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N-Methyl-D-Aspartate Receptors: Different Subunit Requirements for Binding of Glutamate Antagonists, Glycine Antagonists, and Channel-Blocking Agents

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SUMMARY

Expression of the NR-1 subunit in *Xenopus* oocytes produces channels that respond to glutamate and are blocked by competitive and noncompetitive antagonists of the *N*-methyl-D-aspartate (NMDA) receptor. Ionic conductances through these channels are increased by coexpression with NR-2 receptor subunits. We have characterized the pharmacological properties of NMDA receptors assembled from combinations of subunits expressed in transfected cells, to determine the minimum subunit requirements for binding of competitive glutamate antagonists, glycine antagonists, and channel-blocking agents, as detected by ligand-binding experiments. Expression of NR-1a alone produced glycine antagonist binding, whereas the combination of NR-1a and NR-2A was needed to produce binding sites for glutamate antagonists and channel-blocking agents. These results suggest that functional NMDA receptors assemble from these subunits. However, differences in the pharmacological effects of NMDA and polyamines show that not all characteristics of native NMDA receptors are reproduced by this combination of subunits.

The NMDA receptor/ionophore is a major mediator of the postsynaptic excitatory effects of glutamate. It is important in long term potentiation and a variety of pathophysiological events, including chronic neuronal degeneration and acute excitotoxicity (1). The NMDA receptor cation channel is opened by binding of glutamate or NMDA with glycine as an obligate coagonist. However, the channel is blocked by magnesium at normal resting membrane potential, so that calcium enters physiologically only if the membrane is sufficiently depolarized (2).

The NMDA receptor contains binding sites for glutamate and glycine, as well as sites for noncompetitive antagonists such as MK-801 that block the open channel (2, 3). Polyamines such as spermidine modulate the receptor (4). Given this complex pharmacology, it is not surprising that a variety of experiments suggest heterogeneity in brain NMDA receptors (5–7). Recently, multiple cDNAs that are proposed to function as subunits of the NMDA receptor/ionophore complex have been cloned. The initial subunit, designated NR-1, is related in sequence to other glutamate receptors and exists in eight distinct forms (designated a-h) generated by differential mRNA splicing (8, 9). Expression of these subunits in oocytes produces receptors that conduct NMDA-stimulated currents. However, the currents are smaller than expected. Three additional subunits, called NR-2A, -2B, and -2C, each increase the size of NMDA currents when transfected into cells in conjunction with NR-1 (10–13). A fourth homologous subunit has also been described (12). Radioligand binding has been complementary to electrophysiology in analyzing the composition of multisubunit ligand-gated ion channels. Many electrophysiological properties are similar for single subunits or multiple subunits expressed in oocytes. Using transfected cells, we have characterized the minimal number of subunits required for reconstitution of binding activity for the glycine antagonist site (using [^{3}H]DCK), the competitive glutamate antagonist site (using [^{3}H]CGP-39653), and the open channel site (using ^{125}I-MK-801). The pharmacological characteristics of such binding reveal differences between NMDA receptors assembled from NR-1a and -2A subunits and brain NMDA receptors.

Experimental Procedures

Materials. [^{3}H]CGP-39653, [^{3}H]DCK, and ^{125}I-MK-801 were purchased from DuPont-NEN. Dextromethorphan and (±)-ketamine were obtained from Sigma. NMDA and MK-801 were from Research Bio-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; DCK, 5,7-dichlorokynurenic acid; CGP-39653, DL-(*E*)-2-amino-4-propyl-5-phosphonopentenoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

chemicals. Unlabeled iodinated MK-801 was the generous gift of Dr. Keith Williams, Department of Pharmacology, University of Pennsylvania.

cDNA construction and cell transfection. Full length cDNAs encoding the NR-1a and -2A receptors were subcloned from Bluescript/ZAP into eukaryotic expression vectors (pRC/CMV or pRK5) containing cytomegalovirus promoters.

Human embryonic kidney 293 cells were transfected with eukaryotic expression plasmids containing cDNAs encoding NMDA subunits (14, 15). Combinations of the NR-1a and -2A subunits were coexpressed in cells by transfection of equimolar amounts of the individual plasmids. Cells were grown to 50–75% confluence and transfected using calcium phosphate precipitation. After 20–24 hr the medium was replaced with fresh medium, and cells were grown for 24 hr. The cells were then rinsed with phosphate-buffered saline and harvested using a rubber policeman. To prevent NMDA receptor-mediated cell death, cells were routinely grown in the presence of the NMDA receptor antagonists 2-amino-5-phosphonovaleric acid (10 μM) or MK-801 (10 μM) after transfection.

Electrophysiological experiments. The whole-cell patch-clamp technique was used to measure functional NMDA receptor channels. The extracellular solution consisted of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 μM glycine, and 5 mM HEPES, pH 7.2, and the pipet was filled with a solution containing 140 mM CsCl, 10 mM Cs-EGTA, and 10 mM HEPES, pH 7.3. Levels of expression were determined by measuring the amplitudes of currents induced by 100 μM L-glutamate in clusters of 20–50 electrically coupled cells. Single cells were also examined. L-Glutamate was dissolved in extracellular solution and applied to cells using a piezo-driven application pipet made of θ-tubing, at a holding potential of −60 mV. Currents were filtered at 1000 Hz (−3 dB, eight-pole Bessel filter) before being digitized on-line for later display and analysis (16).

Binding experiments. For binding experiments, cells were harvested 48 hr after transfection and washed membranes were prepared. Harvested membranes were frozen and then thawed as needed for binding assays. The membranes were tested for binding of the NMDA receptor ligands [^{3}H]CGP-39653 (17), [^{3}H]DCK (18), and ^{125}I-MK-801 (19, 20), as modified below. When comparisons of different subunits were made, the subunits were compared from the same transfection.

[^{3}H]CGP-39653 binding. Rat forebrain or transfected cell homogenates were prepared by homogenization in 10 mM Tris, pH 8.0. Homogenates were centrifuged at 48,000 × g for 10 min and the pellet was resuspended in 10 mM Tris, pH 8.0. The homogenization and suspension process was repeated four times. Additional washes did not change binding characteristics. Homogenates were assayed by incubation of 200 μl of tissue suspension (50–150 μg of protein) with [^{3}H] CGP-39653 (25 μl) and either buffer or displacing drug (25 μl), in a total volume of 250 μl. For experiments other than saturation analyses, the [^{3}H]CGP-39653 concentration was 30 nM. Nonspecific binding was defined in the presence of 100 μM glutamate. Incubations were for 1 hr at 0° and filtration was performed with a Brandel cell harvester. Radioactivity retained on the filters was determined by scintillation counting.

[^{3}H]DCK binding. [^{3}H]DCK assays were performed as described previously (18). Tissue was homogenized in 50 mM Tris, pH 7.4. The suspension was centrifuged at 48,000 × g, and the pellet was resuspended in 50 mM Tris, pH 7.4. This process was repeated five times. Incubations contained 200 μl of tissue (100 μg of protein) in a total volume of 250 μl. Nonspecific binding was defined with 100 μM glycine, and the concentration of [^{3}H]DCK was varied for saturation analysis. Incubation was for 15 min at 0°.

^{125}I-MK-801 binding. MK-801 was removed from the transfected cell cultures as described previously for neuronal cell cultures (20). Harvested cells (50 mg of tissue) were resuspended in 25 ml of 20 mM HEPES, 100 μM glycine, 100 μM glutamate, 300 μM $MgCl_2$, pH 7.5. The samples were incubated for 30 min at 32° to allow dissociation of MK-801 and were then centrifuged for 10 min at 48,000 × g. This washing was repeated two more times, and samples were resuspended in 20 mM HEPES, 1 mM EDTA, and incubated for 30 min. The samples were centrifuged as stated previously. One additional wash with 20 mM HEPES was performed for experiments testing magnesium concentrations. Additional washes did not change the binding characteristics significantly. ^{125}I-MK-801 binding was performed by resuspension of the tissue in 20 mM HEPES, 100 μM glycine, 100 μM glutamate, 100 μM $MgCl_2$, pH 7.5. The concentration of ^{125}I-MK-801 was 300 pM for standard assays, and nonspecific binding was determined in the presence of 10 μM MK-801. Incubations were for 3 hr at 32°. Saturation analysis were performed using multiple concentrations of ^{125}I-MK-801. The specific activity was diluted with unlabeled iodinated MK-801 to achieve higher concentrations.

For all binding assays, results were normalized per mg of protein. For [^{3}H]CGP-39653 and [^{3}H]DCK assays protein was determined by the method of Lowry *et al.* (21), whereas the Pierce bicinchoninic acid reagent was used for samples from ^{125}I-MK-801 assays. Best-fit curves were determined using CricketGraph and InStat software on a Macintosh II computer, in combination with nonlinear regression analysis on the National Institutes of Health-based PROPHET system. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (22).

Results

Electrophysiological experiments. To ensure expression of NMDA subunits, transfected cells were studied electrophysiologically for the presence of glutamate-mediated ionic currents. Glutamate (100 μM) produced currents of 93 ± 53 pA in cells transfected with NR-1a plus -2A but did not produce detectable currents after transfection with only NR-1a (data not shown).

Binding of [^{3}H]DCK in transfected cells. Cells transfected with DNA encoding combinations of NMDA receptor subunits were tested for the ability to bind the glycine site ligand [^{3}H]DCK. Untransfected 293 cells and cells transfected with control plasmid showed no binding (data not shown). Membranes from cells transfected with NR-1a alone bound [^{3}H]DCK specifically, whereas no significant binding was found in membranes from cells transfected with NR-2A only (Fig. 1). Significant binding was also present in membranes from cells transfected with both NR-1a and NR-2A. Specific binding was typically 80% of total binding at 30 nM ligand concentration. Saturation analysis with [^{3}H]DCK revealed a K_d of 41 ± 11 nM and a B_{max} of 8.7 ± 2.0 pmol/mg of protein (Fig. 1). Transfection of NR-1a and NR-2A also produced saturable binding, with a

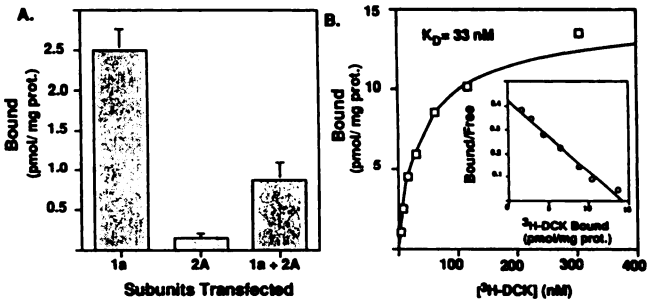

Fig. 1. Saturation analysis of [^{3}H]DCK binding. A, [^{3}H]DCK bound specifically to membranes from cells transfected with NR-1a, or NR-1a and -2A, but not NR-2A alone. The [^{3}H]DCK concentration was 30 nM in these experiments, and means ± standard errors are shown. B, Saturation isotherm for NR-1a with [^{3}H]DCK concentrations of 4–300 nM is shown. When plotted as a Scatchard plot (*inset*), the best-fit line yielded a K_d of 33 nM and a B_{max} of 14 pmol/mg of protein in this preparation. One of three experiments with similar results is shown in B.

K_d (29 nM) similar to that obtained in cells transfected with NR-1a alone (data not shown).

Binding of glutamate antagonists. Transfection of cells with NR-1a DNA in association with NR-2A DNA produced saturable specific binding of [^{3}H]CGP-39653 to cell membranes (Fig. 2). Specific binding was typically 75% of total binding at 30 nM ligand concentration. No binding was detected in cells transfected with control plasmid, NR-1a alone, or NR-2A alone (Fig. 2A). Saturation binding analysis showed a K_d of 34 ± 8 nM, consistent with the K_d of 23 ± 3 nM in rat brain membranes tested in parallel (Fig. 2B). The maximal number of binding sites was 1.8 ± 1.1 pmol/mg of protein for transfected cells.

The inhibition profile of [^{3}H]CGP-39653 binding to membranes from cells transfected with NR-1a and -2A DNA was similar to but distinct from that seen in rat brain membranes (Fig. 3, A and B). Glutamate potently inhibited binding, with a K_i of 131 ± 45 nM in brain and 124 ± 48 nM in transfected cells. Hill coefficients were 0.86 ± 0.14 for brain and 0.97 ± 0.12 for transfected cells (not significantly different from a Hill coefficient of 1.0). Glycine inhibited binding but with shallow curves and Hill coefficients much less than 1.0. The Hill coefficient was 0.21 for transfected cells. This value is similar to the value of 0.38 obtained in brain membranes tested in parallel experiments. However, NMDA inhibited [^{3}H]CGP-39653 binding differently in transfected cells than in brain (Fig. 3C). NMDA inhibited binding in brain with an IC_{50} value of 7.9 μM (K_i = 3.4 ± 0.8 μM) and a Hill slope of 0.75. In transfected cells, NMDA was significantly ($p < 0.01$) less potent, with an IC_{50} value of 46 μM (K_i = 24 ± 6 μM) and a Hill slope of 0.60.

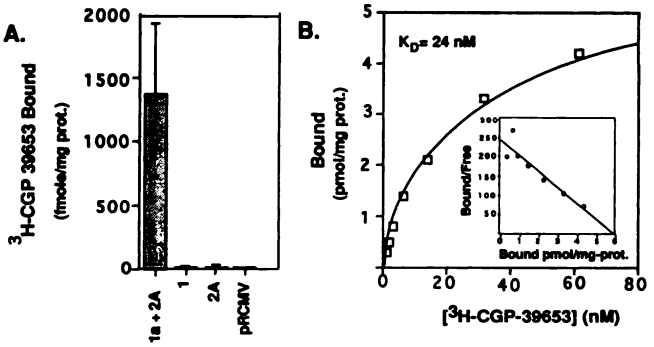

Fig. 2. Saturation analysis of [^{3}H]CGP-39653 binding to transfected cells. Binding of [^{3}H]CGP-39653 to cells transfected with NR-1a plus -2A was specific. Binding was seen only with the combination of NR-1a and -2A (A). Saturation analysis (B) and a Scatchard plot (*inset*) demonstrated a K_d of 24 nM in this preparation of transfected 293 cells. The maximal number of binding sites was 5.8 pmol/mg of protein. A, Results from three experiments (mean ± standard error) with a ligand concentration of 30 nM; B, results from one of four experiments with similar results.

Binding of NMDA channel-blocking agents. Cells transfected with DNA encoding subunits 1a and 2A bound ^{125}I-MK-801 with high affinity (Fig. 4), but no binding was detected in cells transfected with NR-1a alone or NR-2A alone (data not shown). Binding was saturable, with a K_d of 600 ± 93 pM in brain and 540 ± 120 pM in transfected cells. The maximal number of binding sites in transfected cells was 840 ± 320 fmol/mg of protein. Specific binding represented approximately 75% of total binding at 200 pM ligand concentration. The pharmacological profile of binding to transfected cells resembled that seen in brain homogenates. Dextromethorphan, ketamine, and MK-801 potently inhibited binding of ^{125}I-MK-801 to both brain and transfected cell membranes (Fig. 5; Table 1). The K_i values and Hill coefficients for these agents were not significantly different between brain and transfected cells. Spermidine and magnesium also inhibited ^{125}I-MK-801 binding similarly in brain and transfected cells at concentrations in the high micromolar/millimolar range (Fig. 6). However, ^{125}I-MK-801 binding in brain was stimulated by spermidine or magnesium at low micromolar concentrations, whereas binding in transfected cells was not significantly changed by spermidine and was only slightly stimulated by magnesium.

Discussion

The present results show that NR-1a is sufficient to recreate the glycine antagonist binding site, but the co-transfection of NR-1a and -2A subunits is required to produce NMDA receptors with high affinity glutamate antagonist binding and binding of channel-blocking agents, as detected in ligand binding assays. Saturation analysis of [^{3}H]DCK binding to NR-1a from transfected cells reveals it to be of affinity similar to that of the native receptors (18). The pharmacological profile and saturation characteristics of glutamate antagonist and open channel sites are generally similar for native subunits and receptors produced in transfected cells (23, 24). Binding levels of [^{3}H]DCK are higher than those of ^{125}I-MK-801 or [^{3}H]CGP-39653, suggesting that [^{3}H]DCK can bind to unassembled subunits or to receptors containing only the NR-1a subunit. Our

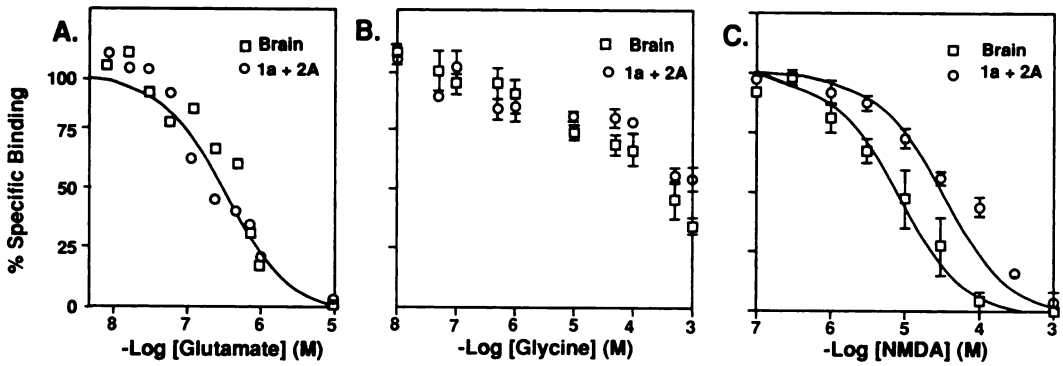

Fig. 3. Inhibition profile of [^{3}H]CGP-39653 binding. Binding of [^{3}H]CGP-39653 to membranes from brain and 293 cells transfected with NR-1a and -2A was inhibited by various concentrations of glutamate (A), glycine (B), or NMDA (C). Results in A are from one representative experiment replicated twice; B and C are combined results from three to five experiments with similar results. Values are shown as mean ± standard error. Glutamate and glycine inhibited binding similarly in brain and transfected cells, whereas NMDA was less potent in transfected cells. The *lines* drawn in A and C are theoretical lines for a one-site fit through the IC_{50}.

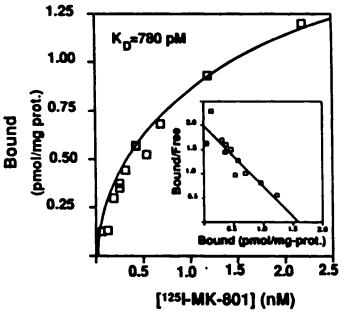

Fig. 4. ^{125}I-MK-801 binding in transfected cell membranes. 293 cells transfected with NR-1a plus -2A were tested for ^{125}I-MK-801 binding. Saturation analysis of binding to receptors in cells transfected with NR-1a plus -2A demonstrated a K_d in this preparation of 780 pM, with a B_{max} of 1.54 pmol/mg of protein (*inset*). This experiment is representative of three similar experiments.

results in brain tissue resemble those reported previously for binding of glutamate antagonists and channel-blocking agents (17, 24). *In situ* hybridization studies showed that NR-1a and -2A are abundantly and widely expressed throughout the brain (10, 11, 25). Coassembly of NR-1a and -2A in the brain is thus one mechanism by which receptors with glutamate antagonist sites and channel-blocking agent sites may be produced.

Some of our results were unexpected, compared with previous studies. The observation that coassembly of subunits is required to detect binding sites for ^{125}I-MK-801 and [^{3}H]CGP-39653 apparently conflicts with the reported action of these and similar drugs and glutamate agonists on homomeric receptors expressed after injection of NR-1a subunit mRNA into oocytes (8, 26, 27). One possible explanation is that oocytes express other factors or subunits that facilitate assembly of NR-1a homomeric receptors. Reduced affinity of NR-1a homomeric receptors for MK-801 may not have been detected in oocyte experiments, which typically use drug concentrations 100-fold higher than ligand-binding K_d values to block currents half-maximally. Alternatively, we would not expect to detect binding sites in 293 cells if the affinity was substantially reduced (>10-fold) or if the receptor levels were low due to inefficient assembly. Similarly, electrophysiological responses in oocytes are many times greater when NR-1 and -2 subunits are present. If responses were similarly low for homomeric receptors in 293 cells, they could be undetectable if receptor assembly for homomeric receptors was inefficient. It seems from our [^{3}H]DCK binding studies and from Western blots (28) that 293 cells synthesize large amounts of NR-1a subunit, but electrophysiological experiments reported here and in other studies indicate that this subunit does not assemble into a detectable number of receptors on the cell surface (11). This fact argues strongly for incomplete assembly of homomeric receptors in transfected 293 cells. Inefficient assembly of multimeric receptors lacking one subunit has been suggested for other ligand-gated ion channels (15, 29). Our results agree with affinity labeling experiments that show that NR-1a can be photoaffinity labeled with a glutamate antagonist only when transfected in combination with NR-2A (30). Cumulatively, these results suggest that the presence of NR-1a and -2A is necessary for efficient production of the glutamate antagonist and ^{125}I-MK-801 ligand binding sites in transfected 293 cells.

However, the binding activities produced in cells transfected with NR-1a and -2A differ from native receptors in specific ways. First, NMDA inhibited [^{3}H]CGP-39653 binding less potently in transfected cells than in brain. The lower affinity for NMDA may be analogous to the unmasking of lower affinity acetylcholine binding sites that occurs after transfection of cells with acetylcholine receptors lacking one subunit (31, 32).

In addition, at low micromolar concentrations spermidine stimulated ^{125}I-MK-801 binding to brain but not to transfected cells. Magnesium was slightly less effective in stimulating binding to transfected cells than to brain. These effects of magnesium on ^{125}I-MK-801 binding in brain have been noted previously (19). A polyamine-insensitive NMDA receptor has previously been reported in cerebellum (33); however, that receptor is insensitive to ketamine, unlike the receptor we demonstrate in transfected cells. The effects of polyamines on the NMDA receptor are very complex (34–37). Polyamines have at least three effects; they increase the affinity of the receptor for glycine at low glycine concentrations, they potentiate channel opening at saturating glycine concentrations, and they act as voltage-dependent blockers. In ^{125}I-MK-801 binding studies they stimulate binding at low micromolar concentrations and inhibit binding at millimolar concentrations (19). In studies of NR-1a homomeric receptors in oocytes, electrophysiological responses are potentiated by polyamines (26, 27). Effects of polyamines on heteromeric receptors produced by coexpression of NR-1a and -2A in oocytes have not yet been reported. However, the effects of zinc and the noncompetitive NMDA receptor antagonist ifenprodil on homomeric NR-1 receptors expressed in oocytes differ from the effects seen with heteromeric receptors (27, 38). A similar situation may exist for polyamine effects, where only the combination of NR-1a with the proper NR-2 subunit might produce polyamine-sensitive receptors. In conjunction with our results, it seems likely that another combination of NMDA subunits or splice variants

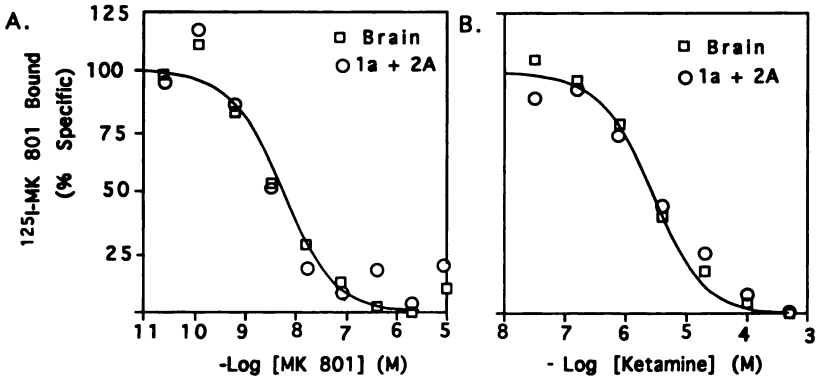

Fig. 5. Comparison of ^{125}I-MK-801 binding in transfected cell membranes and brain. Parallel samples of brain and 293 cells transfected with NR-1a plus -2A were tested for ^{125}I-MK-801 binding in the presence of multiple concentrations of MK-801 (A) or ketamine (B). MK-801 and ketamine inhibited binding similarly in brain and transfected cells. Ligand concentration for all experiments was 300 pM, and *curves* in A and B are best-fit lines through the IC_{50}, assuming a single site.

TABLE 1

Inhibition profile of ^{125}I-MK-801 binding

^{125}I-MK-801 binding was performed at multiple concentrations of inhibiting drug, with a ligand concentration of 300 pM. Data were graphed as inhibition curves, and IC_{50} values and Hill coefficients (n_H) were computed. IC_{50} values were converted to K_i values as described by Cheng and Prusoff (22). Data are mean ± standard error of at least three experiments.

Inhibitor	Brain		1a + 2A	
	K_i	n_H	K_i	n_H
	nM		nM	
MK-801	8.1 ± 1.8	0.89 ± 0.06	8.2 ± 1.7	0.85 ± 0.07
Dextromethorphan	1600 ± 500	0.92 ± 0.05	2000 ± 800	0.82 ± 0.08
(±)-Ketamine	1140 ± 570	0.97 ± 0.07	1030 ± 490	0.81 ± 0.10

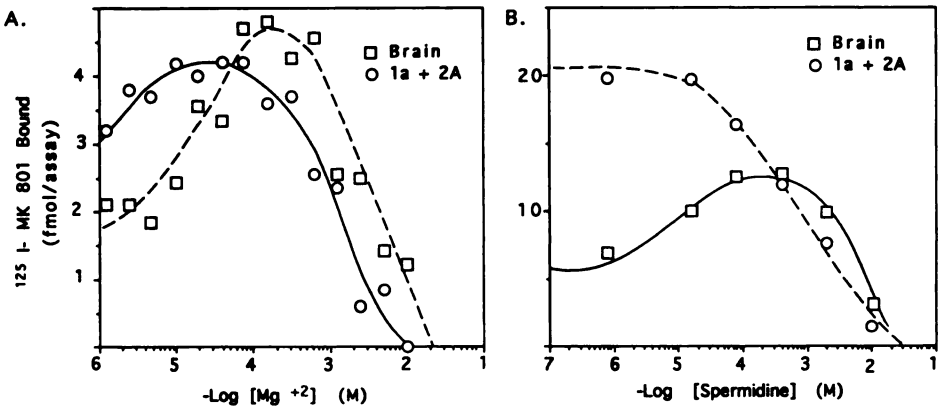

Fig. 6. Comparison of magnesium and spermidine actions in brain and transfected cells. Parallel samples of brain and 293 cells transfected with NR-1a plus -2A were tested for ^{125}I-MK-801 binding in the presence of varying concentrations of magnesium (A) or spermidine (B). Both inhibited binding at millimolar concentrations in brain and transfected cells. Magnesium and spermidine increased binding to the brain at low micromolar levels. Magnesium increased binding and spermidine had no effect at low micromolar concentrations in transfected cells. Ligand concentration for all experiments was 300 pM.

other than those we used mediates stimulatory effects of polyamines on MK-801 binding.

In conclusion, our data show that the heterodimeric combination of NR-1a and -2A accounts for many, but not all, characteristics of native NMDA receptors. Other combinations, including additional subunits or splice variants, may be required to reconstitute receptors with all of the pharmacological properties of native NMDA receptors.

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