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Up-regulation of *N*-Methyl-D-aspartate Receptors on Cultured Cortical Neurons after Exposure to Antagonists

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SUMMARY

The density of *N*-methyl-D-aspartate (NMDA) receptors on membranes prepared from cultured cortical neurons was determined using binding assays with [^{125}I]I-MK-801 after exposure of cultures to antagonists of the NMDA receptor complex. The density of binding sites for [^{125}I]I-MK-801 was increased by 40–80% after exposure to D-2-amino-5-phosphonopentanoic acid (D-AP5), with no change in the number or viability of neurons. The effect of D-AP5 was concentration dependent, with an EC_{50} of 10 μM. Up-regulation of NMDA receptors was observed after 2–7 days but not after 1 day of exposure to 100 μM D-AP5. The density of NMDA receptors was also increased after exposure of cells to CGS 19755 and MK-801 but not after exposure to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione. The binding of [^{3}H]AMPA was unaltered after exposure to D-AP5. These results demonstrate that the density of NMDA receptors on cultured neurons can be selectively up-regulated by exposure to NMDA receptor antagonists. Increases in the density of NMDA receptors occurring *in vivo* could complicate therapeutic approaches to the treatment of neurological disorders.

The NMDA subtype of excitatory amino acid receptor is involved in synaptic transmission and in the generation of various forms of synaptic plasticity, including processes thought to underlie some types of learning and memory (1, 2). Excessive or abnormally prolonged activation of NMDA receptors has been implicated in ischemic neurotoxicity, epilepsy, and several chronic neurodegenerative diseases (3–5). The NMDA receptor is a ligand-gated ion channel complex that contains distinct recognition sites for endogenous and exogenous ligands, including glutamate, glycine, Mg^{2+}, Zn^{2+}, polyamines, and open-channel blockers such as MK-801 (1, 2, 6). It has been suggested that antagonists acting at some of these recognition sites will be clinically useful in the treatment or prevention of epilepsy or ischemic neuronal cell death and ameliorates the progression of neurodegenerative diseases (3–5). However, chronic treatment with receptor antagonists can lead to increases in the densities of receptors, which may complicate therapeutic strategies. For example, increases in the density of receptors have been implicated in the supersensitive responses observed after abrupt discontinuation of β-adrenergic receptor antagonists and in the movement disorders occurring after chronic administration of neuroleptics (7, 8). Currently, little is known about the potential consequences of chronic blockade of NMDA receptors.

Cultured neurons provide a useful model system with which to investigate the cellular and biochemical mechanisms that regulate the expression of neurotransmitter receptors. Studies of NMDA receptors expressed on cultured neurons can be designed to circumvent some of the problems inherent in studies involving the administration of antagonists to animals, which may be complicated by metabolism or the limited bioavailability of the antagonists and by the fact that such compounds may severely disrupt normal central nervous system function. Studies of the expression and regulation of NMDA receptors on dissociated cultured neurons have been facilitated by the availability of [^{125}I]I-MK-801, a selective ligand for the NMDA receptor complex, which is labeled to a high specific activity (9). In the work reported in this paper, we have used [^{125}I]I-MK-801 to look for changes in the density of NMDA receptors on cultured neurons after exposure to excitatory amino acid receptor antagonists.

Materials and Methods

Primary culture of cortical cells. Neuronal/glial co-cultures were prepared from neocortex of embryonic rats at 16–17 days of gestation,

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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; I-MK-801, (+)-3-iodo-MK-801; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; D-AP5, D-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CGS 19755, (*cis*)-4-phosphonomethyl-2-piperidine carboxylic acid; TCP, *N*-(1-[2-thienyl]cyclohexyl)piperidine; 5,7-DCK, 5,7-dichlorokynurenic acid.

as previously described (9, 10). Cortical cells were plated in culture medium (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10% Ham's F-12 nutrient mixture, 50 units/ml penicillin, and 50 μg/ml streptomycin), at a density of $1.0–1.3 \times 10^5$ cells/cm², in 100-mm plastic dishes that had been precoated with poly-D-lysine (20 μg/ml). Cells were maintained at 37° in a humidified atmosphere containing 7% CO_2. Medium was replaced every 2–4 days. Under these conditions, neurons grow predominantly in a dispersed monolayer, with only rare clumps, on a layer of dividing glial cells. After 6–7 days in culture, when the glial cell layer had become confluent, cytosine arabinoside (10 μM) was added for 24 hr to prevent further proliferation of non-neuronal cells. Cells were treated with antagonists beginning at 15–18 days of culture. The medium was removed and replaced with fresh medium (10 ml) containing an antagonist as appropriate. When treatments were carried out for longer than 48 hr, the medium was replaced every 48 hr with fresh medium containing antagonist as appropriate. In these cultures, neurons can be recognized by their phase-bright cell bodies and dendritic extensions. Such cells have been identified as neurons by a variety of techniques, including tetanus toxin staining, dye filling, and extensive electrophysiological analyses (10).

Membranes were prepared from cortical cells for binding assays with [^{125}I]I-MK-801. Cultures were washed and cells were homogenized in EDTA buffer (5 mM K-EDTA, pH 7.0), with a Polytron homogenizer. Homogenates were centrifuged (100,000 × *g*, 30 min), and pellets were resuspended in 25 ml of the same buffer, incubated for 30 min at 32°, and then centrifuged (100,000 × *g*, 30 min). Pellets were resuspended in EDTA buffer, and the washing procedure, including the 30-min incubation, was repeated two more times. The pellets were resuspended in HEPES buffer (20 mM K-HEPES, 1 mM K-EDTA, pH 7.0), centrifuged, resuspended in the same buffer, and stored at −80° until assayed (within 7 days). In experiments in which cells had been treated with MK-801, cells were initially homogenized and the membranes were washed twice (including incubation at 32°) in buffer without EDTA but containing 100 μM glutamate, 100 μM glycine, and 300 μM Mg^{2+}. This washing procedure was developed to promote removal of residual MK-801, which otherwise may remain trapped in the ion channel of NMDA receptors and interfere with subsequent binding assays. The rate of dissociation of MK-801 is greatly enhanced in the presence of glutamate, glycine, and Mg^{2+} (11, 12). Membranes were subsequently washed in EDTA buffer and processed as described above.

Binding assays with [^{125}I]I-MK-801. Membranes were thawed, diluted in assay buffer, centrifuged (100,000 × *g*, 30 min), and resuspended in assay buffer (20 mM K-HEPES, 1 mM K-EDTA, pH 7.0). Binding assays with [^{125}I]I-MK-801 (0.15 nM, 2200 Ci/mmol) were carried out as previously described (9), using 10–15 μg of membrane protein/tube. For saturation binding curves, the specific activity of [^{125}I]I-MK-801 was reduced 5–10-fold by the addition of unlabeled ^{127}I-MK-801 (220–400 Ci/mmol final specific activity), and assays were carried out using eight or nine concentrations (0.01–3.0 nM) of [^{125}I]I-MK-801 at this specific activity. All saturation binding assays were carried out in the presence of 100 μM glutamate, glycine, and spermidine. Under these conditions, binding of [^{125}I]I-MK-801 reaches equilibrium within 2–3 hr (9). Duplicate samples were incubated at 32° for 3 hr. Nonspecific binding was determined in the presence of 10 μM (+)-MK-801. At a concentration of 0.2 nM [^{125}I]I-MK-801, nonspecific binding represented 10–20% of total binding. Assays were terminated by rapid filtration, as described previously (9).

Binding assays with [^{3}H]AMPA. Cells were homogenized and membranes were washed in EDTA buffer, as described above. Membranes were then washed twice, including a 30-min incubation at 32° followed by centrifugation (100,000 × *g*, 30 min), in 5 mM Tris·HCl (pH 7.4). Pellets were resuspended in 50 mM Tris·HCl (pH 7.4) and stored at −80° for 2–7 days. For binding assays, membranes were thawed, washed once, and resuspended in assay buffer (50 mM Tris·HCl, pH 7.4). Membranes (300–400 μg of protein) were incubated with [^{3}H]AMPA (10 nM), in a total volume of 400 μl, for 60 min at 4°. All assays contained 100 mM NaSCN (13). Nonspecific binding was determined in the presence of 1 mM L-glutamate and represented 20–30% of total binding. Assays were terminated by the addition of 10 ml of ice-cold assay buffer, followed by rapid filtration over glass fiber filters. Filters were washed with another 10 ml of buffer, and radioactivity retained on the filters was determined by liquid scintillation counting, at an efficiency for tritium of 30–35%.

Materials. (+)-[3-^{125}I]I-MK-801 (specific activity, 2200 Ci/mmol) and DL-α-[5-*methyl*-^{3}H]AMPA (specific activity, 60 Ci/mmol) were purchased from New England Nuclear/DuPont (Boston, MA). (+)-MK-801 was a gift from Merck, Sharp, and Dohme Division of Merck and Co., Inc. (West Point, PA). Unlabeled (+)-3-^{127}I-MK-801 was a gift from Dr. Paul Anderson, Merck, Sharp, and Dohme (West Point, PA). 5,7-DCK was a gift from Marion Merrell Dow Research Institute, Marion Merrell Dow Inc. (Cincinnati, OH). Fetal calf serum was purchased from Hyclone (Logan, UT). L-Glutamate and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Spermidine was purchased from Aldrich Chemical Co. (Milwaukee, WI). D-AP5 and CNQX were purchased from Cambridge Research Biochemicals (Wilmington, DE). CGS 19755 was a gift from Ciba-Geigy Corporation (Summit, NJ). All other reagents were of the highest purity available and were from commercial sources.

Results and Discussion

Characterization of the binding of [^{125}I]I-MK-801. The characteristics of the binding of [^{125}I]I-MK-801 to membranes prepared from cultured cortical cells were determined. In the absence of spermidine, the specific binding of [^{125}I]I-MK-801 was low even in the presence of glutamate and glycine (Fig. 1). This is due to the slow rate of association of the radioligand (9). Binding was enhanced in the presence of spermidine, which accelerates the rate of association of [^{125}I]I-MK-801 (9), or in the presence of spermidine plus glutamate and glycine (Fig. 1). The specific binding of [^{125}I]I-MK-801 was reduced or abolished by inclusion of the glutamate site antagonist D-AP5 or the glycine site antagonist 5,7-DCK (Fig. 1). This is presumably due to blockade of the effects of residual endogenous amino acids. The specific binding of [^{125}I]I-MK-801 is thus consistent with binding to the NMDA receptor complex, and the characteristics of the binding are similar to those seen in studies of NMDA receptors on membranes prepared from rat brain (9). NMDA receptors appear to be localized exclusively on neurons in these cultures. We have not been able to detect specific

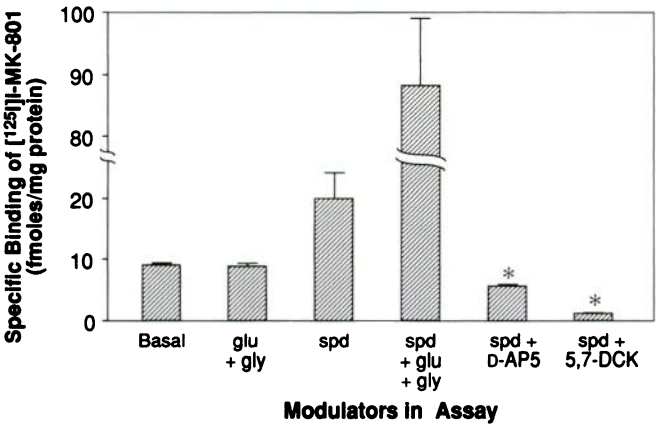

Fig. 1. Effects of modulators on the binding of [^{125}I]I-MK-801. The binding of [^{125}I]I-MK-801 (0.15 nM) to membranes prepared from cultured cortical cells was determined in the presence of various combinations of glutamate (*glu*) (100 μM), glycine (*gly*) (100 μM), spermidine (*spd*) (100 μM), D-AP5 (100 μM), and 5,7-DCK (100 μM), as indicated. *, $p < 0.05$, compared with spermidine (one-way analysis of variance with *post hoc* paired *t* test). Values are mean ± standard error (three experiments).

binding of [^{125}I]I-MK-801 to membranes prepared from cultured glia or from glial/neuronal co-cultures in which the neurons have been destroyed by exposure to high concentrations of glutamate or have degenerated after prolonged culture.[1]

Up-regulation of NMDA receptors on cultured neurons. Treatment of cultured neurons for 4 days with the glutamate site antagonist D-AP5 (100 μM) resulted in a 65 ± 13% increase (mean ± standard error; 12 experiments; $p < 0.001$, paired t test) in the density of NMDA receptors determined in binding assays with [^{125}I]I-MK-801, with no change in the affinity of the receptors for [^{125}I]I-MK-801 (Fig. 2).

The ability of D-AP5 to increase the density of NMDA receptors was concentration dependent, with an EC_{50} of approximately 10 μM and a maximal effect being seen at concentrations of D-AP5 above 30 μM (Fig. 3). The effect of D-AP5 was also time dependent. The density of binding sites for [^{125}I]I-MK-801 was unchanged after 1 day of exposure to D-AP5 but was increased after 2–7 days of exposure (Fig. 4). A maximal increase of 60–80% was seen after 4–7 days (Fig. 4).

It is possible that neuronal degeneration, mediated by tonic stimulation of NMDA receptors, occurs in control cultures and that exposure to D-AP5 protects against this neurotoxicity. This could result in an apparent increase in the density of NMDA receptors. To investigate this possibility, the number of neuronal cell bodies (as determined by phase-contrast appearance) and the number of viable neurons (assessed by trypan blue exclusion) were determined before and after exposure of cells to D-AP5 (Fig. 5). There was no difference in the number of phase-bright neuronal cell bodies (Fig. 5A) or in the number of viable neurons (Fig. 5B) after treatment with D-AP5, compared with controls. This suggests that the increase in the binding of [^{125}I]I-MK-801 after exposure of cells to D-AP5 reflects an increase in the density of NMDA receptors per neuron, rather than protection by D-AP5 against ongoing neuronal degeneration. Alternatively, a time-dependent loss of dendritic processes expressing NMDA receptors could be occurring (14). If this loss was prevented by exposure to D-AP5, an apparent increase in receptors per neuron would be observed. A change in dendritic processes would not be detected by assessing the number of neurons. However, the density of NMDA receptors in control cultures did not decrease over the

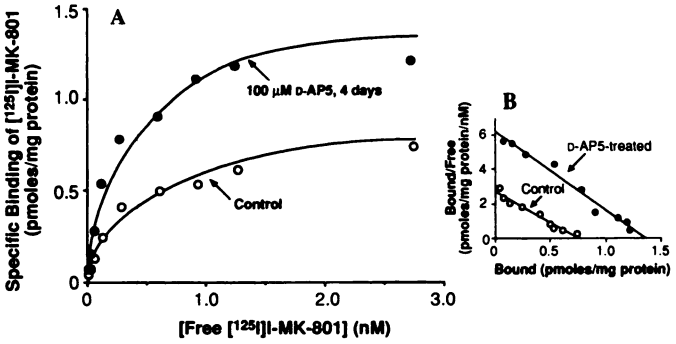

Fig. 2. Effects of exposure of cultured neurons to D-AP5. A, Saturation binding curves for [^{125}I]I-MK-801 were determined using membranes prepared from control cells and from a sister culture exposed to 100 μM D-AP5 for 4 days. B, Scatchard analyses of the same data. Results are from a single representative experiment. Values for the density of binding sites (B_{max}) were 0.76 pmol/mg of protein (control) and 1.36 pmol/mg of protein (D-AP5-treated) and for the equilibrium dissociation constant (K_d) were 0.27 nM (control) and 0.22 nM (D-AP5-treated).

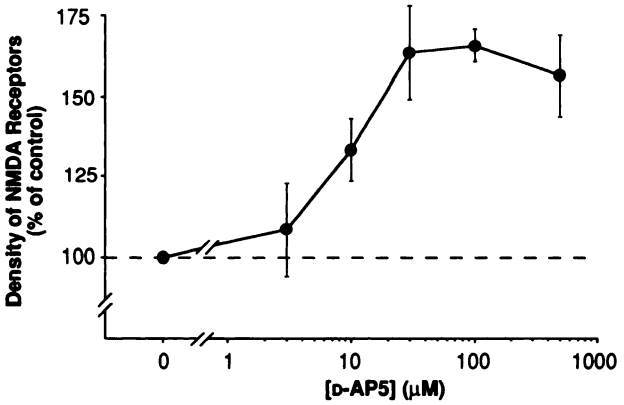

Fig. 3. Effects of exposure of cultured neurons to D-AP5 for 4 days. The density of NMDA receptors was determined by Scatchard analysis of the binding of [^{125}I]I-MK-801 after exposure of cells to 3–500 μM D-AP5, and data are expressed as a percentage of the density in control cultures (757 ± 61 fmol/mg of protein). Values are mean ± standard error (four experiments).

[1] K. Williams and P. B. Molinoff, unpublished observations.

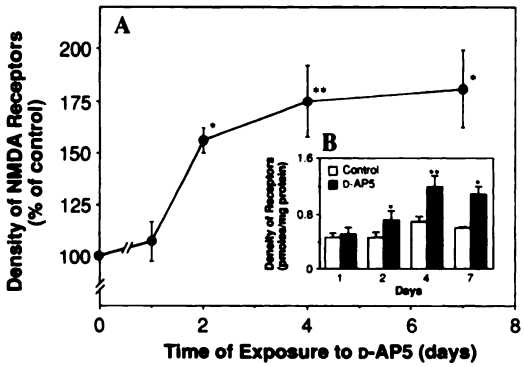

Fig. 4. Time-dependence of exposure to D-AP5 on the density of NMDA receptors. A, The density of receptors was determined from saturation binding curves with [^{125}I]I-MK-801 in cultures exposed for 1–7 days to 100 μM D-AP5. Data are expressed as a percentage of the binding of [^{125}I]I-MK-801 in control cultures. B, The same data (expressed as pmol/mg of protein) are shown. Values are mean ± standard error (four to six experiments). *, $p < 0.05$, **, $p < 0.01$ (paired t test).

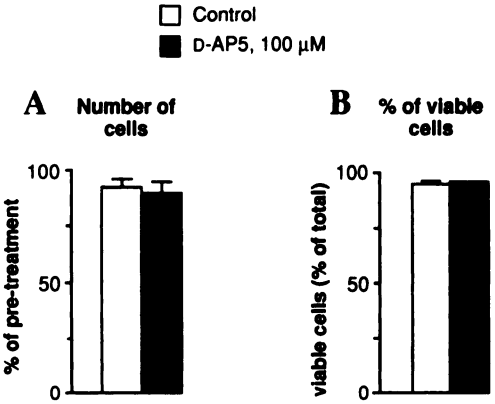

Fig. 5. Number and viability of neurons after exposure to D-AP5. The number of neuronal cells (A) and the proportion of viable neurons (B) were determined in five randomly selected fields in culture dishes before and after exposure of cultures to D-AP5 and in control sister cultures. Values are mean ± standard error (five experiments).

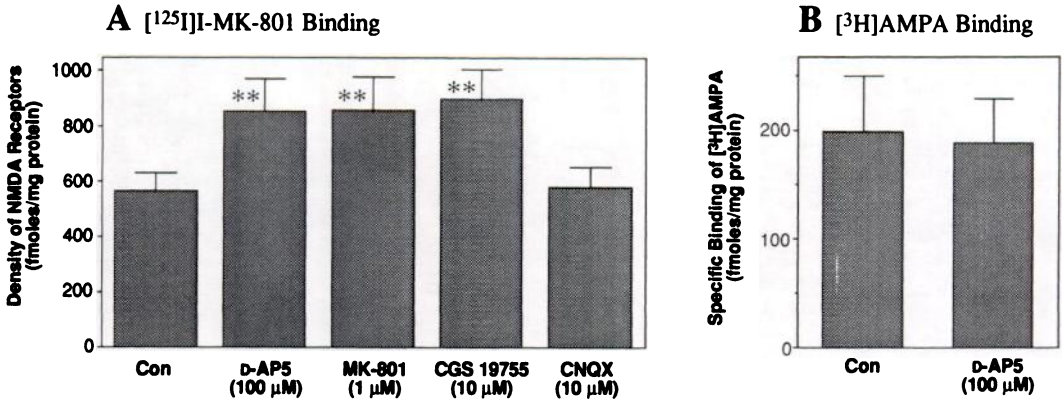

Fig. 6. Effects of exposure of cultured neurons to glutamate receptor antagonists. A, The density of binding sites for [^{125}I]I-MK-801 was determined from saturation binding curves in control (*Con*) cultures and in cultures exposed for 4 days to the NMDA receptor antagonists D-AP5 (100 μM), MK-801 (1 μM), or CGS 19755 (10 μM) or the AMPA/kainate receptor antagonist CNQX (10 μM). **, $p < 0.01$, compared with control (block-design analysis of variance with *post hoc* paired *t* test). B, The binding of [^{3}H]AMPA (10 nM) was determined after exposure to D-AP5 (100 μM) for 4 days. Values in A and B are mean ± standard error (five experiments).

course of the experiment (Fig. 4B), suggesting that loss of dendrites is not responsible for the observed effect of D-AP5. In addition, dendritic pruning that occurs in the presence of glutamate appears to be mediated by activation of AMPA/kainate receptors, rather than NMDA receptors (14).

An increase in the number of receptors per neuron may reflect an increase in the surface density of receptors on a constant dendritic architecture or an enhanced dendritic arborization with an unchanged density of receptors per unit area. In either case, however, an increase in excitability and, potentially, in excitotoxic cell death could result after removal of antagonists.

The specificity of the effect of D-AP5 was investigated by exposing cells to the selective NMDA receptor antagonists D-AP5, MK-801, and CGS 19755 and to the AMPA/kainate receptor antagonist CNQX (15–17). There was a 40–50% increase in the density of NMDA receptors after exposure to D-AP5, MK-801, or CGS 19755 (Fig. 6A). Thus, up-regulation of NMDA receptors occurs after exposure to competitive glutamate site antagonists or to open-channel blockers such as MK-801. This suggests that the effect is due to blockade of the activation of NMDA receptors. In contrast, there was no change in the density of NMDA receptors after exposure to CNQX (Fig. 6A), suggesting that tonic activation of AMPA and kainate receptors is not involved in the regulation of expression of NMDA receptors under the conditions used in these studies. Furthermore, the binding of [^{3}H]AMPA, which selectively labels the AMPA subtype of glutamate receptor, was unchanged after exposure of cells to D-AP5 (Fig. 6B). The effects of D-AP5 are thus selective for NMDA receptors, and blockade of these receptors does not lead to a change in the density of at least one other subtype of glutamate receptor.

There is considerable interest in the potential clinical use of NMDA receptor antagonists to reduce ischemic brain damage and to treat epilepsy and various neurodegenerative diseases (2–5). It was reported that prolonged (2–20-week) exposure of cultured hippocampal neurons to the nonselective glutamate receptor antagonist kynurenic acid or to elevated concentrations of Mg^{2+} led to increases in the excitability of neurons, with cells showing "epileptic-like" activity, and increases in their susceptibility to excitotoxicity, after removal of the antagonists (18). These effects may involve increases in the density of NMDA receptors, because kynurenic acid and Mg^{2+} reduce activation of NMDA receptors (18–20). An increase in the density of NMDA receptors *in vivo* would be predicted from the results reported in the present work and could contribute to increased neuronal excitability after abrupt discontinuation of antagonist therapy. Such changes could complicate drug regimens and could lead to serious side effects, such as increased susceptibility to seizures and neurodegeneration after withdrawal of antagonists. In this regard, an increase in the density of NMDA receptor-specific binding sites for L-[^{3}H]glutamate and an increase in the severity of NMDA-induced neurotoxicity in rat brain after treatment of 6-day-old rats with MK-801 have been reported (21). However, in the same study (21), the apparent density of binding sites for [^{3}H]TCP (which labels the MK-801 binding site) in slices prepared from rat brain was decreased by 60–80%. This may be a consequence of the presence, in tissue slices, of residual MK-801 that inhibited the binding of [^{3}H]TCP.

The up-regulation of many types of receptors by antagonists is a reflection of blockade of the tonic effects of endogenous agonists. Our results suggest that NMDA receptors on neurons in culture are tonically activated. This could result from spontaneous synaptic activity between synaptically coupled neurons or from the presence of amino acids in the culture medium. Glutamate and aspartate are nominally present at concentrations of 10 μM in the medium, but their concentrations in culture are reduced to <1 μM within 24 hr of changing the medium, presumably due to cellular uptake.[2] Estimates of the concentration of glutamate in extracellular fluid *in vivo* range from 1 to 50 μM, which may be sufficient to cause tonic activation of NMDA receptors even in the absence of pronounced synaptic activity (22).

References

1. Mayer, M. L., and G. L. Westbrook. The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog. Neurobiol.* **28**:197–276 (1987).
2. Collingridge, G. L., and R. A. J. Lester. Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.* **41**:143–210 (1989).
3. Choi, D. W. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**:623–634 (1988).
4. Dingledine, R., C. J. McBain, and J. O. McNamara. Excitatory amino acid receptors in epilepsy. *Trends Pharmacol. Sci.* **11**:334–338 (1990).

[2] K. Williams and M. B. Robinson, unpublished observations.

5. Dichter, M. A., and D. W. Choi. Excitatory neurotransmitters and excitotoxins. *Curr. Neurol.* **9**:1–26 (1989).
6. Williams, K., C. Romano, M. A. Dichter, and P. B. Molinoff. Minireview: modulation of the NMDA receptor by polyamines. *Life Sci.* **48**:469–498 (1991).
7. Aarons, R. D., A. S. Nies, J. Gal, L. R. Hegstrand, and P. B. Molinoff. Elevation of β-adrenergic receptor density in human lymphocytes after propranolol administration. *J. Clin. Invest.* **65**:949–957 (1980).
8. Jenner, P., and C. D. Marsden. Neuroleptics and tardive dyskinesia, in *Neuroleptics: Neuorchemical, Behavioral, and Clinical Perspectives* (J. T. Coyle and S. J. Enna, eds.). Raven Press, New York, 223–253 (1983).
9. Williams, K., J. L. Hanna, and P. B. Molinoff. Developmental changes in the sensitivity of the *N*-methyl-D-aspartate receptor to polyamines. *Mol. Pharmacol.* **40**:774–782 (1991).
10. Dichter, M. A. Cerebral cortex tissue culture, in *Current Methods in Cellular Neurobiology* (J. Barker, ed.), Vol. 4. John Wiley and Sons, New York (1983).
11. Reynolds, I. J., and R. J. Miller. Multiple sites for the regulation of the *N*-methyl-D-aspartate receptor. *Mol. Pharmacol.* **33**:581–584 (1988).
12. Williams, K., V. L. Dawson, C. Romano, M. A. Dichter, and P. B. Molinoff. Characterization of polyamines having agonist, antagonist, and inverse agonist effects at the polyamine recognition site of the NMDA receptor. *Neuron* **5**:199–208 (1990).
13. Honoré, T., and J. Drejer. Chaotropic ions affect the conformation of quisqualate receptors in rat cortical membranes. *J. Neurochem.* **51**:457–461 (1988).
14. Mattson, M. P., P. Dou, and S. Kater. Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J. Neurosci.* **8**:2087–2100 (1988).
15. Wong, E. H. F., J. A. Kemp, T. Priestley, A. R. Knight, G. N. Woodruff, and L. L. Iversen. The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA* **83**:7104–7108 (1986).
16. Lehmann, J., A. J. Hutchinson, S. E. McPherson, C. Mondadori, M. Schmutz, C. M. Sinton, C. Tsai, D. E. Murphy, D. J. Steel, M. Williams, D. L. Cheney, and P. L. Wood. CGS 19755, a selective and competitive *N*-methyl-D-aspartate-type excitatory amino acid receptor antagonist. *J. Pharmacol. Exp. Ther.* **246**:65–75 (1988).
17. Honoré, T., S. N. Davies, J. Drejer, E. J. Fletcher, P. Jacobsen, D. Lodge, and F. E. Nielsen. Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science (Washington, D. C.)* **241**:701–703 (1988).
18. Furshpan, E. J., and D. D. Potter. Seizure-like activity and cellular damage in rat hippocampal neurons in cell culture. *Neuron* **3**:199–207 (1989).
19. Nowak, L., P. Bregestovski, P. Ascher, A. Herbet, and A. Prochiantz. Magnesium gates glutamate-activated channels in mouse central neurones. *Nature (Lond.)* **307**:462–465 (1984).
20. Mayer, M. L., G. L. Westbrook, and P. B. Guthrie. Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurons. *Nature (Lond.)* **309**:261–263 (1984).
21. McDonald, J. W., F. S. Silverstein, and M. V. Johnston. MK-801 pretreatment enhances *N*-methyl-D-aspartate-mediated brain injury and increases brain *N*-methyl-D-aspartate recognition site binding in rats. *Neuroscience* **38**:103–113 (1990).
22. Sah, P., S. Hestrin, and R. A. Nicoll. Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurons. *Science (Washington, D. C.)* **246**:815–817 (1989).

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