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Distinct [^{3}H]MK-801 Binding Profiles with the Agonist, Partial Agonist, and Antagonist Acting at the Glycine Binding Site of the *N*-Methyl-D-Aspartate Receptor

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Abstract – The *N*-methyl-D-aspartate (NMDA) receptor-ion channel complex is activated by the simultaneous presence of L-glutamate and glycine, allowing the binding of MK-801 to the phencyclidine (PCP) site of the receptor. The [^{3}H]MK-801 binding assay system was established for determination of pharmacological functions of test compounds acting at the glycine site of the receptor. The binding in the presence of 0.1 μM L-glutamate was increased by an agonist (glycine) in a dose-dependent fashion, while decreased by either partial agonist (R-(+)-HA-966) or antagonist (5,7-dichlorokynurenic acid: 5,7-DCKA). To distinguish partial agonism from antagonism, various concentrations of 7-chlorokynurenic acid (7-CKA) were added in the assay to eliminate the interference of the endogenous glycine present in the membrane preparations. The bindings in the presence of L-glutamate (0.1 μM) and 7-CKA (1, 5, or 10 μM) were increased by R-(+)-HA-966. Being a weak partial agonist, the extent of potentiation was much less than that by the agonist. These binding profiles were clearly distinguishable from those by the antagonist, 5,7-DCKA, which exhibited no intrinsic activity. The binding assays established in the present study are a useful system to classify ligands acting at the glycine site of the NMDA receptor by their pharmacological functions.

Keywords □ NMDA receptor, glycine modulatory site, MK-801, radioligand binding

The *N*-methyl-D-aspartate (NMDA) receptor is a subclass of excitatory amino acid receptors which mediate excitatory neurotransmission and synaptic plasticity in the mammalian central nervous system. Over the past decades evidence has been accumulated indicating that the excessive activation of the NMDA receptor is involved in the development of a variety of neuropathological conditions such as convulsive disorders, hypoxic/ischemic neuronal damage or cell death, and acute or chronic neurodegenerative disorders. Up to date, intensive efforts have been made to develop various types of potent and selective NMDA receptor antagonists possessing anticonvulsive and neuroprotective actions (for reviews, Bigge and Malone, 1993; Leeson and Iversen, 1994; Muir and Lees, 1995).

The activity of NMDA receptor-ion channel complex is modulated through several pharmacologically distinct recognition sites. These include glutamate (NMDA) recognition site, glycine site, site recognizing ion channel blockers such as MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine) or phencyclidine (PCP), and sites for other proposed regulatory factors such as polyamines, Mg^{2+} and Zn^{2+}, respectively. Since the glycine site has been discovered by Johnson and Ascher in 1987, it has received much attention in the development of potential pharmacological agents, because the modulatory action of the NMDA receptor activity at this site is believed to be more physiological and may show less untoward behavioral effects (Huettner, 1990; Kemp and Leeson, 1993; Bigge and Malone, 1993; Leeson and Iversen, 1994; Muir and Lees, 1995).

NMDA receptor ligands were reported to regulate MK-801 binding by controlling the access to the PCP binding site located within the receptor-ion channel complex (Ransom and Stec, 1988). This finding validates the use of [^{3}H]TCP (N-[1-(thienyl)cyclohexyl]piperidine) or [^{3}H]MK-801 binding as a measure of the functional activation of the NMDA receptor-coupled ion channel in isolated membrane preparations (Bonhaus and McNa-

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mara, 1988). The abilities of L-glutamate and glycine to stimulate [^{3}H]MK-801 binding, as reported previously (Foster and Wong, 1987; Reynolds *et al.*, 1987; Wong *et al.*, 1987), are direct evidence that MK-801 binds to the activated state of the receptor-ion channel complex. The stimulation of [^{3}H]MK-801 binding by these agonists are reversed by antagonists acting at the respective recognition sites of the receptor. Therefore, analysis of the [^{3}H]MK-801 binding profiles in the presence of various concentrations of test compounds will provide a simple biochemical assay system for pharmacological characterizations of ligands.

In the present study, [^{3}H]MK-801 binding assays with the glycine site agonist, partial agonist, and antagonist were performed in an attempt to establish an assay system for the determination of pharmacological functions of test compounds. Glycine, R-(+)-HA-966 (R-(+)-3-amino-1-hydroxypyrrolid-2-one) and 5,7-dichlorokynurenic acid (5,7-DCKA) were employed as a typical agonist, partial agonist and antagonist at this site, respectively. In the presence of 0.1 μM L-glutamate, glycine showed distinct, concentration-dependent enhancement of [^{3}H]MK-801 binding as expected, while both R-(+)-HA-966 and 5,7-DCKA showed inhibition of the binding. To distinguish the binding profile of R-(+)-HA-966 from that of 5,7-DCKA, the assay system was modified. Various concentrations of 7-chlorokynurenic acid (7-CKA) were included in the [^{3}H]MK-801 binding assay system to eliminate the influence of the residual endogenous glycine present in membrane preparations. In the presence of 5 μM 7-CKA, R-(+)-HA-966 showed a concentration-dependent increase in the binding, whereas 5,7-DCKA showed no significant changes. These binding assay conditions can be utilized to determine pharmacological functions of test ligands potentially acting at the glycine site of the NMDA receptor.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats were obtained from Experimental Animal Laboratory, Korea Research Institute of Chemical Technology (KRICT), Korea. Glycine, polyethylenimine, 7-CKA, L-glutamic acid, Trizma base, sucrose, Triton X-100, were purchased from Sigma Chemical Co. (St. Louis, MO). 5,7-DCKA, R-(+)-HA-966, (+)-MK-801 were from Research Biochemicals International (Natick, MA). Bio-Rad D_C protein assay reagent was from Bio-Rad. (+)-[3-^{3}H]MK-801 (20.3 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Whatman GF/B was from Whatman. Lumagel scintillation cocktail was purchased from Lumac* LSC B.V. (Olen, Belgium). All other reagents were reagent grade or better.

Rat cortical membrane preparation

Cerebral cortices from male Sprague-Dawley rats (300-400 g) were dissected, chopped with scalpel and homogenized in 10 volumes of 0.32 M sucrose using a Teflon-glass homogenizer by 5 strokes. Following centrifugation at 1,000×g for 10 min in a Beckman J2/21 centrifuge (rotor: JA20), the supernatant was collected and centrifuged at 20,000×g for 20 min. The pellet was resuspended in 20 volumes of ice-cold distilled-water using Brinkman Polytron homogenizer (setting No. 5, 30 sec), incubated at 4℃ for 30 min, and followed by centrifugation at 8,000×g for 20 min. The supernatant and buffy uppercoat were collected and centrifuged at 39,800×g for 25 min and the pellet was stored at −70℃ overnight.

On the next day, the pellet was thawed at room temperature for 10 min, resuspended with 20 volumes of 50 mM Tris-acetate (pH 7.1 at 4℃) containing 0.04% Triton X-100, incubated at 37℃ for 20 min, and centrifuged at 39,800×g for 20 min as above. The pellet was washed three times by centrifugation as above with 20 volumes of 50 mM Tris-acetate, pH 7.1, without detergent. The final pellet was suspended in 50 mM Tris-acetate, pH 7.1, and protein concentration was determined using Bio-Rad D_C protein assay kit. The resuspending buffer volume was adjusted to give a membrane protein concentration of 1 mg/m*l*, and aliquots were stored at −70℃.

[^{3}H]MK-801 binding assay

[^{3}H]MK-801 binding assays were carried out as described by Wong *et al.* (1986) with some modifications. In brief, rat cortical membranes (200-300 μg of membrane protein per tube) were incubated at 30℃ for 60 min in a final volume of 1 m*l* reaction mixture, containing 50 mM Tris-acetate buffer, pH 7.1, 5 nM [^{3}H]MK-801, 0.1 μM L-glutamate, and various concentrations of test compound. The incubation was terminated by the addition of 2.5 ml of ice-cold, 50 mM Tris-acetate buffer, pH 7.1, and the bound radioactivity was separated using a Brandel cell harvester (Brandel M-

12R) by rapid filtration through Whatman GF/B glass fiber filter, which was presoaked in 0.3% polyethylenimine in the assay buffer. The filters were washed twice with 2.5 *ml* of cold buffer within 10 s, and trapped radioactivity on the filter was measured by a liquid scintillation counter (Beckman LS 6000TA) using 3 *ml* Luma gel at a counting efficiency of 50~55%. Non-specific binding was determined in the presence of 0.1 mM (+)-MK-801.

Test compounds were dissolved in either assay buffer (glycine), saline (R-(+)-HA-966), or dimethyl sulfoxide (5,7-DCKA), and serially diluted to appropriate concentrations for binding assays. The final concentration of DMSO in the assay mixture was 2%, and apparently did not affect the binding of radioligands at this concentration.

Assays were performed in triplicate and repeated at least two times.

RESULTS AND DISCUSSION

The NMDA receptor-ion channel complex has a number of sites for pharmacological intervention. Among these, particularly glycine binding site has drawn much attention as a drug target because of the findings that glycine site antagonists were effective as neuroprotective and anti-convulsant drugs. Their side effect profile was found to be more desirable compared to other NMDA antagonists (Leeson and Iversen, 1994). In addition, several more possible advantages were suggested (Huettner, 1991). Both the onset of inhibition and recovery from block may be faster than ion-channel blockers. Finally, a partial blockade of the glycine site, or saturation with a partial agonist, could prevent excessive channel activation while still maintaining normal synaptic transmission.

For development of new drugs acting at the glycine binding site of the NMDA receptor, it was necessary to establish efficient pharmacological evaluation methods to screen potential compounds. Initially, glycine site binding assays using [^{3}H]glycine were employed to determine their binding affinities for the target site (data not shown). To evaluate compounds with considerable binding affinities for their pharmacological functions in the receptor activation, the following assay system was investigated.

As reported previously (Foster and Wong, 1987, Ransom and Stec, 1988) the binding of [^{3}H]MK-801 to the rat cortical membrane, prepared by the methods described in the Materials and Methods, was markedly stimulated by L-glutamic acid (data not shown). In agreement with published reports (Reynolds *at al.*, 1987,

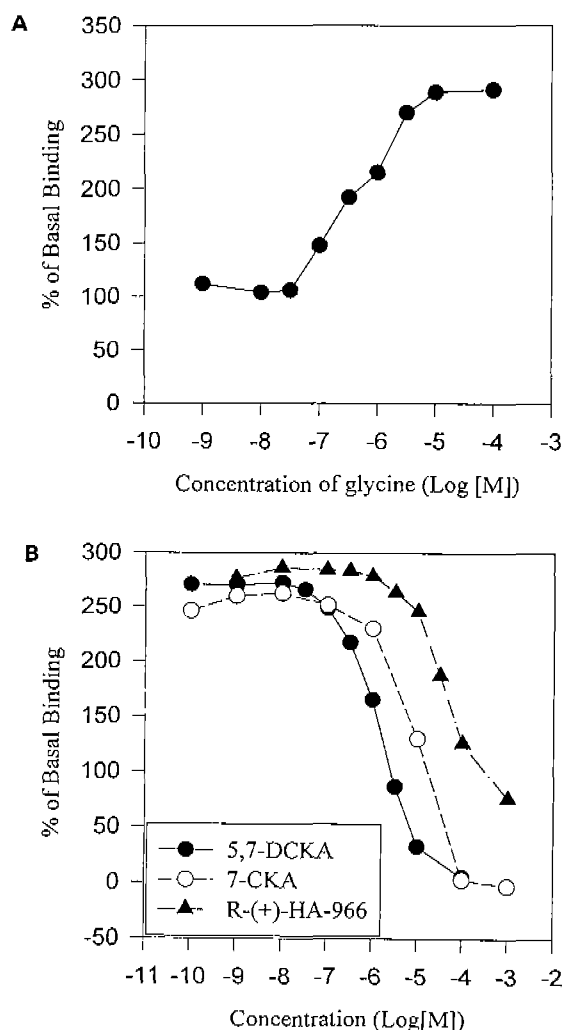

Fig. 1. A: Effect of glycine on [^{3}H]MK-801 binding. Various concentrations of glycine were incubated with [^{3}H]MK-801 (5 nM), rat cortical membrane (200-300 μg) prepared as described in the Materials and Methods, and 0.1 μM L-glutamate in a total volume of 1 *ml* of 50 mM Tris-acetate, pH 7.1 for 60 min at 30℃. Non-specific binding was defined in the presence of 0.1 mM MK-801. Data are expressed as % of basal [^{3}H]MK-801 binding, which was measured in the absence of glycine added to the reaction mixture. Representative data from 3 separate experiments, each performed in triplicate, are shown. B: Inhibition by 7-CKA, 5,7-DCKA, and R-(+)-HA-966 of [^{3}H]MK-801 binding stimulated by glycine. Various concentrations of 7-CKA or 5,7-DCKA or R-(+)-HA-966 were incubated as above, but in the presence of glycine (1 μM) added to the reaction mixture. Representative data from 2-3 separate experiments are shown.

Wong *et al.*, 1987, Ransom and Stec, 1988), glycine produced a large enhancement (>3-fold) of [^{3}H]MK-801 binding performed in the presence of 0.1 μM L-glutamic acid (Fig. 1A). This result confirms the cooperative interactions between glutamate binding site and glycine binding site, resulting in the activation of the receptor-coupled ion channel. The enhanced [^{3}H]MK-801 binding by glycine was reversed by selective glycine site antagonists 5,7-DCKA or 7-CKA, and by a partial agonist R-(+)-HA-966 (Fig. 1B). Based on these findings as well as previous findings reported by Bonhaus and McNamara (1988), the [^{3}H]MK-801 binding assay system can be utilized to assess pharmacological functions of test ligands interacting with the specific recognition sites (glycine site in this case) of the NMDA receptor.

To test whether the [^{3}H]MK-801 binding profiles can be directly used to distinguish test ligands by their functions, three ligands that have pharmacologically distinct functions in the receptor activation were employed as typical examples. As described above and shown in Fig. 1A, glycine, a typical agonist at the glycine site of NMDA receptor, produced a concentration-dependent increase in [^{3}H]MK-801 binding profile in the presence of 0.1 μM L-glutamate. However, as shown in Fig. 2, both R-(+)-HA-966 and 5,7-DCKA showed inhibition of [^{3}H] MK-801 basal binding. One can explain these observations that R-(+)-HA-966 and 5,7-DCKA functioned as inverse agonists to actively suppress responses to 0.1 μM L-glutamate in the absence of glycine (Kemp *et al.*, 1988; Ascher *et al.*, 1988; Foster and Kemp, 1988). An alternative possibility, however, is that responses obtained in the presence of 0.1 μM L-glutamate without the addition of exogenous glycine (expressed as "basal binding" in all figures) may be due to the presence of the low concentrations of endogenous glycine contaminated in the cortical membrane preparations. Therefore, we examined whether the cortical membranes used for the [^{3}H]MK-801 binding assays in the present study contain residual endogenous glycine. Based on electrophysiological studies using cultured neurons with minimized contamination of glycine and *Xenopus* oocyte system, it is now generally accepted that R-(+)-HA-966 acts as a weak partial agonist and 5,7-DCKA as an antagonist rather than being inverse agonists (Huettner, 1990; Bigge and Malone, 1993; Kemp and Leeson, 1993; Leeson and Iversen, 1994; and references therein). If this is the case, the residual glycine may have contributed to

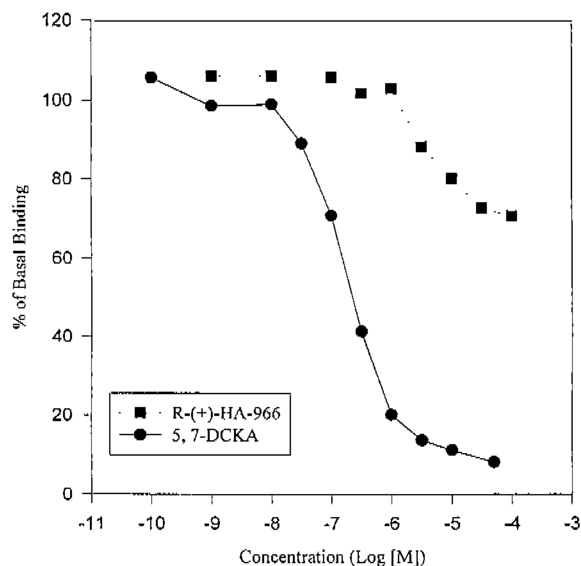

Fig. 2. Inhibition by 5,7-DCKA and R-(+)-HA-966 of the [^{3}H] MK-801 basal binding. Various concentrations of R-(+)-HA-966 or 5,7-DCKA were incubated and % of basal [^{3}H]MK-801 binding was measured as described in Fig. 1. A. Representative data from 3 separate experiments are shown.

the receptor activation in the presence of 0.1 μM L-glutamate, and the basal binding could be inhibited by R-(+)-HA-966 or 5,7-DCKA as shown in Fig. 2.

To test this hypothesis, various concentrations of 7-CKA were included in the [^{3}H]MK-801 binding assay conditions to titrate the action of glycine which may be present in the cortical membrane preparation. In the presence of 1, 5, 10 μM 7-CKA, R-(+)-HA-966 behaved exactly as a weak partial agonist, producing concentration-dependent increases in [^{3}H]MK-801 binding profiles as shown in Fig. 3. In contrast, 5,7-DCKA still produced a concentration-dependent inhibition of [^{3}H]MK-801 binding in the presence of 1 μM 7-CKA (Fig. 4). In the presence of 5 or 10 μM 7-CKA, however, 5,7-DCKA produced no significant changes in [^{3}H]MK-801 binding (Fig. 4). These results indicated that 5,7-DCKA acts as an antagonist without intrinsic activity under these conditions.

In the presence of 1 μM 7-CKA without R-(+)-HA-966 or 5,7-DCKA, the [^{3}H]MK-801 binding was decreased to 32~44% of basal binding. In the presence of 5 μM 7-CKA, the binding without test ligands was decreased to 10% of basal binding, which was maintained with 10 μM 7-CKA. Based on these results, the contaminating glycine was indeed present in our membrane preparations and the action of the residual glycine was appropriately titrated by 5 or 10 μM 7-CKA. Whether the

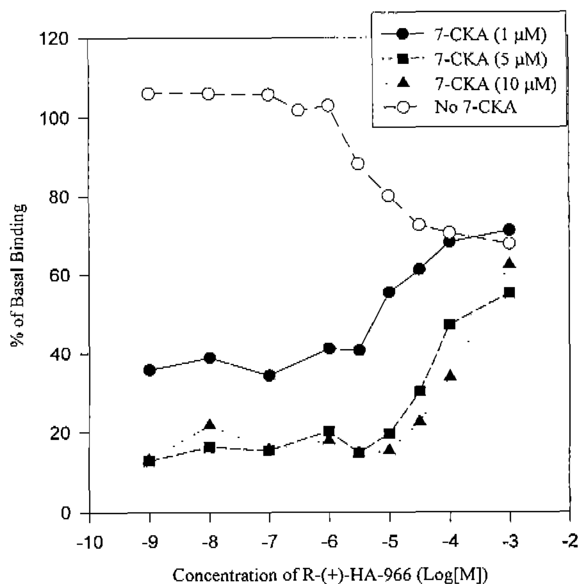

Fig. 3. Effects of 7-CKA on [^{3}H]MK-801 binding with R-(+)-HA-966. Various concentrations of R-(+)-HA-966 were incubated in the presence of fixed concentrations of 7-CKA as indicated (0, 1, 5, 10 μM), and % of basal [^{3}H]MK-801 binding was measured as described in Fig. 2. Representative data from 2-3 separate experiments are shown.

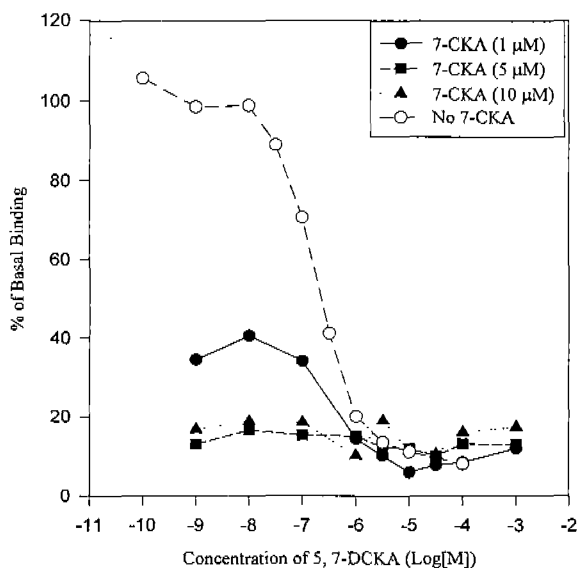

Fig. 4. Effects of 7-CKA on [^{3}H]MK-801 binding with 5,7-DCKA. Various concentrations of 5,7-DCKA were incubated in the presence of fixed concentrations of 7-CKA as indicated (0, 1, 5, 10 μM), and % of basal [^{3}H]MK-801 binding was measured as described in Fig. 2. Representative data from 2-3 separate experiments are shown.

membrane preparations are contaminated with other endogenous ligands such as L-glutamate, and whether the 10% of basal binding detected in the presence of 5 or 10 μM 7-CKA is due to the additional contamination need

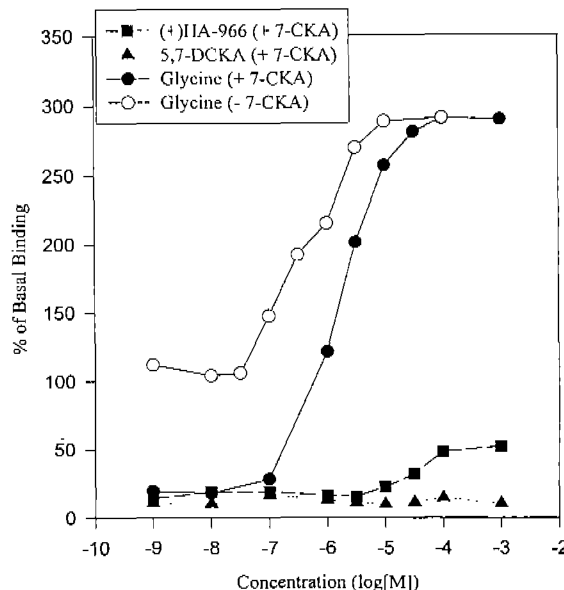

Fig. 5. Distinct [^{3}H]MK-801 binding profiles with glycine, R-(+)-HA-966, and 5 ,7-DCKA in the presence of 5 μM 7-CKA. Various concentrations of glycine, R-(+)-HA-966, and 5,7-DCKA were incubated as described in Fig. 2., with the addition of 5 μM 7-CKA, and % of basal [^{3}H]MK-801 binding was measured. The binding with glycine in the absence of 7-CKA is shown for comparison. Representative data from 2-3 separate experiments are shown.

to be examined.

In the presence of 50 μM 7-CKA, R-(+)-HA-966 could not significantly enhance [^{3}H]MK-801 binding (data not shown). Danysz *et al.* (1989) found that HA-966 increased [^{3}H]MK-801 binding in the presence of 50 μM 7-CKA using crude synaptic membranes. This may be due to the differences in the amount of contaminated glycine in membrane preparations because they prepared membranes for the binding without the detergent-treated step.

Finally, Fig. 5 shows the distinct [^{3}H]MK-801 binding profiles with the typical glycine site agonist, partial agonist, and antagonist assayed in the presence of minimal concentration of 7-CKA (5 μM). The binding was again dramatically increased with glycine. Due to the addition of 7-CKA, the "actual" basal binding was decreased to 10% of basal binding. However, the maximal potentiation of the binding achieved by glycine was similar regardless of the presence of 7-CKA. Having low efficacy (Donald *et al.*, 1988, Kemp *et al.*, 1988, Foster and Kemp, 1989), the extent of potentiation by R-(+)-HA-966 was much less than that by glycine. The antagonist exhibited no intrinsic activity as expected in this

assay condition.

Thus, the [^{3}H]MK-801 binding assay system performed under conditions described in the present study may prove to be a useful tool for the quick evaluation of pharmacological functions of test ligands interacting with the glycine site of the NMDA receptor.

The functional characterization of compounds is an important step in new drug development since further pharmacological evaluation methods to examine their *in vivo* activities can be designed based on their functional properties. If we consider the advantages of partial agonists in treating neuropathological conditions while maintaining normal actions, discriminating partial agonists from antagonists may be very useful. In fact, several partial agonists such as D-cycloserine (Monahan *et al.*, 1989), L-687,414 (Tricklebank *et al.*, 1994), and 1-aminocyclopropane carboxylic acid (Trullas *et al.*, 1991) were shown to exhibit facilitated learning performance in rats, and anticonvulsant, antidepressant, neuroprotective and anxiolytic actions. These are useful experimental tools to study biochemistry of the NMDA receptor as well as potential candidates for new drugs to treat stroke and other neurodegenerative disorders (Muir and Lees, 1994).

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