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[^{3}H]MK-801 Binding to the Synaptic Membranes of Rat Forebrains: Age-related Regulation by Glutamate, Glycine and Spermine

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The N-methyl-D-aspartate (NMDA) receptor-mediated glutamatergic neurotransmission is involved in synaptic plasticity, developmental processes, learning and memory and many neuropathological disorders including age-related diseases. In the present study, regulation of the NMDA receptor properties by various ligands was investigated using [^{3}H]MK-801 binding studies in the synaptic membranes of young and aged rat forebrains. The binding in the presence of glutamate and glycine increased dramatically with growth between 1 and 6 weeks old, and thereafter declined gradually with aging. Glutamate, glycine or spermine respectively increased the binding with growth. Glutamate maintained the binding during aging, while glycine or spermine significantly decreased the binding in the aged brain. The maximum stimulation by glycine varied depending on the ages of brains. Greater sensitivity to glycine was observed at 1 week and 3 months and the sensitivity was significantly reduced in the aged brain. In contrast, spermine showed similar stimulation patterns in young and aged rats. These results indicated that the functional properties of the NMDA receptor-ion channel complex in young and aged rat forebrains are differentially regulated by agonists, and the reduction of the receptor function with normal aging may be, in some degree, due to the reduction of the receptor sensitivity to glycine.

Key Words: NMDA receptor, Aging, Synaptic plasticity, Radioligand binding, MK-801

Abbreviations: NMDA, N-methyl-D-aspartate; EAA, excitatory amino acid; LTP, long-term potentiation; PCP, phencyclidine; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; 5,7-DCKA, 5,7-dichloro-kynurenic acid.

INTRODUCTION

L-glutamic acid is a predominant excitatory amino acid (EAA) neurotransmitter in the mammalian central nervous system. Evidence has been accumulated during the last 15 years indicating that EAA receptors play important roles in synaptic plasticity, developmental processes, and learning and memory. Hyperactivity of EAA receptors is associated with neuronal cell injuries in cerebral ischemia, anoxia and hypoglycemia, and involved in the etiology of certain neurodegenerative disorders such as Huntington's chorea, epilepsy and Alzheimer's disease (for reviews, Huettner, 1990; Kemp & Leeson, 1993; Bigge & Malone, 1993; Leeson & Iversen, 1994; Herrling, 1994; Muir & Lees, 1995).

N-methyl-D-aspartate (NMDA) receptor, a subtype of EAA receptors, has been found to be an essential component in the induction and maintenance of long-term potentiation (LTP) that underlies learning and memory processes (Collingridge et al, 1983; Harris et al, 1984; Bliss & Collingridge, 1993). The NMDA receptor has multiple recognition sites through which the receptor function is regulated. These include sites for glutamate, glycine, phencyclidine (PCP), polyamine and divalent cations such as Mg^{2+} and Zn^{2+}. Although

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precise regulation mechanisms by these ligands are not clearly elucidated to date, it is now widely accepted that both glutamate and glycine are required for the activation of this receptor (Johnson & Ascher, 1987; reviewed by Thomson, 1990). Polyamines such as spermine and spermidine are believed to modulate the receptor activity through the association with polyamine binding site and through the control of the affinity of glycine for its binding site (reviewed by Rock & McDonald, 1995).

PCP and MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine) block NMDA receptor-mediated responses by binding to the PCP site located within the receptor-ion channel. The binding is use-dependent and potentiated dose-dependently by glutamate and glycine (Foster & Wong, 1987; Reynolds et al, 1987; Wong et al, 1987; Ransom & Stec, 1988; Cho et al, 1996). The potentiation of MK-801 binding by these agonists is reversed by competitive antagonists. Based on these findings, [^{3}H] MK-801 binding studies are considered to be a biochemical marker of the NMDA receptor function (Ransom & Stec, 1988), and therefore, utilized in the present study as well as in studies previously reported to assess the functional properties of the receptor (Foster & Wong, 1987; Reynolds et al, 1987; Wong et al, 1987; Ransom & Stec, 1988; Cho et al, 1996).

Cloning studies have shown that pharmacologically distinct populations of NMDA receptor subtypes with different regulatory properties are present in various regions of brain (Monaghan & Buller, 1994). In addition, distinct NMDA receptor isoforms with reduced sensitivity to Mg^{2+} are preferentially expressed early in development (Ben-Ari et al, 1988; Morrisett et al, 1990). These findings imply fine-tuned regulation of the receptor properties during the whole life span including development and aging.

It has been suggested that the age-related decline of cognition and memory may be associated with the reduction of the NMDA receptor function. Radioligand binding studies (Tamaru et al, 1991; Cohen & Mueller, 1992; Serra et al, 1994) using [^{3}H]glutamate, [^{3}H]glycine, [^{3}H]CGP 39653 and [^{3}H]MK-801 indicated that the bindings of these ligands were decreased in several areas of aged rat brains, and the decreases were due to losses in the number of binding sites rather than changes in the binding affinity. Similar results were obtained with the aged cat visual cortex (Gordon et al, 1991) and human brains (Slater et al, 1993). These observations strongly suggest that the decrease of the NMDA receptor density in the aged brain might be one of the causative factors of age-related cognitive impairment (Cohen & Mueller, 1992; Mueller et al, 1994). In contrast, AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor is less likely to be involved in these processes (Miyoshi et al, 1991a; Tamaru et al, 1991).

In the present study, we investigated regulation of the receptor properties by glutamate, glycine and spermine in young and aged rat forebrains to examine if the receptor function is differentially regulated depending on the stages of growth and aging. All three agonists contributed to the increases in [^{3}H]MK-801 binding between 1～3 weeks and 3 months, but the reduction of the binding observed in the aged brain appeared to be contributed by the actions of glycine and spermine rather than glutamate. The reduction of the receptor function observed with normal aging may be, in part, due to the reduction of the receptor sensitivity to glycine.

METHODS

Materials

Glycine, polyethylenimine, L-glutamic acid, Trizma base, sucrose, Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Spermine 4HCl, ifenprodil tartrate, 5,7-dichlorokynurenic acid (5,7-DCKA), and (+)-MK-801 were from Research Biochemicals International (Natick, MA). Bio-Rad D_C protein assay reagent was from Bio-Rad. (+)-[3-^{3}H] MK-801 (20.3 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Whatman GF/B was from Whatman. Lumagel scintillation cocktail was purchased from Lumac*LSC B.V. (Olen, Belgium). All other reagents were reagent grade or better.

Animals

Male Sprague-Dawley rats were obtained from Experimental Animal Laboratory, Korea Research Institute of Chemical Technology, and maintained under standard laboratory conditions with food and water *ad libitum*. The animals were decapitated at the ages of 1 week, 3 weeks, 6 weeks, 3 months, 6 months, 12 months and 20 months, and their forebrains were

frozen in liquid nitrogen until used.

Synaptic membrane preparation

Synaptic membranes for binding studies were prepared from rat forebrains of different ages according to the methods previously described (Cho et al, 1996). The volumes of final resuspension of the membrane preparations in 50 mM Tris-acetate buffer, pH 7.1, were adjusted to 1 mg of protein/ml, determined by Bio-Rad D_c protein assay kit.

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

[^{3}H]MK-801 binding assays were carried out as described by Cho et al. (1996) with minor modifications. In brief, synaptic membranes (300 μg of membrane protein per tube) were incubated at 30°C for 60 min in a final volume of 1 ml reaction mixture, containing 50 mM Tris-acetate buffer, pH 7.1, 5 nM [^{3}H]MK-801, and appropriate concentrations of test ligands. The incubation was terminated and the bound radioactivity to the membranes was separated and measured as described (Cho et al, 1996). Non-specific binding was determined in the presence of 0.1 mM (+)-MK-801.

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

Data analysis

The experimental results are expressed as the means ± SEM. Statistical significance was assessed by Student's *t*-test in comparison with the results obtained from 3 months old rat forebrain (* for $p<0.05$; ** for $p<0.01$; and *** for $p<0.001$).

RESULTS

Age-related changes in [^{3}H]MK-801 binding in the presence of glutamate and glycine

In order to examine age-related overall changes in NMDA receptor function, [^{3}H]MK-801 binding studies were performed in the presence of glutamate and glycine, using synaptic membranes prepared from 1 week, 3 weeks, 6 weeks, 3 months, 6 months, 12 months and 20 months old rat forebrains. The concentrations of glutamate and glycine were 100 μM and 30 μM, respectively, which are shown to maximally stimulate the binding (unpublished data). As shown in Fig. 1, the specific binding of [^{3}H]MK-801 in the simultaneous presence of glutamate and glycine was dramatically increased from 1 week to 6 weeks. The maximum binding observed at 6 weeks was almost 6-fold of that at 1 week. The overall binding decreased gradually between 6 weeks and 20 months. The reduction at 20 months was approx. 30% of the maximum binding.

Effects of several agonists on the age-related changes in NMDA receptor function

The effects of glutamate interacting with the neurotransmitter site of the NMDA receptor, glycine interacting with the glycine site, and spermine interacting with the polyamine site were respectively examined on the changes in the receptor function.

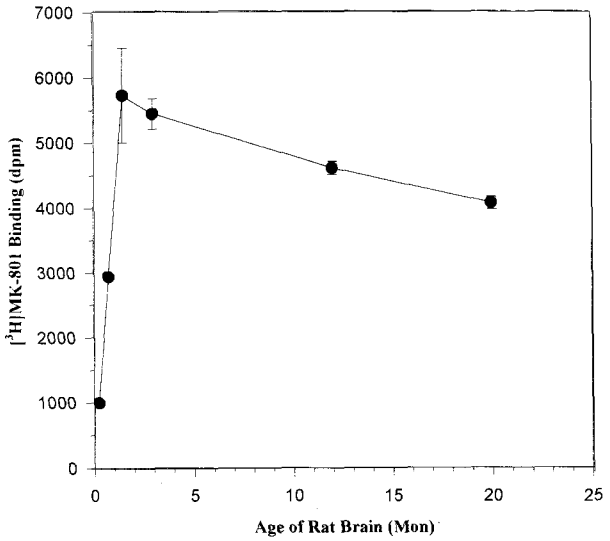

Fig. 1. Age-related changes in [^{3}H]MK-801 binding in the presence of L-glutamate and glycine. Specific bindings of [^{3}H]MK-801 to synaptic membranes, prepared from 1 week, 3 weeks, 6 weeks, 3 months, 12 months and 20 months old rat forebrains, were determined in the presence of 100 μM glutamate and 30 μM glycine, as described in the Materials and Methods. Data represent the mean ± SEM from 3 separate experiments, each performed in triplicate.

The dramatic increase in [^{3}H]MK-801 binding during growth as shown in Fig. 1 was also observed by 100 μM glutamate alone (Fig. 2A), with the maximum binding at 3 months. The increases in bindings at 3 weeks, 6 weeks, and 3 months were about 5-fold, 8-fold, and 10-fold, respectively, compared to that at 1 week. Statistical significance of the binding in young and old rats in Figs. 2A, 2B and 2C was determined in comparison to the adult rat (3 months old). Similar patterns of increases were obtained with 30 μM glycine (Fig. 2B) or 100 μM spermine (Fig. 2C) during this period. The gradual decrease in binding between 6 weeks and 20 months observed in Fig. 1 was also seen with glycine or spermine with 25~30% reduction at 20 months (Figs. 2B and 2C). However, the decrease with aging was not prominent with glutamate alone (Fig. 2A). The extent of potentiation in [^{3}H]MK-801 binding by spermine was greater than those by glutamate or glycine (Figs. 2A, 2B and 2C). Although the exact molecular mechanisms

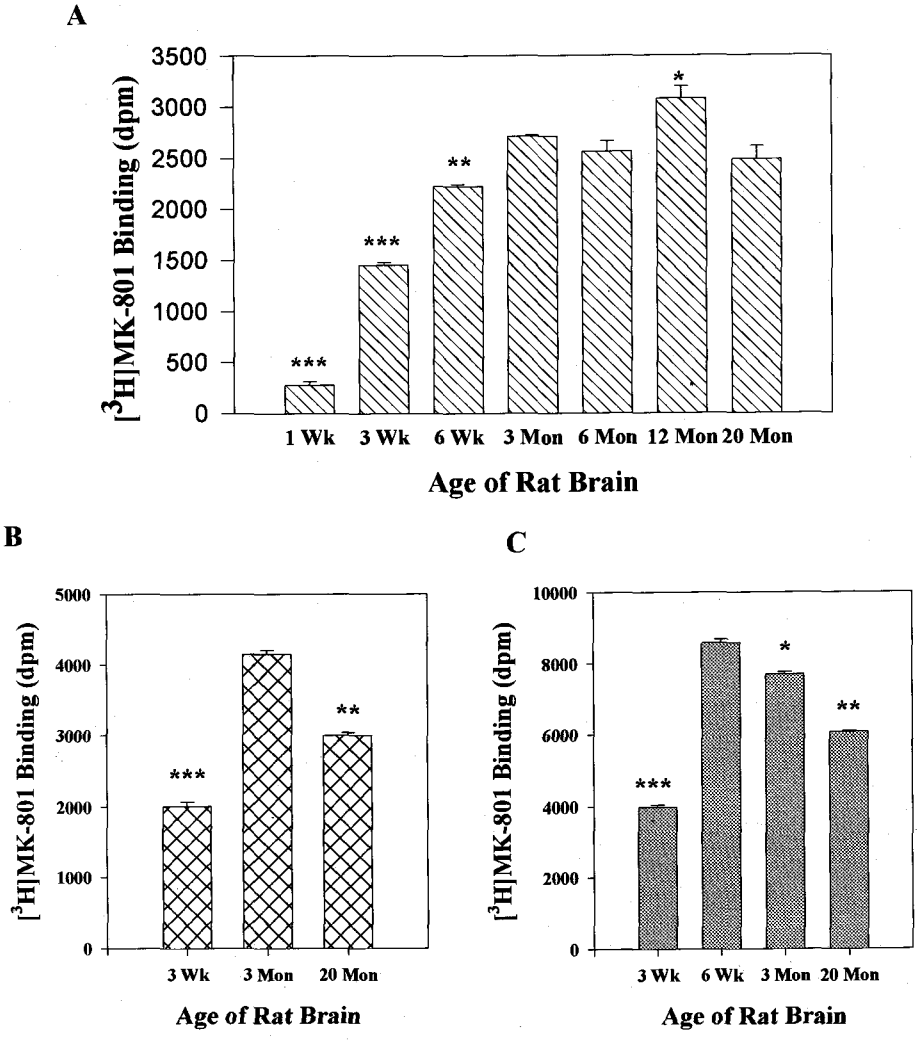

Fig. 2. Effects of L-glutamate, glycine, or spermine on age-related changes in NMDA receptor function. [^{3}H]MK- 801 bindings to synaptic membranes, prepared from rat forebrains with different ages as indicated, were determined in the presence of maximally stimulating concentration of glutamate (100 μM: A), glycine (30 μM: B) or spermine (100 μM: C). Data represent the mean $\pm$ SEM from 2-3 separate experiments, each performed in triplicate. Statistical significance was determined in comparison to 3 months old by Student's *t*-test (*$p<0.05$; ** $p<0.01$; *** $p<0.001$).

of potentiation by polyamines are not clearly understood yet, this phenomenon was consistently observed in previous studies (unpublished data).

The results shown in Fig. 2 indicate that all three ligands tested in this study participate in the modulation of NMDA receptor function in young and aged rats, and particularly in the aged rat, glycine and spermine appear to have more influence on the reduction of the receptor function than glutamate.

Differential sensitivity to glycine in the regulation of NMDA receptor function

Since the reduction of the receptor properties in the aged forebrain appeared to be due to the actions by glycine and spermine (Figs. 1, 2B and 2C), we now examined the modulation patterns by glycine at various ages. As shown in Fig. 3, the [^{3}H]MK-801 bindings at all ages tested were increased dose-dependently by glycine, reaching maximum bindings at the glycine concentration of approx. 30 μM. However, the extent of maximum stimulation was different depending on the ages of brains. The maximum binding at 1 week reached ~220% of basal binding, and decreased at 3 weeks to ~190% and decreased even further at 6 weeks to ~170%. At 3 months, however, the maximum binding in the presence of glycine restored to the similar level of 1 week, and then decreased again extensively at 20 months to ~140% of basal binding. These results demonstrated that the NMDA receptors at 1 week and 3 months are highly sensitive to glycine, and the reduction of NMDA receptor function with normal aging may be, in some degree, due to the decreases in the sensitivity of the receptor to glycine.

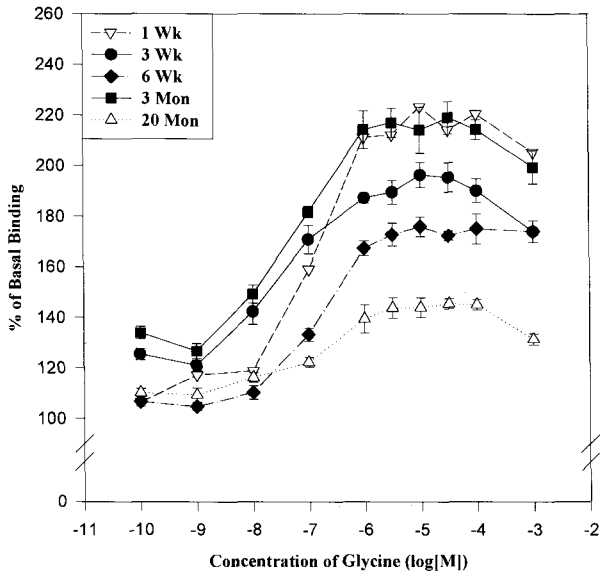

Fig. 3. Age-related differential sensitivity to glycine in the regulation of NMDA receptor function. [^{3}H]MK-801 binding assays were performed in the presence of glutamate (100 μM) and various concentrations of glycine, using synaptic membranes prepared from rat forebrains with different ages as indicated. The basal binding was measured with 100 μM glutamate, but in the absence of exogenous glycine. Potentiation of [^{3}H]MK-801 binding by various concentrations of glycine is expressed as % of basal binding. Data represent the mean±SEM from 2-3 separate experiments of triplicates.

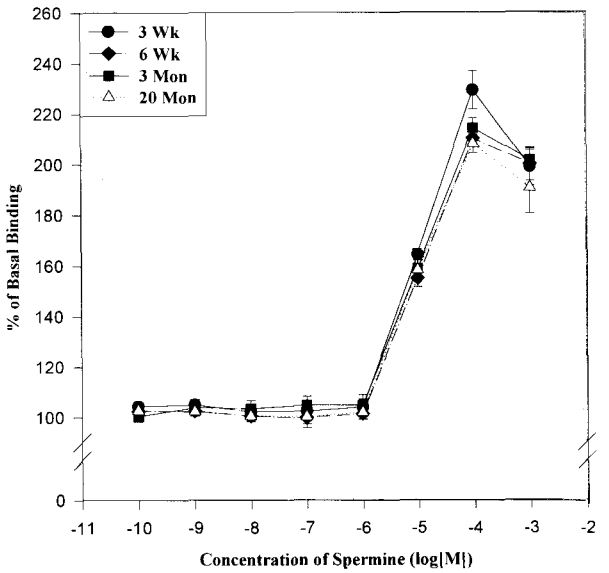

Fig. 4. Sensitivity to spermine in the regulation of NMDA receptor function. [^{3}H]MK-801 binding assays were performed in the presence of glutamate (100 μM), glycine (30 μM) and various concentrations of spermine. The basal binding was measured with glutamate (100 μM) and glycine (30 μM), but in the absence of spermine. Potentiation of [^{3}H]MK-801 binding by various concentrations of spermine is expressed as % of basal binding. Data represent the mean±SEM from 3 separate experiments of triplicates.

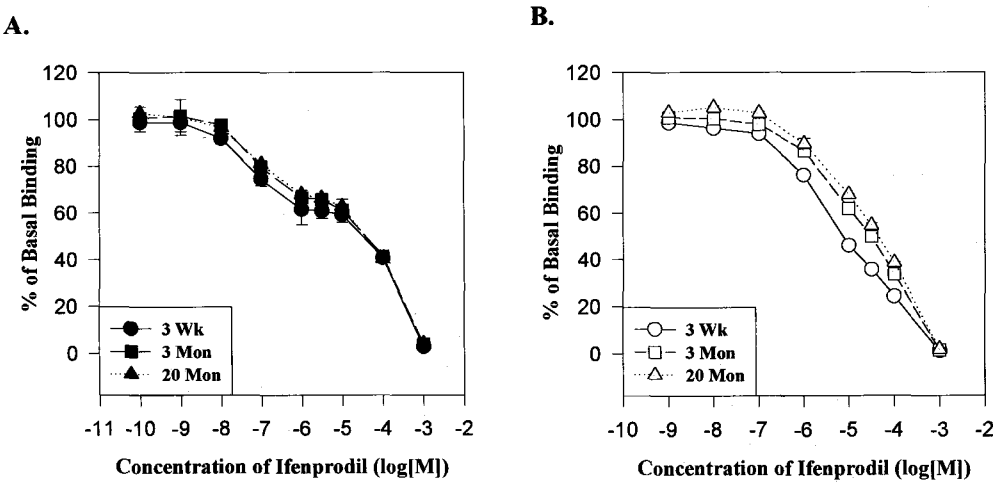

Fig. 5. Effects of ifenprodil on age-related NMDA receptor function. [^{3}H]MK-801 binding assays were performed in the presence of: (A) glutamate (100 μM), glycine (30 μM) and various concentrations of ifenprodil; (B) glutamate (100 μM), glycine (30 μM), spermine (100 μM) and various concentrations of ifenprodil. The basal bindings were measured in the absence of ifenprodil. Blockade of [^{3}H]MK-801 binding by various concentrations of ifenprodil is expressed as % of basal binding. Data represent the mean$\pm$SEM from 3 separate experiments of triplicates.

Sensitivity to spermine in the regulation of NMDA receptor function

Since the reduction of the [^{3}H]MK-801 binding in the aged brain may be in part due to the action of spermine (Figs. 1 and 2C), the modulation patterns of the receptor function by spermine were examined in young and aged rat forebrains. Fig. 4 showed that the bindings increased by spermine in dose-dependent manners, reaching maximum bindings at the concentration of 100 μM. However, there were no differential modulations observed with spermine within the ages tested.

Effects of antagonists acting at the glycine or polyamine site of NMDA receptor

The age-related differential sensitivity of the receptor properties to glycine (Fig. 3) suggested the possibilities of differential regulation by glycine site antagonists. The maximally stimulated [^{3}H]MK-801 binding by glutamate and glycine was reversed dose-dependently by glycine site antagonist 5,7-DCKA, but there was no significant difference in the receptor sensitivity to 5,7-DCKA within the ages tested (data not shown). Similarly, the [^{3}H]MK-801 binding maximally stimulated by either glutamate/glycine (Fig. 5A) or glutamate/glycine/spermine (Fig. 5B) was reversed dose-dependently by polyamine site antagonist ifenprodil. But, again, there was no significant difference in the sensitivity to ifenprodil within the ages tested (Figs. 5A and 5B).

DISCUSSION

It is now widely recognized that NMDA receptors are closely involved in several forms of activity-dependent synaptic plasticity including LTP, a synaptic model of memory (Harris et al, 1984; Bliss & Collingridge, 1993). Memory and cognitive functions are known to decline during normal aging and, more rapidly, in Alzheimer's type dementia (Greenamyre et al, 1987). Previous studies reported that functional properties of the NMDA receptor decreased during aging and that may be closely related to the age-related cognitive impairment (Cohen & Mueller, 1992; Mueller et al, 1994). The reduction of the receptor function is shown to be due to the decreases in the receptor density without changes in the binding affinity (Tamaru et al, 1991; Cohen & Mueller, 1992;

Serra et al, 1994; Gordon et al, 1991; Slater et al, 1993).

NMDA receptor is subject to complex regulation by various endogenous ligands including glutamate, glycine and polyamine. Given the heterogeneous populations of the receptor throughout the brain and differential expression in various regions, it may be possible that the receptor properties are regulated differentially by these ligands during growth and aging. These possibilities were investigated in the present study using [^{3}H]MK-801 binding assay system as a functional marker of the receptor.

Glycine is required for NMDA receptor activation in response to NMDA or glutamate, and now considered to be a co-agonist (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988; Thomson, 1990). Therefore, the [^{3}H]MK-801 binding in the presence of both glutamate and glycine represents the overall function of NMDA receptor (Fig. 1). Although it was small, [^{3}H]MK-801 binding was detected in 1 week old rat forebrain. In fact, functional NMDA receptors in rat cerebral cortex as well as in cerebellar granule cells are expressed before the appearance of synapses (LoTurco et al, 1991) or from the very early period in their differentiation (Rossi & Slater, 1993). During this period, NMDA receptor may participate in the differentiation and migration of neurons before they receive any synaptic contact (Pearce et al, 1987; Komuro & Rakic, 1993). The maximum receptor function in rat forebrain was observed at 6 weeks. In granule cells, however, near adult levels are reached at 3 weeks (Garthwaite et al, 1987), showing differential expression of the receptor in various regions.

The overall receptor function was gradually decreased thereafter, which is consistent with the previous findings. However, the loss of the total binding sites was not the sole reason for the reduction of the receptor function during aging. The ability of glycine to potentiate [^{3}H]MK-801 binding was decreased significantly at 20 months ($\sim$140%) relative to 3 months ($\sim$220%). Therefore, decreased sensitivity of NMDA receptor to glycine may also contribute, in some degree, to the reduction of the receptor function associated with aging.

Our study found the increased sensitivity of the receptor to glycine in 1 week and 3 months old rat forebrains. The receptor with the greater glycine sensitivity at 1 week may be required for postnatal developmental processes. Following this period, rapid increases in the receptor expression may be compromised by slight decreases in the sensitivity as seen at 3 weeks and 6 weeks (Fig. 3). The functional significance of high overall receptor function (Fig. 1) and greater sensitivity to glycine at 3 months (Fig. 3) may correlate with the high activities in learning and memory in adult stage.

In contrast to glycine, the sensitivity of the receptor to spermine was maintained consistently during growth and aging. Similar results were obtained with polyamine site antagonist, ifenprodil. However, Palmer and Burns (1994) reported the declined modulatory activity by polyamine in the superior frontal and the superior temporal cortex of patients with Alzheimer's disease. The discrepancy may reside in the differences in species (rat *vs* human), regions of brain studied, normal aging *vs* disease-induced state, or antagonists employed in the studies (ifenprodil *vs* arcaine). Interestingly, the binding profiles with ifenprodil in the presence of glutamate/glycine (Fig. 5A) were different from those in the presence of glutamate/glycine/spermine (Fig. 5B). Further investigation to uncover the mechanisms of potentiation by polyamines and antagonism by polyamine site antagonists will explain this discrepancy.

In conclusion, the functional properties of the NMDA receptor-ion channel complex are regulated by glutamate, glycine and spermine during growth and aging. In the aged brain, the regulatory effects of glycine and spermine appeared to have more influence on the age-related reduction of the receptor function. The receptor showed lower sensitivity to glycine in the aged brain, which may be an additional cause for the reduction of the receptor function with aging. These findings may provide information for the development of pharmacological interventions, which potentially manipulate NMDA receptor properties under specified clinical disorders such as age-related dementia.

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