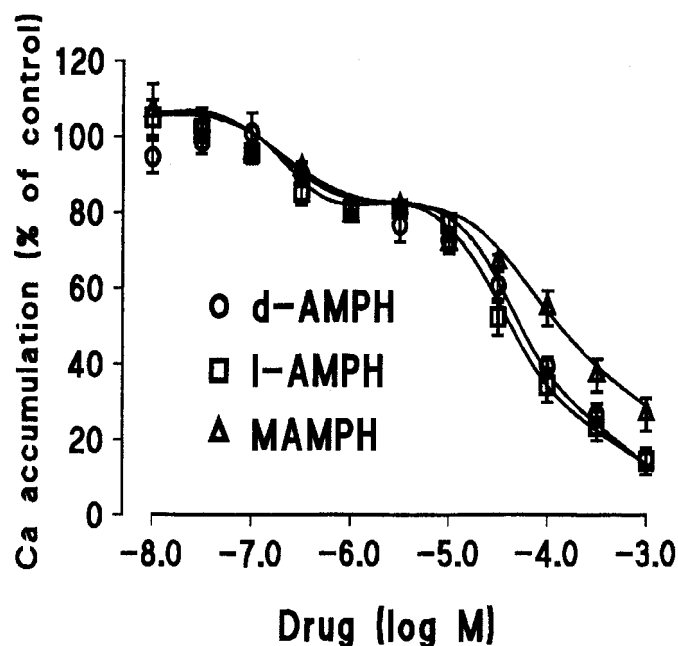
$0.08 \mu\text{M}$ , respectively. The  $\text{IC}_{50}$  for low-potency effect of MAMPH and *l*-AMPH was  $294 \pm 27$  and  $71 \pm 12 \mu\text{M}$ , respectively. Again, no significant difference in the  $\text{IC}_{50}$  of high-potency effect was found between these three AMPH analogs, but the  $\text{IC}_{50}$  of low-potency effect of MAMPH was significantly higher than that of *l*-AMPH or *d*-AMPH ( $p < 0.05$ , one-way ANOVA with Newman-Keuls test).

**AMPH Inhibited NMDA- and Glycine-Induced LDH Release in Primary Rat Cortical Cell Cultures.** We subsequently examined the effects of AMPH analogs on the NMDA-induced cell death in the culture (Choi, 1990). A 30-min incubation of NMDA induced apparent cell death as visualized by phase microscopy (data not shown). Addition of *d*-AMPH attenuated the NMDA-induced cell loss in a concentration-dependent manner, and *d*-AMPH alone did not produce apparent cell death. We used the LDH assay as a quantitative measurement for cell death. NMDA induced LDH release in a concentration-dependent manner with  $\text{EC}_{50}$  of  $35 \pm 15 \mu\text{M}$ . Addition of  $10 \mu\text{M}$  glycine did not further increase the NMDA-induced LDH release. The effect of NMDA ( $100 \mu\text{M}$ ) could be abolished by NMDA receptor antagonists  $10 \mu\text{M}$  MK-801,  $10 \mu\text{M}$  TCP,  $100 \mu\text{M}$  dextromethorphan, or  $500 \mu\text{M}$  D-APV (data not shown). The inhibitory potency of MK-801, TCP, or dextromethorphan was  $0.11 \pm 0.04 \mu\text{M}$  ( $n = 3$ ),  $0.21 \pm 0.07 \mu\text{M}$  ( $n = 3$ ), and  $33 \pm 5 \mu\text{M}$  ( $n = 3$ ), respectively (data not shown). AMPH analogs alone did not increase the LDH release, but concentration-dependently inhibited NMDA- and glycine-induced increase in the LDH concentration (Fig. 6). The  $\text{IC}_{50}$  of *d*-AMPH and *l*-AMPH was  $217 \pm 32$  and  $192 \pm 24 \mu\text{M}$ , respectively. The  $\text{IC}_{50}$  of MAMPH could not be calculated because the maximal inhibition for  $1 \text{ mM}$  MAMPH was less than 50%. It is noted that low concentrations ( $< 10 \mu\text{M}$ ) of these three AMPH analogs did not have any significant effect on the NMDA- and glycine-induced LDH release.

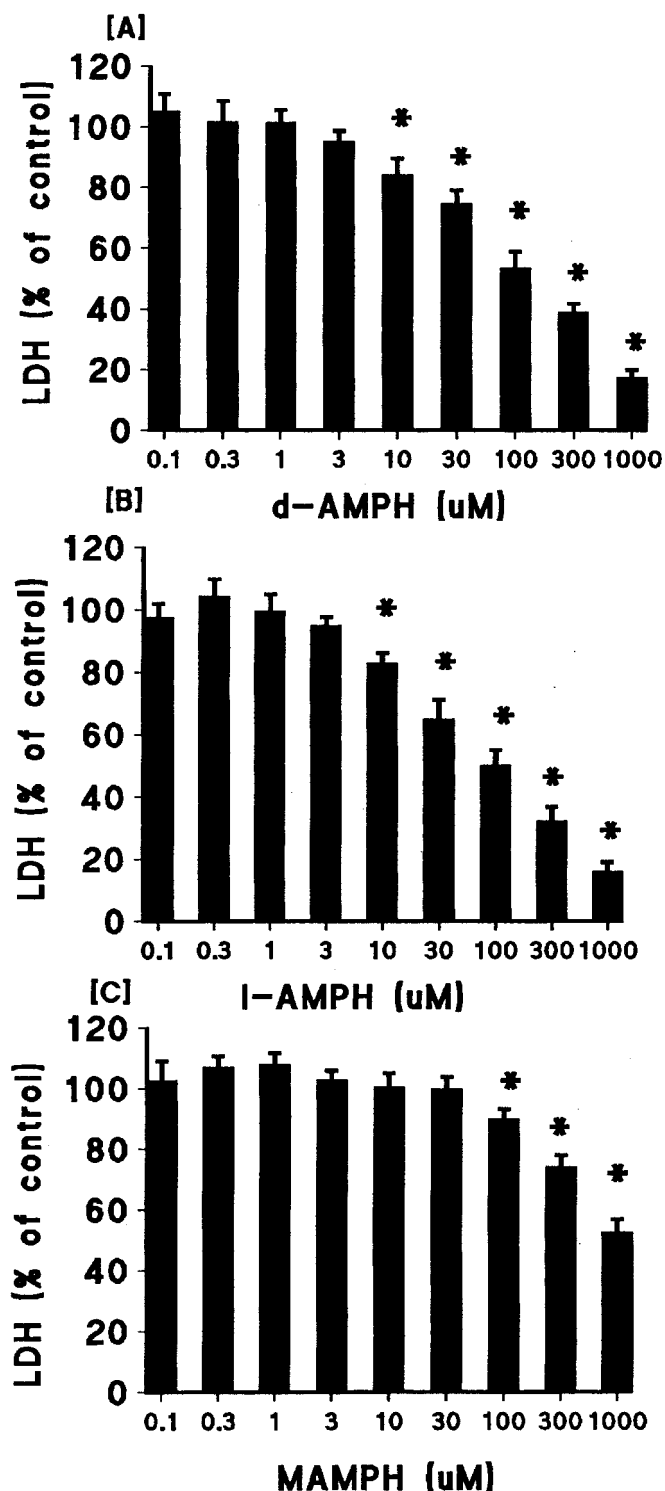
**Addition of Dopamine Receptor Antagonists, Chlorpromazine, Haloperidol, and *S*(-)-Sulpride Did Not Reverse the Inhibitory Effect of AMPH on the NMDA- and Glycine-Elicited  $^{45}\text{Ca}^{+2}$  Accumulation and NMDA-Elicited LDH Release in the Primary Cortical Cell Cultures.** To exclude the possibility that activation of the dopamine receptor may contribute to the inhibitory effect of AMPH on the NMDA receptor, we examined whether  $30 \mu\text{M}$  chlorpromazine, a nonselective dopamine receptor antagonist;  $10 \mu\text{M}$  haloperidol, a D1/D2 receptor antagonist; and  $10 \mu\text{M}$  *S*(-)-sulpride, a D2 receptor selective antagonist, could reverse the inhibitory effect of *d*-AMPH on the NMDA- and glycine-elicited  $^{45}\text{Ca}^{+2}$  accumulation and LDH release in the primary cortical cell cultures. All these dopamine receptor antagonists did not affect the inhibitory effect of AMPH on the NMDA receptor-mediated responses (Fig. 7).

## Discussion

The principle finding in this report is that AMPH could inhibit the NMDA receptor-mediated biochemical responses in vitro, including [ $^3\text{H}$ ]TCP binding in membrane preparation, and  $^{45}\text{Ca}^{2+}$  accumulation and cell death in primary cortical neuronal cultures. The finding that dopamine antagonists could not reverse the inhibitory effects of AMPH excludes the involvement of dopamine and its receptor. Therefore, these inhibitory effects are specific for AMPH, and are



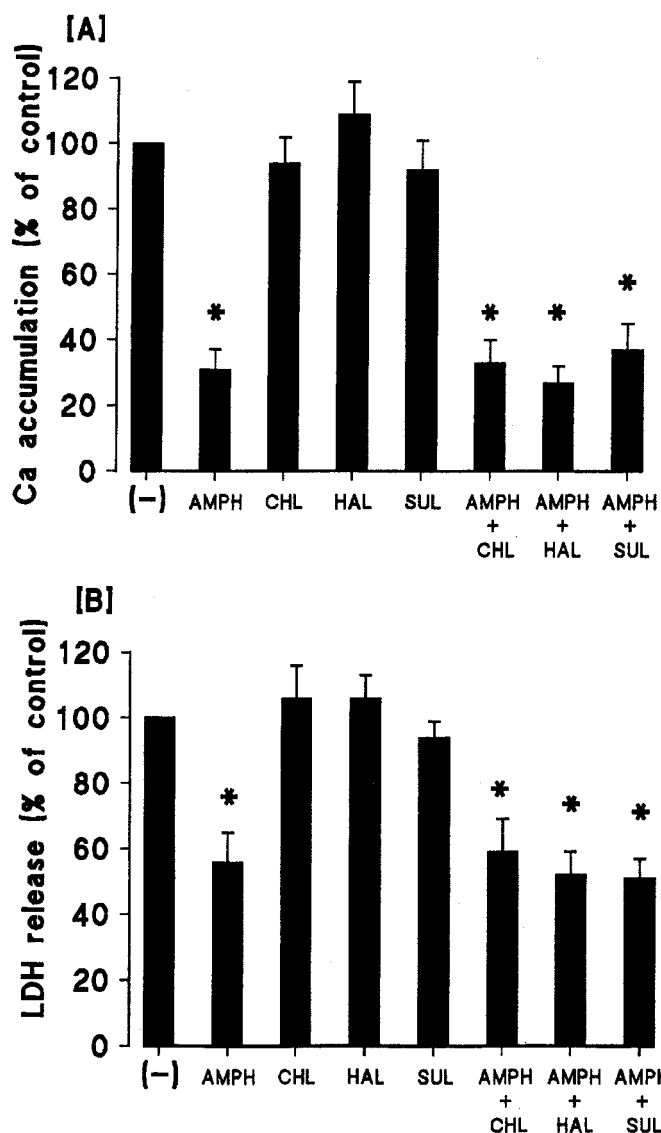
**Fig. 5.** Effects of *d*-AMPH (○), *l*-AMPH (□), and methamphetamine (MAMPH) (△) on the NMDA- and glycine-elicited  $^{45}\text{Ca}^{2+}$  accumulation in the primary cortical cell culture. The cells were incubated with these compounds following the described experiment methods. Values are mean  $\pm$  S.E.M. of six experiments from six different batches of cultures. Nonspecific values were the values in the presence of  $100 \mu\text{M}$  NMDA,  $10 \mu\text{M}$  glycine, and  $10 \mu\text{M}$  MK-801 and were subtracted from each collected value. Control values were the specific values of  $^{45}\text{Ca}^{2+}$  accumulation in cells incubated with  $100 \mu\text{M}$  NMDA and  $10 \mu\text{M}$  glycine, and the values were ranging from 2457 to 3650 cpm per culture well.



**Fig. 6.** The effects of *d*-AMPH, *l*-AMPH, and MAMPH on the NMDA- and glycine-elicited LDH release in rat primary cortical cell cultures. Cultured cells were incubated with 100  $\mu$ M NMDA and 10  $\mu$ M glycine in the absence and presence of AMPH analogs for 30 min following experimental methods described above. Values are mean  $\pm$  S.E.M. of four experiments from four different batches of cells. The control values were  $54 \pm 8$  unit/ml per cultured well. \*, significantly different to that values in the absence of AMPH ( $p < 0.05$ , paired *t* test).

due to direct action of AMPH on the NMDA receptor/channel complex.

The inhibition of the NMDA receptor-mediated responses



**Fig. 7.** The effects of antagonists of dopamine receptor on the *d*-AMPH induced inhibition on the NMDA- and glycine-elicited (A)  $^{45}\text{Ca}^{2+}$  accumulation and (B) LDH release in the primary rat cortical cell culture. Values are mean  $\pm$  S.E.M. of four experiments from four different batches of cells. Control cultures were exposed 100  $\mu$ M NMDA and 10  $\mu$ M glycine. The additional concentration of chlorpromazine (CHL), haloperidol (HAL), S(-)-sulpride (SUL), and *d*-AMPH (AMPH) was 10, 10, 10, and 100  $\mu$ M, respectively. \*, significantly different to that values in the absence of AMPH ( $p < 0.05$ , paired *t* test).

by AMPH is marked by the presence of two distinct potencies: a minor inhibitory effect with high potency (the high-potency effect) and a major inhibitory effect with low potency (the low-potency effect). This phenomenon was first demonstrated by the study of the [ $^3\text{H}$ ]TCP binding and then by the studies of the  $^{45}\text{Ca}^{2+}$  accumulation in primary cortical cell cultures. The inhibitory potencies measured by these two different biochemical assays are quite similar. Such correlation strengthens the idea that AMPH acts as an antagonist on the NMDA receptor. However, the effects of AMPH analogs in inhibiting NMDA- and glycine-elicited LDH release were much weaker than their effects in inhibiting the [ $^3\text{H}$ ]TCP binding or  $^{45}\text{Ca}^{2+}$  accumulation, and no multiple inhibitory phases was shown within the examined concentration range.



The reason for this disparity is not clear. However, the potencies of MK-801, TCP, and dextromethorphan in inhibiting NMDA- and glycine-elicited LDH release were also at least 10-fold lower than their potencies to inhibit [<sup>3</sup>H]TCP binding, suggesting that the weaker potency for AMPH to inhibit LDH release was likely due to the relative insensitivity of LDH assay to estimate the potency of the noncompetitive antagonists. In addition, the lack of inhibition on LDH release by low concentrations of AMPH may be related to its relatively low efficacy of inhibition, which could not be revealed by the LDH assay.

According to the results of the [<sup>3</sup>H]TCP binding study, the low-potency effect is a result of competitive inhibition on the [<sup>3</sup>H]TCP binding, because high concentration of AMPH decreased the affinity without significant effect on the density of [<sup>3</sup>H]TCP binding. From this aspect, the effect of AMPH is similar to that of MK-801 and dextromethorphan (Bonhaus and McNamara, 1989; Stirling et al., 1989; Franklin and Murray, 1992; Berman and Murray, 1996), which have been shown to compete with [<sup>3</sup>H]TCP binding in membrane preparation. Thus, our result suggests that AMPH at high concentrations may inhibit NMDA receptor-mediated responses by blocking the NMDA channel in its opening status, like noncompetitive antagonists of the NMDA receptor. The binding site for AMPH in the channel might be close to or at the PCP site. On the hand, the high-potency inhibitory effect of AMPH is likely a result from decreasing the accessibility of [<sup>3</sup>H]TCP to the NMDA receptor-coupled ion channel. This action is partially similar to that of competitive antagonists, like D-APV (Bonhaus and McNamara, 1988). However, AMPH did not affect the glutamate or glycine binding to the NMDA receptor, and AMPH could only partially decrease the accessibility of [<sup>3</sup>H]TCP, suggesting that AMPH, at low concentration, may act as a partial antagonist with a unique inhibitory mechanism on the agonist-induced activation of NMDA receptor-coupled ion channel.

The rank order of the potency for AMPH analogs in producing each of the two inhibitory effects on the NMDA receptor-mediated responses is quite different to each other. Although there is no significant rank order of the AMPH potency for the high-potency inhibitory effect, the rank order for the low-potency inhibitory effect is *d*-AMPH ≈ *l*-AMPH ≫ MAMPH. This finding suggests that the interacting sites for these two inhibitory effects of AMPH are independent to each other. Thus, we tentatively propose that AMPH could directly interacting with two different sites on the receptor/channel complex; namely a high-affinity site in partially inhibiting the activation of NMDA receptor channel and a low-affinity site in blocking the channel. However, our result could not exclude the possibility that the multiple potencies for AMPH to inhibit NMDA receptor-mediated responses are due to two different populations of the NMDA receptor with different sensitivity to inhibition by amphetamine, because existence of heterogeneity of the NMDA receptor population in the brain has been reported previously (Audinat et al., 1994; Farrant et al., 1994). But, the difference in the underlying mechanism between the high- and low-potency effect of AMPH suggests that this possibility is less likely than the suggestion for two distinct interacting sites for AMPH on the NMDA receptor.

Previous reports using [<sup>3</sup>H]AMPH ligand binding in either intact brain tissues or brain membrane preparation have

demonstrated specific [<sup>3</sup>H]AMPH binding sites with two different affinities (Paul and Hulihan-Giblin, 1982; Hauger et al., 1984). In general, the binding affinities of [<sup>3</sup>H]AMPH were all within a few-hundred nanomolar range. These affinities were close to that of the high-affinity site for AMPH on the NMDA receptor suggested by the present study. However, the density of [<sup>3</sup>H]AMPH binding is highest in the hypothalamus and brainstem and is quite low in the cortex and hippocampus. This regional distribution is in contrast to the distribution of the NMDA receptor in the brain, in which the cortex and hippocampus are enriched with the NMDA receptor, whereas the hypothalamus has a very low density of this receptor. Such lack of correlation in the regional distribution suggests that the majority of the specific [<sup>3</sup>H]AMPH binding sites are not identical with the AMPH interacting sites on the NMDA receptors.

Whether AMPH can inhibit the NMDA receptor-mediated neurotransmission in vivo will depend on the ambient concentration of AMPH in the brain circuits using the NMDA receptor. Theoretically, the ambient concentration of AMPH in brain circuit is primarily decided by the dosage of AMPH injected, and by the repetition of injection. So far, there is very limited information regarding the concentration of AMPH in vivo. One particular study had estimated that the extracellular concentration of AMPH in striatum could reach about 2.8, 5.6, and 11.9 μM after single injection of 1, 2.5, and 5 mg/kg AMPH, respectively. The concentration of AMPH could further approach to 30 μM after repeated injection of 15 mg/kg AMPH (Clausing et al., 1995). If this measurement of AMPH concentration is applicable, and our estimation of potencies of AMPH on the NMDA receptor is correct, single low dose AMPH (1–2.5 mg/kg) injection should provide sufficient ambient concentration of AMPH, at least in striatum, to saturate the high-affinity site of AMPH on the NMDA receptor. On the other hand, repeated injection of high dose AMPH (up to 15 mg/kg) should able to provide sufficient concentration of AMPH to interact with the lower-affinity site on the NMDA receptor to a significant extent. Nevertheless, one should be aware that the inhibitory potencies of AMPH revealed by the present biochemical assays might be not the true potencies for AMPH once applied into neuron in vivo. For more accurate estimation, electrophysiological assay using whole cell or patch clamp will be necessary.

In general, a single injection of 1 to 2.5 mg/kg AMPH could induce behavioral change, and repeated injections of these dosages will lead to behavioral sensitization. Single or repeated injections of 5 to 10 mg/kg will be neurotoxic, especially in the dopaminergic system. This raises the possibility that the antagonism property on NMDA receptor might contribute to the AMPH-induced neuropathologies if sufficient concentration of AMPH could be in the brain circuit using the NMDA receptor. However, this possibility seems to be incompatible with the general idea that activation of the NMDA receptor is required for AMPH-induced responses (Karler et al., 1989; Lowy, 1990; Hemrick-Luecke et al., 1992; Bristow et al., 1994; Wang et al., 1994; Konradi et al., 1996). Alternatively, our result may suggest that AMPH, by acting as a NMDA receptor antagonist, is neuroprotective against other neuropathologies related to over-activation of the NMDA receptor, like hypoxia-ischemia insult in brain circuits enriched with this receptor. For instance, one report has shown that repeated treatment of AMPH (2 mg/kg every 3 days) in rats



after neocortical infarction induced by ischemia accelerated neocortical neural sprouting, synaptogenesis, and behavioral recovery (Stroemer et al., 1998). The underlying mechanism for this specific effect of AMPH was not yet identified. Thus, it is worthy to determine whether the direct inhibition of AMPH on the NMDA receptor may contribute to the enhancement of neural recovery by attenuating the progression of neurodegeneration processes mediated by the NMDA receptor after the insult.

In summary, our study disclosed a novel pathway for AMPH to influence the neuronal functions in the brain by directly inhibiting the NMDA receptor, and two distinct interacting sites on this receptor/channel complex for AMPH are strongly suggested. Further investigations toward the verification of this inhibition *in vivo* and identification of the functional significance of this specific antagonism effect of AMPH are necessary.

#### Acknowledgments

We thank J. O. McNamara kindly provided the protocol of cell culture and Y. R. Chen for checking the draft of the manuscript.

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