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Developmental Changes in the Sensitivity of the *N*-Methyl-D-Aspartate Receptor to Polyamines

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SUMMARY

The binding of [^{125}I]I-MK-801 to *N*-methyl-D-aspartate (NMDA) receptors on membranes prepared from cultured cerebral cortical neurons and from forebrain of rats of different ages was investigated. Specific binding of [^{125}I]I-MK-801 was enhanced by glutamate, glycine, and polyamines and was inhibited by divalent cations and open-channel blockers of the NMDA receptor, indicating that [^{125}I]I-MK-801 selectively labels a component of the NMDA receptor/ion channel complex. The ability of spermine to enhance the binding of [^{125}I]I-MK-801 was lower in membranes prepared from cultured cerebral cortical neurons or from neonatal rat brain than in membranes prepared from adult rat brain. There was a progressive increase in the potency of spermine and in the magnitude of the stimulatory effect of spermine in rat forebrain between days 3 and 10 of postnatal life. In contrast, the apparent affinity of the NMDA receptor for spermine remained unchanged in cerebral cortical neurons maintained in culture for up to 5 weeks. Mg^{2+} also enhanced the binding of [^{125}I]I-MK-801 and was more potent in membranes prepared from adult than from 3-day-old rat forebrain. The potency of glutamate for enhancing the binding of [^{125}I]I-MK-801 was not altered in 3-day-old, compared with adult, brain tissue. The increase in the affinity of the polyamine recognition site on the NMDA receptor complex in rat forebrain during the first 2 weeks of postnatal life suggests that the macromolecular properties of the NMDA receptor are altered during development. This may suggest that the subunit composition of the NMDA receptor is under developmental control. Cultured cortical neurons may represent a useful system for investigating factors that regulate developmental changes in the properties of the NMDA receptor.

The NMDA subtype of excitatory amino acid receptor is a ligand-gated ion channel that is involved in the generation of various forms of synaptic plasticity, including learning and memory, in both the developing and mature central nervous system (1–3). In the developing nervous system, activation of NMDA receptors may be involved in defining neuronal architecture and synaptic connectivity (2, 4, 5). For example, activation of NMDA receptors is involved in experience-dependent synaptic modifications, including the development of ocular dominance patterns in the visual cortex (4–6). Excessive or abnormally prolonged activation of NMDA receptors can lead to neuronal degeneration. This excitotoxicity may be involved in brain damage following ischemia and in a number of chronic neurodegenerative diseases (7). Activation of NMDA receptors has also been implicated in the development of epileptic states and in the occurrence of seizure activity (8).

The NMDA receptor/ion channel complex contains a number of distinct recognition sites for endogenous and exogenous ligands. These include binding sites for glutamate (or NMDA), glycine (9, 10), polyamines (11), Mg^{2+} (12, 13), Zn^{2+} (14, 15), and open-channel blockers such as MK-801 and TCP (16–18). Glutamate and glycine, which cause opening of the ion channel, greatly increase the rates of both association and dissociation of binding of [^{3}H]MK-801 (18–20). This is consistent with the proposal that MK-801 binds to an open-channel state of the receptor complex, probably to a site within the ion channel, and that glutamate and glycine increase the accessibility of this binding site. Mg^{2+} causes a voltage-dependent block of the ion channel and inhibits the binding of [^{3}H]MK-801 measured in the presence of glutamate and glycine (12, 13). In the nominal absence of glutamate and glycine, low concentrations of Mg^{2+} can stimulate the binding of [^{3}H]MK-801 (19, 21). Zn^{2+} acts at one or more distinct sites to inhibit NMDA-induced ion flux and to inhibit the binding of [^{3}H]MK-801 (14, 15, 19).

The endogenous polyamines spermine and spermidine have been shown to increase the affinity of a binding site for [^{3}H]MK-801 and [^{3}H]TCP above that seen in the presence of maximally effective concentrations of glutamate and glycine (22–25). These polyamines increase the rates of both associa-

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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; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; K_D, equilibrium dissociation constant; IBPA, 3,3′-iminobispropylamine; DA10, 1,10-diaminodecane; TCP, *N*-[1-(2-thienyl)cyclohexyl]piperidine; GABA, γ-aminobutyric acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid.

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tion and dissociation of binding of [^{3}H]MK-801, suggesting that they act by increasing the accessibility of the binding site for this ligand (26). Polyamines appear to act at a specific recognition site that is distinct from the binding sites for glutamate, glycine, Mg^{2+}, and Zn^{2+}. Spermine and spermidine have been termed agonists at this site (11, 23, 26). Compounds that have been classified as partial agonists, antagonists, and inverse agonists at the polyamine recognition site have also been described (11, 23–26). It has recently been suggested that Mg^{2+} can act as a partial agonist at the polyamine recognition site (27, 28), which could explain the stimulatory effects of Mg^{2+} on the binding of [^{3}H]MK-801 and [^{3}H]TCP observed in the nominal absence of glutamate and glycine (21, 27, 28).

Effects of polyamines on the function of NMDA receptors have also been reported. Spermine increased the current elicited by NMDA in cultured hippocampal neurons (26) and in *Xenopus* oocytes expressing NMDA receptors after injection of RNA prepared from rat brain (29). The effects of spermine on NMDA receptors in hippocampal neurons were blocked by the polyamine antagonist diethylenetriamine, which by itself had no effect on NMDA-induced currents (26). Effects of polyamines were not voltage dependent, indicating that polyamines do not act at the voltage-dependent binding sites for Mg^{2+} or Zn^{2+} (26, 29). These data are consistent with the suggestion that polyamines such as spermine and spermidine are allosteric modulators of the NMDA receptor, acting at a distinct polyamine recognition site associated with the NMDA receptor/ion channel complex.

The density of NMDA receptors in rat forebrain has been shown to be increased during the first 3 weeks of postnatal life, as determined in binding assays with [^{3}H]TCP, [^{3}H]MK-801, [^{3}H]glycine, and [^{3}H]glutamate (30–33). Developmental changes in the conductance properties and affinities of a number of multisubunit receptors that contain an integral ion channel, including the nicotinic acetylcholine receptor on skeletal muscle (34) and the inhibitory glycine receptor in the brain and spinal cord (35), have been reported. These changes result from alterations in the subunit composition of the receptor complex during development (34, 35). It is possible that changes in the macromolecular properties of other ligand-gated ion channels, including the NMDA receptor, occur during development. In this regard, the voltage-dependent blockade of the NMDA receptor complex has been shown to be less pronounced in the hippocampus of neonatal rats than in adult rats (36–38).

We have recently initiated a series of biochemical studies to investigate the properties and regulation of the NMDA receptor complex and its associated polyamine recognition site on cultured neurons. Studies of receptors on cultured neurons require the use of a radioligand labeled to a high specific activity. An early goal of the present work was to characterize the properties of the binding of [^{125}I]I-MK-801 to NMDA receptors. It was observed that the sensitivity of NMDA receptors to polyamines was 7–10-fold lower in membranes prepared from cultured cerebral cortical neurons than in membranes prepared from adult rat brain. This observation led us to investigate the effects of polyamines on NMDA receptors in rat brain during postnatal development. The results indicate that the sensitivity of the NMDA receptor to polyamines increases progressively *in vivo* during the first 2 weeks of postnatal development. In contrast, NMDA receptors expressed on cultured neurons prepared from fetal rat brain remain relatively insensitive to the effects of polyamines for up to 5 weeks *in vitro*. Cultured neurons may provide a useful model system with which to study the developmental control of the expression of NMDA receptors.

Materials and Methods

Primary culture of cortical cells. Embryos were removed from pregnant rats at 16–17 days of gestation. All subsequent procedures were carried out under aseptic conditions. The embryos were rinsed with sterile buffer A (containing 145 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Na-HEPES, and 8 mM glucose, pH 7.4), and the brains were removed and rinsed in buffer A. Cerebral cortices were dissected, placed in Dulbecco's modified Eagle's medium containing 0.1% (w/v) trypsin, and incubated for 45 min at 37°. The tissue was then washed twice in buffer (containing 145 mM NaCl, 3 mM KCl, 10 mM Na-HEPES, and 8 mM glucose, pH 7.4). Cortical tissue was dissociated 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) by trituration through a Pasteur pipette, and undissociated material was allowed to settle for 10 min. The cell suspension was removed, and the suspension and undissociated material were triturated separately. Undissociated material was allowed to settle for 10 min, and the resulting cell suspensions, consisting of >95% single cells, were removed and combined. The number of viable cells was determined by trypan blue exclusion. Cells were diluted in culture medium and plated at a density of 1.0–1.3 × 10^5 cells/cm^2 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. Culture medium was changed every 2–4 days. In this culture paradigm, neurons grow 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.

Membranes were prepared from cortical cells after 2–5 weeks in culture for binding assays with [^{125}I]I-MK-801. Cultures were washed three times with 10 ml of buffer A. Cells were removed from the dish by vigorous pipetting with 5–10 ml of buffer B (5 mM K-EDTA, pH 7.0) and homogenized in buffer B (25 ml) with a Polytron (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged (100,000 × g, 30 min), and pellets were resuspended in 40 ml of buffer B, incubated for 30 min at 32°, and then centrifuged (100,000 × g, 30 min). Pellets were resuspended in buffer B, and the washing procedure, including the 30-min incubation, was repeated three more times. The final pellets were resuspended in buffer C (20 mM K-HEPES, 1 mM K-EDTA, pH 7.0), at a concentration of 350–600 µg of protein/ml, and used for binding assays with [^{125}I]I-MK-801.

Preparation of membranes from rat brain. Sprague-Dawley rats, 1–21-days old or adults (14–16 weeks old), were killed by decapitation, and the forebrain (minus cerebellum and brainstem) was removed. Unless otherwise stated, all procedures were carried out at 4°. Brains (1–3 g of wet weight) were chopped with scissors and then homogenized, with a Polytron, in 10 volumes (w/v) of 0.32 M sucrose containing 5 mM K-EDTA (pH 7.0). Homogenates were centrifuged for 10 min at 1,000 × g, and supernatants were removed and centrifuged at 34,000 × g for 30 min. Pellets were resuspended in 250 ml of buffer B, incubated at 32° for 30 min, and centrifuged at 50,000 × g for 30 min. The washing procedure, including the 30-min incubation, was repeated three times. The final pellets were resuspended (1–1.5 mg of protein/ml) in buffer B and stored in aliquots at −80°.

Binding assays with [^{125}I]I-MK-801. Aliquots of brain membranes were thawed, diluted in buffer C, and washed twice by incubation at 32° for 30 min, followed by centrifugation (34,000 × g, 30 min). Membranes were resuspended in buffer C (100–150 µg of protein/ml). For experiments in which the effects of Mg^{2+} or Zn^{2+} were to be determined, membranes were thawed, washed twice in buffer without EDTA (20 mM K-HEPES, pH 7.0), and resuspended in EDTA-free buffer. Binding assays were carried out in polypropylene test tubes

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containing brain membranes (3–8 μg of protein) or membranes prepared from cultured cortical cells (15–30 μg of protein), [^{125}I]I-MK-801, and glutamate, glycine, and polyamines as indicated. The final incubation volume was 200 μl. For most experiments, a concentration of [^{125}I]I-MK-801 of 0.13 nM was used (specific activity, 2200 Ci/mmol). For saturation binding curves, the specific activity of [^{125}I]I-MK-801 was reduced 10-fold, to 220 Ci/mmol, by the addition of unlabeled ^{127}I-MK-801, and assays were carried out using nine concentrations (0.01–3.0 nM) of [^{125}I]I-MK-801 at this specific activity. 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 0.13 nM (specific activity, 2200 Ci/mmol), nonspecific binding of [^{125}I]I-MK-801 was <10% of total binding in the presence of 100 μM glutamate and glycine plus 30 μM spermine. About half of the nonspecific binding of [^{125}I]I-MK-801 was due to binding of the ligand to glass fiber filters. Binding to filters was not inhibited by unlabeled MK-801 or unlabeled ^{127}I-MK-801 and was not reduced by pretreatment of the filters with polyethyleneimine.

For studies of the association of binding of [^{125}I]I-MK-801, membranes were incubated in a total volume of 12 ml, and aliquots (200 μl; 3 μg of protein) were removed at various times (2 min to 24 hr). For studies of the dissociation of binding of [^{125}I]I-MK-801, membranes were incubated for 3 hr with [^{125}I]I-MK-801 in a total volume of 8 ml, followed by the addition of unlabeled MK-801 (10 μl; 10 μM final concentration), and aliquots (200 μl; 3 μg of protein) were removed at various times.

All assays were carried out at 32°. Assays were terminated by the addition of 10 ml of ice-cold assay buffer, followed by rapid filtration over glass fiber filters (Schleicher & Schuell no. 30). The filters were washed with another 10 ml of buffer, and radioactivity retained on the filters was determined in a Beckman γ-counter. EC_{50} values for the stimulatory effects of spermine were determined from concentration-effect curves and represent the concentration of spermine calculated to produce 50% of the maximal increase in binding of [^{125}I]I-MK-801 caused by spermine in membranes prepared from rats of a given age.

Materials. (+)-[3-^{125}I]I-MK-801 (specific activity, 2200 Ci/mmol) was provided by New England Nuclear/DuPont (Billerica, 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). Fetal calf serum was purchased from Hyclone (Logan, UT). Cytosine arabinoside was purchased from Calbiochem Corp. (La Jolla, CA). L-Glutamate and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Polyamines were purchased from Aldrich Chemical Co. (Milwaukee, WI). (−)-Ketamine hydrochloride was a gift from Parke-Davis Pharmaceuticals Division of Warner-Lambert Co. (Ann Arbor, MI). (+)-SKF 10,047 hydrochloride was provided by the National Institute on Drug Abuse. Magnesium acetate and zinc acetate were purchased from Fisher Scientific (Philadelphia, PA). All other reagents were of the highest purity available and were from commercial sources.

Results

Characterization of the binding of [^{125}I]I-MK-801. The binding of [^{125}I]I-MK-801 was determined in membranes prepared from adult rat brain. In assays containing small amounts of membrane (3–5 μg of protein), little specific binding of [^{125}I]I-MK-801 was observed in the nominal absence of glutamate, glycine, or polyamines. Addition of glutamate or glycine by themselves did not enhance the binding of [^{125}I]I-MK-801 (Fig. 1A). The inclusion of 100 μM glutamate plus 100 μM glycine or of 30 μM spermine increased the binding of [^{125}I]I-MK-801 (Fig. 1A). The effect of spermine was attenuated or abolished by the inclusion of the glutamate site antagonist D-2-amino-5-phosphonopentanoic acid (100 μM) or the glycine site antagonist 7-chlorokynurenic acid (100 μM) (data not shown). Maximal enhancement of the binding of [^{125}I]I-MK-801 was seen in the presence of 100 μM glutamate plus 30 μM spermine (Fig. 1A). Glycine enhanced the binding seen in the presence of glutamate but had no effect on the stimulation of binding caused by spermine. Spermidine (100 μM) was as effective as 30 μM spermine (data not shown). The association (Fig. 1B) and dissociation (Fig. 1C) of binding of [^{125}I]I-MK-801 were determined in the presence of glutamate and glycine and of glutamate and glycine plus spermine. In the presence of glutamate

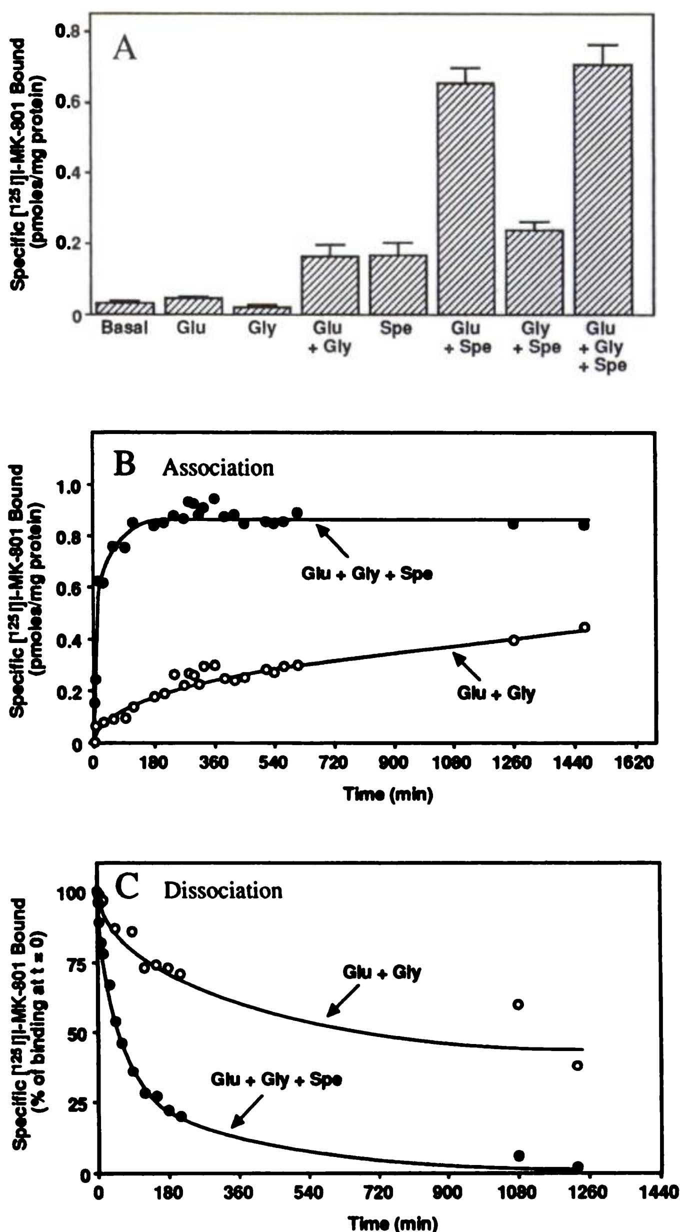

Fig. 1. Binding of [^{125}I]I-MK-801 to membranes prepared from adult rat brain. A, Binding of [^{125}I]I-MK-801 was determined in the absence of added modulators (*Basal*) and in the presence of various combinations of 100 μM L-glutamate (*Glu*), 100 μM glycine (*Gly*), and 30 μM spermine (*Spe*), as shown. Values are mean ± standard error (four experiments). B and C, The association (B) and dissociation (C) of binding of [^{125}I]I-MK-801 were determined in the presence of 100 μM glutamate and glycine and in the presence of 100 μM glutamate and glycine plus 30 μM spermine. Data are representative of results obtained in two similar experiments.

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and glycine, binding of [^{125}I]I-MK-801 did not reach equilibrium for at least 24 hr (Fig. 1B). Spermine greatly enhanced the rate of association of [^{125}I]I-MK-801, and binding reached equilibrium within 2–3 hr when assays were carried out in the presence of spermine (Fig. 1B). The rate of dissociation of [^{125}I]I-MK-801 was also enhanced in the presence of spermine (Fig. 1C). The kinetics of association and dissociation of binding of [^{125}I]I-MK-801 were complex and could not be described by monoexponential rate constants. Pseudo-first-order association and first-order dissociation plots were multiphasic (data not shown).

Saturation binding analyses were carried out in the presence of 100 μM glutamate and glycine plus 100 μM spermidine (Fig. 2) or 30 μM spermine. Scatchard analysis of the binding of [^{125}I]I-MK-801 revealed that the ligand binds with high affinity (K_D = 0.15–0.3 nM) to a single class of binding sites (Fig. 2, *inset*).

When assays were carried out in the presence of 100 μM glutamate and glycine plus 30 μM spermine, binding of [^{125}I]I-MK-801 was inhibited by a number of compounds, including MK-801, ketamine, and SKF 10,047, that are open-channel blockers, thought to act at a common binding site on the NMDA receptor/ion channel complex (Fig. 3). Binding of [^{125}I]I-MK-801 was also inhibited by Mg^{2+} and Zn^{2+}, which act at distinct sites on the receptor complex (Fig. 3). The properties of the NMDA receptor complex identified with [^{125}I]I-MK-801 are

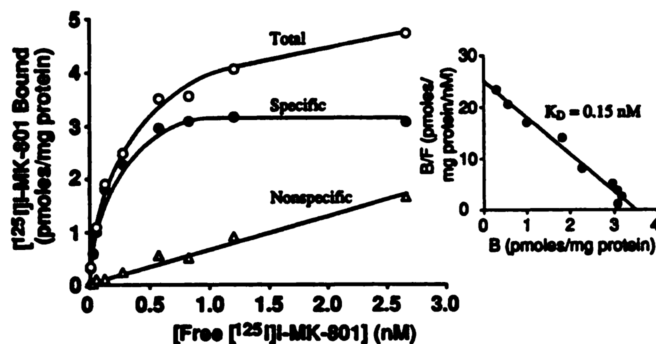

Fig. 2. Binding of [^{125}I]I-MK-801 to membranes prepared from adult rat brain. Binding of [^{125}I]I-MK-801 (specific activity reduced to 220 Ci/mmol) was determined in the presence of 100 μM glutamate, glycine, and spermidine. Data are from a single experiment, representative of five such experiments. *Inset*, Scatchard analysis of the specific binding of [^{125}I]I-MK-801.

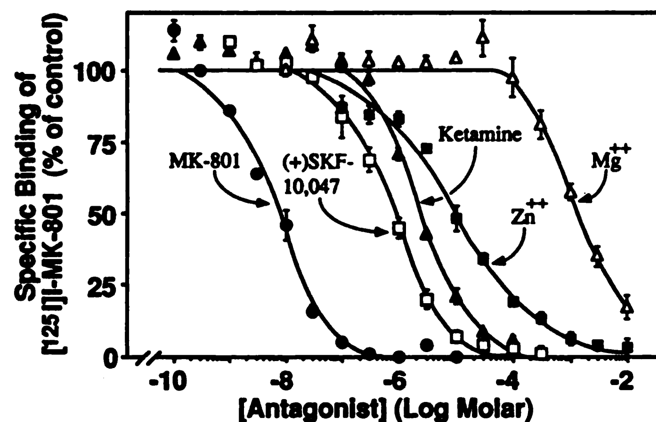

Fig. 3. Inhibition of [^{125}I]I-MK-801 binding by open-channel blockers of the NMDA receptor and by divalent cations. Binding of [^{125}I]I-MK-801 was determined in the presence of 100 μM glutamate and glycine plus 30 μM spermine. Data are mean ± standard error from three or four experiments.

thus similar to those seen in studies with [^{3}H]MK-801 and [^{3}H]TCP (10, 16, 18–25, 39, 40).

Binding of [^{125}I]I-MK-801 to NMDA receptors in cortical neurons. The effects of spermine on the binding of [^{125}I]I-MK-801 to membranes prepared from cerebral cortical neurons maintained for 2–5 weeks in culture were determined and compared with the effects on membranes prepared from adult rat brain (Fig. 4). The density of binding sites was 5–25-fold lower in cultured cortical neurons (Fig. 4C) than in adult rat brain (Fig. 4B). In the presence of glutamate and glycine, spermine enhanced the binding of [^{125}I]I-MK-801 to NMDA receptors in membranes from cerebral cortical neurons and from adult brain. However, the potency of spermine to enhance binding of [^{125}I]I-MK-801 to NMDA receptors on cultured cortical neurons was 7–10-fold lower than in adult rat brain (Fig. 4A). There was also a small change in the inhibitory effects of high concentrations of spermine. Similar results were seen in studies of neurons prepared from hippocampus (data not shown). The relatively low potency of the stimulatory effect of spermine at NMDA receptors on cortical neurons maintained in culture for 13–19 days was not altered after 5 weeks in culture (Fig. 4A).

Developmental studies in postnatal rat brain. The difference in the potency of spermine observed in membranes prepared from cultured neurons derived from fetal rat brain and in membranes prepared from adult rat brain led us to investigate the effects of spermine on binding of [^{125}I]I-MK-801 to NMDA receptors during postnatal development. The density of binding sites for [^{125}I]I-MK-801 increased progressively during postnatal development, with no change in the affinity of the sites for [^{125}I]I-MK-801, measured in the presence of a combination of modulators that maximally activate the receptor/ion channel complex (Fig. 5). The effects of spermine on the binding of [^{125}I]I-MK-801 were compared in membranes prepared from 3-day-old and adult rat forebrain. In the presence of 100 μM glutamate and glycine, the binding of [^{125}I]I-MK-801 was much lower in membranes prepared from 3-day-old than from adult rat brain (Fig. 6). Spermine increased the binding of [^{125}I]I-MK-801 to NMDA receptors in both adult and 3-day-old rat brain. The absolute increase (fmol/mg of protein) in binding of [^{125}I]I-MK-801 seen in the presence of spermine was greater in membranes prepared from adult than from 3-day-old brain. Furthermore, the potency of spermine for stimulating the binding of [^{125}I]I-MK-801 was 7–8-fold higher in adult than in 3-day-old rat brain (Fig. 6, *inset*), with little or no change in the inhibitory effect of spermine.

The difference in the potency of spermine in membranes prepared from adult rats, compared with 3-day-old rats, was also observed after extensive washing of the membranes. Concentration-effect curves for spermine were not altered when membranes prepared from 3-day-old and adult rat brain were washed six times after thawing on the day of an experiment, compared with washing twice (data not shown). This suggests that the difference in the potency of spermine observed in 3-day-old and adult brain tissue is not due to the presence of different concentrations of residual substances that might influence the activation state of the NMDA receptor complex.

The effects of spermine were determined in membranes prepared from rats of various ages, from 1-day postnatally to adult (Fig. 7). There was little change in the level of binding of [^{125}I]I-MK-801 measured in the presence of glutamate and

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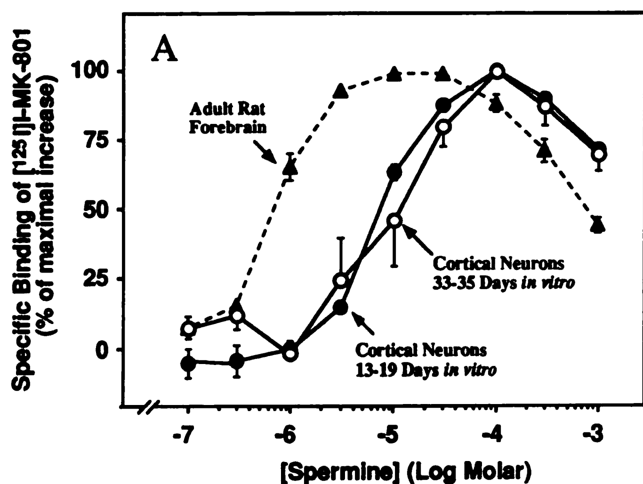


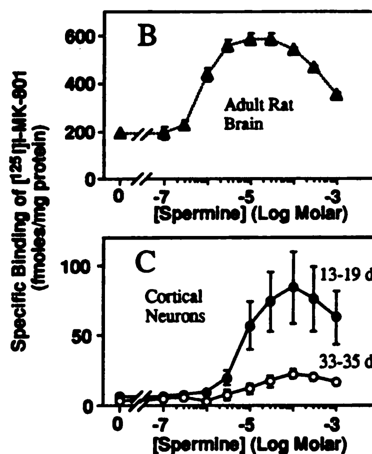


Fig. 4. Effect of spermine on the binding of [^{125}I]I-MK-801 to NMDA receptors in membranes prepared from adult rat forebrain and cultured neurons. The effects of spermine on binding of [^{125}I]I-MK-801 to membranes prepared from adult rat brain or from cerebral cortical neurons maintained for 13–19 or 33–35 days in culture were determined in the presence of 100 μM glutamate and glycine. A, Data are expressed as a percentage of the maximal increase in binding seen with spermine. B and C, Untransformed data showing binding of [^{125}I]I-MK-801 as fmol/mg of protein. Values are mean ± standard error from four or five experiments.

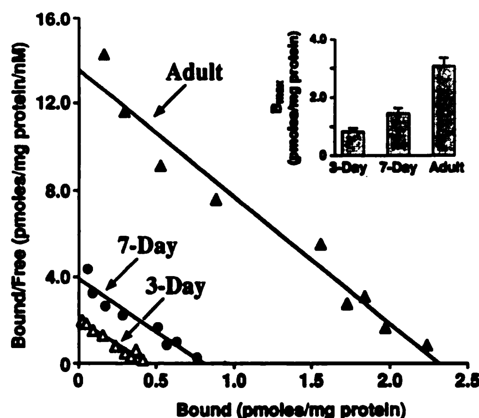
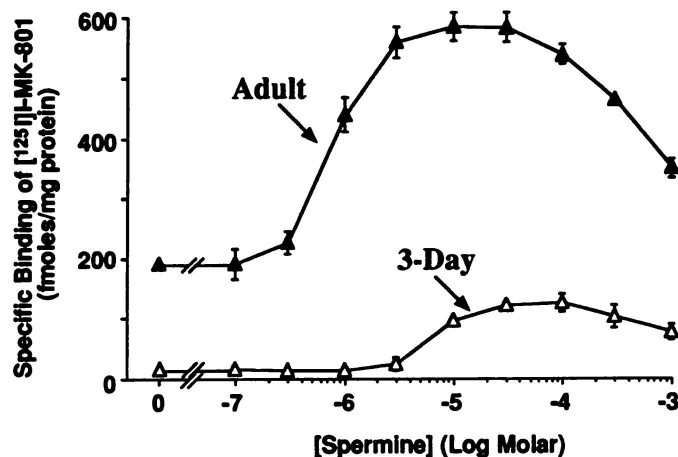


Fig. 5. Binding of [^{125}I]I-MK-801 to membranes prepared from rats of different ages. Scatchard analyses of the binding of [^{125}I]I-MK-801 to membranes prepared from forebrain of 3-day-old, 7-day-old, and adult rats are shown. Experiments were carried out in the presence of 100 μM glutamate, glycine, and spermidine. *Inset*, values for B_{max} (mean ± standard error) from three such experiments at each age.

glycine during postnatal days 1–7, but there was a progressive increase in the stimulation of binding of [^{125}I]I-MK-801 caused by spermine over this time (Fig. 7A). In addition, the potency of spermine increased during postnatal days 3–10, as illustrated by the decrease in the EC_{50} for spermine (Fig. 7B). Little further change occurred after postnatal day 10.

The effects of two other agonists at the polyamine recognition site, spermidine and IBPA (23), and of the inverse agonist DA10 (26) were determined in membranes prepared from 3-day-old and adult rats. The potencies of spermidine and IBPA at NMDA receptors in 3-day-old rat brain were 5–7-fold lower than in adult brain (Fig. 8, A and B). This was similar to the change in sensitivity of the NMDA receptor to spermine. In contrast, there was no difference in the potency of DA10 in membranes prepared from 3-day-old, compared with adult, rat brain (Fig. 8C).

The effects of glutamate and Mg^{2+} on the binding of [^{125}I]I-MK-801 were also investigated in membranes prepared from 3-day-old and adult rats. In the absence of spermine, little binding of [^{125}I]I-MK-801 was observed even in the presence of 100 μM glutamate and glycine (Fig. 1A). Therefore, concentration-effect curves for glutamate were measured in the nominal absence of glycine but in the presence of 30 μM spermine. There was no difference in the potency of glutamate in membranes prepared from 3-day-old, compared with adult, rat brain (Fig. 9A). The potency of glutamate was also the same with membranes prepared from 3-day-old and adult rats when concentration-effect curves for glutamate were measured in the presence of 30 μM spermine plus 100 μM glycine (data not shown). In the presence of glutamate and glycine, Mg^{2+} (3–100 μM) enhanced the binding of [^{125}I]I-MK-801. At a concentration of



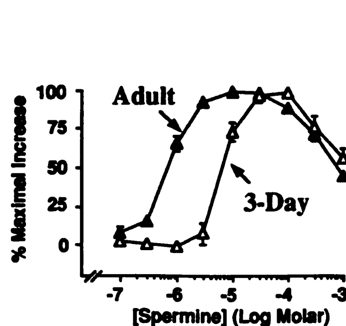


Fig. 6. Effect of spermine on the binding of [^{125}I]I-MK-801 to NMDA receptors in membranes prepared from 3-day-old and adult rat brain. Experiments were carried out in the presence of 100 μM glutamate and glycine. Values are mean ± standard error from five (adult) or three (3-day-old) experiments. *Inset*, The same data are expressed as a percentage of the maximal increase in binding of [^{125}I]I-MK-801 seen with spermine in membranes from adult or 3-day-old rats.

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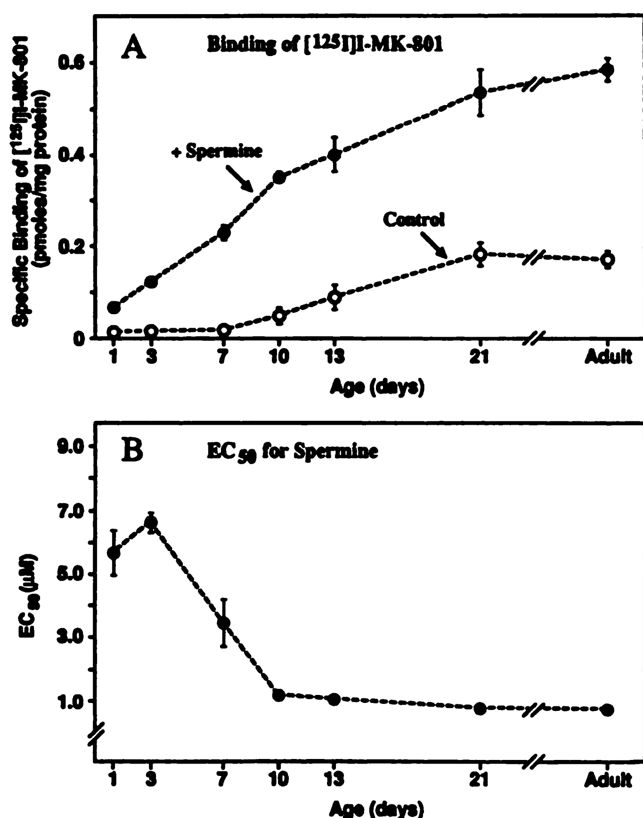

Fig. 7. Effect of spermine on the binding of [^{125}I]I-MK-801 to NMDA receptors in membranes prepared from forebrain of rats of different ages. A, Binding was measured in the presence of 100 μM glutamate and glycine (*Control*) and in the presence of 100 μM glutamate and glycine plus 30 μM spermine. B, EC_{50} values for the stimulatory effects of spermine were derived from concentration-effect curves, similar to those shown in Fig. 5. Data are mean ± standard error from three to five experiments at each time point.

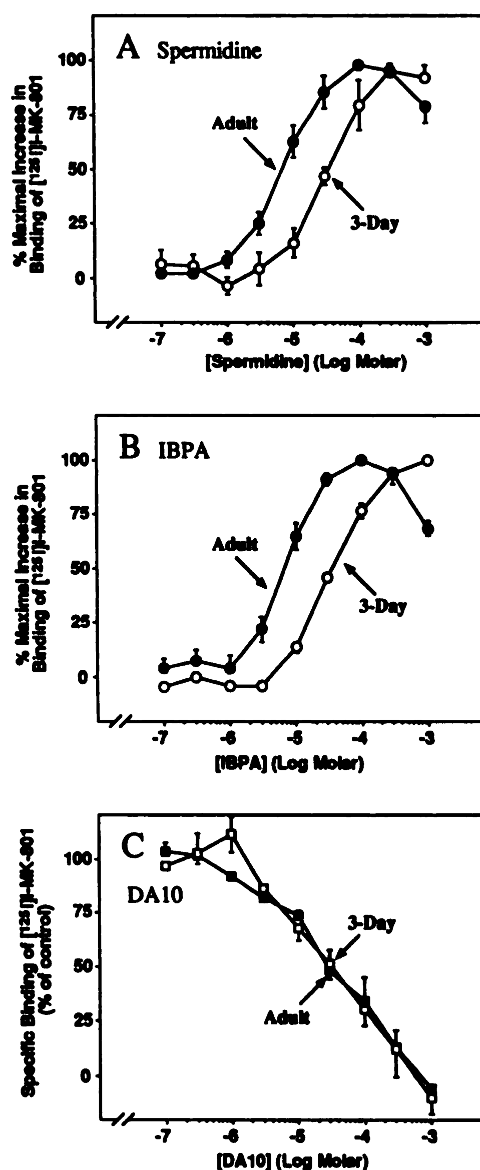

Fig. 8. Effects of polyamines on the binding of [^{125}I]I-MK-801 to membranes prepared from 3-day-old and adult rat forebrain. Effects of spermidine (A), IBPA (B), and DA10 (C) were determined in the presence of 100 μM glutamate and glycine. Data in A and B are mean ± standard error from three experiments and are expressed as a percentage of the maximal increase in binding of [^{125}I]I-MK-801 seen in the presence of spermidine or IBPA. Data in C are mean ± range of two experiments and are expressed as a percentage of the control binding of [^{125}I]I-MK-801 determined in the presence of glutamate and glycine. For the experiments with DA10 (C), incubations were carried out for 24 hr.

100 μM, Mg^{2+} caused a 2–3-fold increase in the level of binding of [^{125}I]I-MK-801. Inhibition of binding was seen at higher concentrations of Mg^{2+} (Fig. 9B). Both the stimulatory and inhibitory effects of Mg^{2+} occurred at lower concentrations in membranes prepared from adult than from 3-day-old rat forebrain (Fig. 9B).

Discussion

In the experiments reported in this paper, we have used a new radiolabeled derivative of MK-801, [^{125}I]I-MK-801, to examine changes in the properties of the NMDA receptor complex in the developing rat brain and in cultured cerebral cortical neurons. Tritium-labeled MK-801 has been widely used to study the properties of the NMDA receptor complex, and experiments carried out with [^{3}H]MK-801 have provided information about allosteric interactions between various sites on the receptor complex, possible mechanisms of action of modulators acting at these sites, and the localization and regulation of NMDA receptors (e.g., Refs. 10, 16, 19–24, 39, and 41). The availability of [^{125}I]I-MK-801, which is labeled to about a 100-fold higher specific activity than [^{3}H]MK-801, now makes it possible to study the properties and regulation of NMDA receptors in tissues where the density of receptors is low or where the amount of material available is limited.

The characteristics of the binding of [^{125}I]I-MK-801 are similar to those of [^{3}H]MK-801. The affinity of binding of [^{125}I]I-MK-801 was 4–20-fold higher than that reported for [^{3}H]MK-801 (22, 23, 26, 28). The specific binding of [^{125}I]I-MK-801 was enhanced in the presence of 100 μM glutamate plus 100 μM glycine or in the presence of spermine or spermine plus one or both of the amino acids. By themselves, neither glutamate nor glycine increased the binding of [^{125}I]I-MK-801. This suggests that the concentrations of residual glutamate and glycine (derived from the membrane preparations) present in the assays are very low, because the effect of glutamate on binding of open-channel blockers is thought to require the presence of glycine, and *vice versa*. However, spermine in-

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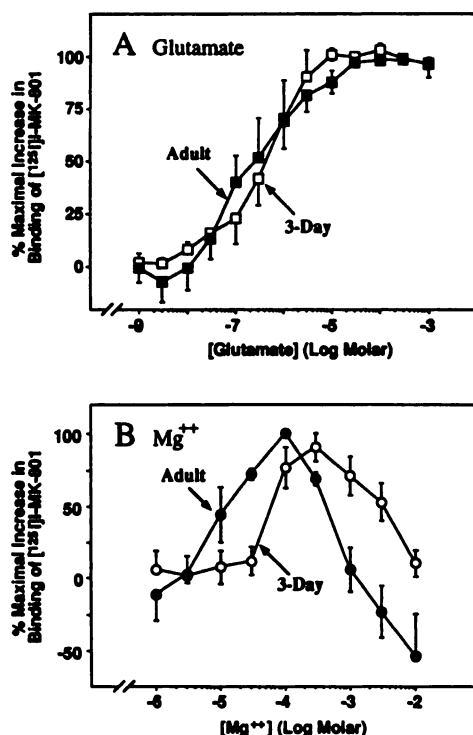

Fig. 9. Effects of glutamate and Mg^{2+} on the binding of [^{125}I]I-MK-801 to membranes prepared from 3-day-old and adult rat forebrain. A, Effects of glutamate were determined in the presence of 30 μM spermine in the nominal absence of glycine. B, Effects of Mg^{2+} were determined in the presence of 100 μM glutamate and glycine. Data in each panel are mean ± standard error from three experiments and are expressed as a percentage of the maximal increase in binding of [^{125}I]I-MK-801 seen in the presence of glutamate or Mg^{2+}.

creased the binding of [^{125}I]I-MK-801 even in the absence of added glutamate and glycine, and this effect was blocked by antagonists at the binding sites for glutamate and glycine. It is possible that spermine increases the affinity of the amino acid binding sites for residual endogenous amino acids (22) and that this accounts for its ability to enhance the binding of [^{125}I]I-MK-801 in the nominal absence of glutamate and glycine. This could also account for the observation that maximal enhancement of the binding of [^{125}I]I-MK-801 was seen with spermine plus glutamate even in the absence of added glycine.

Maximal enhancement of the binding of [^{125}I]I-MK-801 was observed in the presence of glutamate plus spermine, and the level of binding was 200–300% higher than that observed in the presence of glutamate and glycine. The increase in binding of [^{125}I]I-MK-801 observed in the presence of spermine was greater than that previously seen in similar studies using [^{3}H]MK-801. In binding assays with [^{3}H]MK-801, spermine caused a 2-fold increase in the apparent affinity of binding of [^{3}H]MK-801, which was manifest as a 40–50% increase in binding when studies were carried out in the presence of glutamate and glycine using a concentration of [^{3}H]MK-801 close to its K_D value (22, 23, 28). The larger increase in the binding of [^{125}I]I-MK-801 in the presence of spermine that was seen in the present study probably results, at least in part, from the use of a lower concentration of radioligand. Spermine greatly increased the rates of association and dissociation of [^{125}I]I-MK-801, similar to effects seen in assays with [^{3}H]MK-801 (24, 26). The kinetics of binding of [^{125}I]I-MK-801 were complex and could not be described by monoexponential rate constants. Similar effects have been observed in studies of the binding of [^{3}H]MK-801. The complex kinetics of binding of [^{125}I]I-MK-801 are probably a reflection of two or more modes of access of the ligand to its binding site within the ion channel of the NMDA receptor complex.

A difference between the binding of [^{125}I]I-MK-801 and [^{3}H]MK-801 was observed in studies of the effects of Mg^{2+}. In binding assays with [^{3}H]MK-801 carried out in the nominal absence of glutamate and glycine, Mg^{2+} enhances and then inhibits, in a concentration-dependent manner, the binding of [^{3}H]MK-801. Inhibition but not enhancement of binding is seen when assays are carried out in the presence of saturating concentrations of glutamate and glycine (21, 26, 39). In contrast, the binding of [^{125}I]I-MK-801 was enhanced by concentrations of Mg^{2+} from 3–100 μM, even in the presence of 100 μM glutamate and glycine. It is possible that there are differences in the properties of binding of [^{125}I]I-MK-801, compared with [^{3}H]MK-801, or that the differences observed in studies with [^{125}I]I-MK-801 are due to the use of nonequilibrium conditions or of lower concentrations of membranes in experiments with [^{125}I]I-MK-801 (3–8 μg of membrane protein), compared with studies with [^{3}H]MK-801 (80–200 μg of membrane protein). The use of smaller amounts of protein means that the concentrations of residual endogenous modulators of the receptor complex associated with the membranes are also reduced.

The specific binding of [^{125}I]I-MK-801 was inhibited by a number of antagonists [MK-801, ketamine, and (+)-SKF 10,047] that function as open-channel blockers of the NMDA receptor. The rank order of potency of these compounds and their apparent affinities were similar to those determined in binding assays with [^{3}H]MK-801 and [^{3}H]TCP (39, 40). When assays were carried out in the presence of glutamate and glycine plus spermine, binding of [^{125}I]I-MK-801 was also inhibited by Mg^{2+} and Zn^{2+}, which have previously been shown to act at distinct sites on the receptor complex. Taken together, these data are consistent with binding of [^{125}I]I-MK-801 to an open-channel state of the receptor complex, probably to the same site labeled by [^{3}H]MK-801 and [^{3}H]TCP.

The major observations from this study concern the developmental changes in the apparent affinity of the NMDA receptor complex for polyamines. NMDA receptors expressed in cultured cerebral cortical neurons prepared from embryonic rat brain exhibited a relatively low sensitivity to polyamines, similar to the sensitivity of receptors in neonatal rat forebrain. The potency of spermine at NMDA receptors in rat forebrain increased progressively during days 3–10 of postnatal life and by day 21 was identical to the potency seen in adult tissue. During postnatal days 3–10 *in vivo* there was an increase both in the potency of spermine at the NMDA receptor and in the magnitude of the enhancement of binding of [^{125}I]I-MK-801 by spermine. The sensitivities of the NMDA receptor in adult and 3-day-old rats to spermidine and IBPA, also agonists at the polyamine recognition site, and to the stimulatory effects of Mg^{2+} were also different. The latter observation is consistent with recent suggestions that Mg^{2+} can act as a partial agonist at the polyamine recognition site (27, 28). The affinity of the receptor for glutamate determined in binding assays with [^{125}I]I-MK-801 was not different in membranes prepared from 3-day-old, compared with adult, rats. This suggests that the change in the potency of polyamines is not generalized to other recognition sites that are coupled to channel opening and to

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increases in the binding of [^{125}I]I-MK-801. Changes in the effects of polyamines that occur during development may, therefore, be due to alterations in the properties of the polyamine recognition site or of its coupling to the ion channel or other components of the NMDA receptor complex.

We have suggested that DA10 may act as an inverse agonist at the polyamine recognition site on the NMDA receptor (26). DA10 inhibits the binding of [^{3}H]MK-801, an effect that is opposite to that of spermine and is blocked by the polyamine antagonist diethylenetriamine. These findings suggest that spermine, diethylenetriamine, and DA10 act at the same site (26). In the present study, the potency of DA10 was not different in membranes prepared from 3-day-old and adult rats. This is in contrast to the developmental change in sensitivity to polyamine agonists. This suggests that some of the domains on the NMDA receptor that are involved in binding polyamine agonists, but not those involved in binding the inverse agonist DA10, are altered during development. Another possibility is that the stimulatory effects of spermine are mediated at a site distinct from that which mediates inhibitory effects of DA10 (11).

Developmental changes in the properties of a number of receptors that are multisubunit, ligand-gated ion channels have been reported. For example, the conductance properties of the nicotinic acetylcholine receptor are different in fetal, compared with adult, skeletal muscle (34). This is due to the expression of receptors made up of different combinations of polypeptide subunits in adult and fetal muscle. Receptors expressed at both ages contain identical α, β, and δ subunits, but receptors in fetal muscle additionally contain a γ subunit that is replaced by an ϵ subunit in adult muscle (34). The inhibitory glycine receptor present in the brain and spinal cord of neonatal rats is less sensitive to strychnine than is the receptor expressed in adult rats. The difference in the affinity of binding of strychnine is due to a change in the subunit composition of glycine receptors in neonatal and adult rats (35). It has recently been shown that the α subunit expressed in neonates differs by one amino acid from the α subunit expressed in adults, a change that accounts entirely for the difference in sensitivity to strychnine (35). Developmental changes in the levels and regional distributions of mRNAs coding for polypeptide subunits of AMPA-sensitive glutamate receptors and $GABA_A$ receptors have also been reported and may be involved in the expression of different forms of the receptor complexes during development (42–44). mRNAs coding for subunits of AMPA receptors are expressed in two alternative splice forms, termed Flip and Flop, which confer different conductance properties on receptors containing Flip or Flop subunits. Differential changes in the levels and distributions of Flip and Flop mRNA splice variants during postnatal development have recently been reported (44). The NMDA receptor is likely to be a multisubunit complex analogous to the nicotinic acetylcholine, $GABA_A$, inhibitory glycine, and AMPA receptors. It is thus conceivable that there are changes in the subunit composition of the NMDA receptor during development and that these changes are responsible for differences in the sensitivity of the receptor to polyamines.

In contrast to the developmental change in the sensitivity of NMDA receptors to spermine that occurred *in vivo*, the sensitivity of NMDA receptors to spermine remained unchanged for up to 5 weeks in cerebral cortical neurons maintained *in vitro* (equivalent to 4.5 weeks *in vivo*). It may be that the factors responsible for developmental changes in the properties of NMDA receptors *in vivo* are not present or are not operative in the primary culture paradigm used in these studies. Expression of NMDA receptors may be regulated in part by tonic neuronal activity or by neuroactive factors released from neurons or glial cells in the central nervous system. Neurons maintained in primary culture may thus represent a useful system for future studies designed to identify factors that regulate the expression of NMDA receptors during development.

Inhibitory effects of Mg^{2+} determined in binding assays with open-channel blockers such as [^{3}H]MK-801 and [^{125}I]I-MK-801 may be mediated at the voltage-dependent Mg^{2+} binding site characterized in electrophysiological studies of NMDA receptors. The inhibitory effects of Mg^{2+} on the binding of [^{125}I]I-MK-801 were more potent in membranes prepared from adult than from neonatal rat brain. This observation is consistent with results from a number of electrophysiological studies that have led to the conclusion that NMDA receptors in neonatal rat hippocampus have a reduced sensitivity to the voltage-dependent blockade by Mg^{2+}, compared with receptors in adult hippocampus (36–38). These results suggest that there may also be changes in the affinity or properties of the ion channel Mg^{2+} binding site during postnatal development. Again, this could be due to alterations in the subunit composition of the NMDA receptor complex during development.

Polyamines are known to play a role in cellular growth and differentiation, by influencing transcription and translation of nucleic acids and the synthesis of proteins (45, 46). These effects of polyamines are mediated at intracellular sites. However, current evidence suggests that the polyamine recognition site of the NMDA receptor complex is located extracellularly, and endogenous polyamines may be released from neurons or glial cells and act at this site to modulate the properties of the receptor (11). The NMDA receptor/ion channel complex is thought to be involved in the generation of various forms of synaptic plasticity in the developing nervous system, including changes in the signaling properties of synapses and changes in neuronal and synaptic morphology (2, 4–6). The role of the polyamine recognition site on the NMDA receptor complex in normal physiological processes is unknown. If polyamines act as endogenous modulators of the NMDA receptor complex, then it is possible that changes in the sensitivity of the receptor to polyamines during development may be involved in regulating the properties and function of the receptor, its response to endogenously released polyamines, and its role in synaptic transmission and plasticity.

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