Divalent Cations Modulate N-Methyl-d-Aspartate Receptor Function at the Glycine Site

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ABSTRACT

The modulation of the N-methyl-d-aspartate (NMDA) receptor (NMDAR) by divalent cations was examined using (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten 5,10-imine maleate ([3H]MK-801) binding as a functional indicator of NMDAR function. Ca2+ and Mg2+ produce a biphasic effect on the binding of [3H]MK-801 to the NMDAR channel in extensively washed adult rat brain membranes. Concentrations of Ca2+ and Mg2+ between 1 and 600 μM potentiate binding, but higher concentrations inhibit binding. The potentiating effect of Ca2+ and Mg2+ on [3H]MK-801 binding is due to an increase in the maximal number of binding sites (Bmax) with no effect on binding affinity (Kd). Ca2+- and Mg2+-induced potentiation is the result of an apparent increase in the affinity of the NMDAR for glycine. The ontogeny of NMDAR potentiation by Ca2+ and Mg2+ was also investigated. The number of [3H]MK-801 binding sites associated with divalent cation potentiation are present at low levels shortly after birth, and increase to peak level at 17 days of age before declining to adult levels. The potency of Ca2+ and Mg2+ to stimulate [3H]MK-801 binding did not change as a function of age. Lead (Pb2+) and zinc (Zn2+), potent inhibitors of the NMDAR, antagonize NMDAR potentiation by Ca2+ and Mg2+. These findings indicate that divalent cations differentially regulate NMDAR function by modulation of the glycine site. The NMDAR glycine site may be important in the regulation of glutamatergic neurotransmission by physiologically and toxicologically relevant cations.

N-Methyl-d-aspartate receptors (NMDARs) play an important role in neuronal development (Scheetz and Constantine-Paton, 1994) and synaptic plasticity (Collingridge and Singer, 1990). NMDAR activation by the coagonists glutamate and glycine leads to an open state of the Ca2+-permeable ion channel and subsequent activation of second messenger systems. NMDARs have multiple modulatory domains that regulate its function (McBain and Mayer, 1994). Within the channel pore there are recognition sites for noncompetitive channel blockers such as phencyclidine and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten 5,10-imine maleate (MK-801). Because these noncompetitive channel blockers require an open state of the channel to bind, tritted channel blockers such as MK-801 have been used to monitor the functional state of the channel (Reynolds and Miller, 1988; Johnson et al., 1989).

Divalent cations play an important role in the modulation of NMDAR function. Mg2+ at physiological concentrations exerts a voltage-dependent block of the NMDAR at a site thought to be located deep within the ion pore (Mayer et al., 1984; Coan and Collingridge, 1985). An intracellular Mg2+ block of the NMDAR channel exhibiting voltage dependence opposite to the extracellular Mg2+ block has also been described (Kupper et al., 1998). Wang and MacDonald (1995) demonstrated that when the NMDAR glycine site is not saturated, low extracellular concentrations of Mg2+ potentiate NMDAR currents. Potentiating concentrations of Mg2+ are lower than concentrations producing voltage-dependent block of the channel. The Mg2+ potentiation of NMDA-evoked currents was the result of an increase in the affinity of the NMDAR for glycine. Paolletti et al. (1995) also reported a glycine-independent potentiation of the NMDAR by low extracellular concentrations of Mg2+.

Similar to Mg2+, low millimolar Ca2+ concentrations potentiate NMDA responses in trigeminal neurons (Gu and Huang, 1994). The modulatory effect of Ca2+ on NMDA-evoked currents also occurs at subsaturating glycine concentrations and is the result of a Ca2+-mediated increase in the NMDAR affinity for glycine. There is suggestive evidence that Ca2+ and Mg2+ bind at the same site to regulate the affinity of the NMDAR glycine site (Wang and MacDonald, 1995; Paolletti et al., 1995). Consistent with these electrophysiological findings (Gu and Huang, 1994; Paolletti et al., 1995; Premkumar and Auerbach, 1996), radioligand binding studies show that Ca2+ and Mg2+ potentiate [3H]MK-801 (Rajdev and Reynolds, 1992; Enomoto et al., 1992) and [3H]glycine (Marvizon and Skolnick, 1988) binding to the.

ABBREVIATIONS: NMDAR, N-methyl-d-aspartate receptor; NMDA, N-methyl-d-aspartate; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten 5,10-imine maleate; DCKA, dichlorokynurenic acid.
Divalent cations of toxicological importance, in particular the heavy metals Pb\textsuperscript{2+} and Zn\textsuperscript{2+}, are potent inhibitors of the NMDAR (Guilarte, 1997). In vitro or in vivo exposure to Pb\textsuperscript{2+} impairs NMDAR-dependent long-term potentiation in the hippocampus, a cellular model of learning and memory (Altman et al., 1991; Lasley et al., 1993; Zaiser and Miletic, 1997). Pb\textsuperscript{2+}-induced impairment of the NMDAR is emerging as a principal mechanism for learning and memory deficits observed in children exposed to Pb\textsuperscript{2+} in their environment (Guilarte, 1998). The precise mechanism(s) by which Pb\textsuperscript{2+} produces NMDAR inhibition is not known. However, we have suggested that Pb\textsuperscript{2+} may inhibit the NMDAR by interacting at a Zn\textsuperscript{2+} regulatory site (Guilarte et al., 1995). Pb\textsuperscript{2+} may also have properties that mimic Ca\textsuperscript{2+} in some biological systems (Goldstein, 1993). Therefore, this study was undertaken to biochemically characterize NMDAR potentiation by Ca\textsuperscript{2+} and Mg\textsuperscript{2+} to determine whether divergent cations such as Pb\textsuperscript{2+} and Zn\textsuperscript{2+} can influence such effects. Our findings show that in the presence of subsaturating concentrations of NMDAR agonists, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} potentiate NMDAR function by increasing the receptor affinity for glycine, and this effect is antagonized by Pb\textsuperscript{2+} and Zn\textsuperscript{2+}.

Materials and Methods

\[^{3}H\]MK-801 with a specific activity of 23.9 Ci/mmol was purchased from DuPont-NEN (Boston, MA). Unlabeled (+) MK-801 was obtained from RBI (Natick, MA). All other supplies were purchased from commercial sources and were of the highest grade possible.

Rat Brain Membrane Preparation. Adult rat brain membranes were prepared from whole brain without the cerebellum and brain stem due to the low density of NMDARs in these brain structures. The preparation of rat brain membranes and the \[^{3}H\]MK-801 binding assay have been described (Guilarte and Miceli, 1992). Briefly, rat brain tissue was homogenized in 10 volumes of 0.32 M sucrose at 4°C and centrifuged at 1000g for 10 min. The supernatant was centrifuged at 18,000g for 20 min, the pellet resuspended in 10 volumes of 5 mM Tris-HCl (pH 7.7) with a polytron (6 setting) and centrifuged at 8000g for 20 min. The supernatant and upper buffy coat were centrifuged at 40,910g for 20 min. The resulting pellet was homogenized in 10 volumes of 5 mM Tris-HCl buffer with a polytron and centrifuged at 40,910g for 20 min. This washing procedure was done four times and the final pellet stored at −80°C overnight. The following day the pellet was thawed and homogenized in 10 volumes of Tris-HCl buffer with a polytron and centrifuged at 40,910g. The washing procedure was repeated four times and the pellet stored at −80°C until used.

It should be noted that the extensive washing and freeze-thaw cycles described in this preparation of rat brain membranes is to remove endogenous NMDAR modulators, notably glutamate and glycine. The concentration of glutamate and glycine that remains in the rat brain membrane preparation after the extensive washing protocol is in the low nM range.

\[^{3}H\]MK-801 Binding Assay. \[^{3}H\]MK-801 binding assays were performed with adult rat brain membranes unless otherwise indicated. On the day of assay, the rat brain membrane pellet was thawed and homogenized with a polytron in 5 mM Tris-HCl assay buffer, pH 7.5 (sodium-free) and distributed into assay tubes to provide approximately 200 to 300 μg protein/tube. Protein concentration of tissue homogenates was determined by the method of Lowry et al. (1951) using BSA as a standard. The final assay volume was 1 ml and all tubes were kept on ice during preparation. Membrane suspensions were incubated in triplicate and binding was determined with \[^{3}H\]MK-801 concentrations ranging from 0.5 to 20 nM for saturation isotherms or at a 2- to 3-nM concentration for single point assay. Nonspecific binding was measured in the presence of 100 μM unlabeled (+)-MK-801 hydrogen maleate. Assay tubes were incubated for 1 h at 24°C (nonequilibrium conditions). The reaction was terminated by filtration through Whatman GF/F filter paper with a BRANDEL filtering system. Filters were washed three times with 4 ml of ice-cold assay buffer. Radioactivity retained in the filters was measured by liquid scintillation spectrometry using 10 ml of complete counting cocktail (Budget-Solve; RPI Corp., Mt. Prospect, IL).

\[^{3}H\]MK-801 binding parameters (K\textsubscript{d} and B\textsubscript{max}), and Ca\textsuperscript{2+} and Mg\textsuperscript{2+} IC\textsubscript{50} values were estimated using EBDA/LIGAND (Biosoft). EC\textsubscript{50} values for Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Pb\textsuperscript{2+}, Zn\textsuperscript{2+}, glutamate, and glycine were obtained using Sigma Plot (Jandel Scientific, Corte Madera, CA). Maximal enhancement of \[^{3}H\]MK-801 by divalent cations or glutamate and glycine were expressed as a percentage of control (no treatment) or as fmol/mg protein. The maximal enhancement (%) values in Table 5 were obtained using the following formula:

\[
\text{Maximal binding induced by Ca}^{2+} + \text{or Mg}^{2+} + \text{in the presence of Pb}^{2+} + \text{or Zn}^{2+} + \text{) = (Maximal binding in the presence of Pb}^{2+} + \text{or Zn}^{2+} + \text{and no Ca}^{2+} \text{or Mg}^{2+} + \text{)} - \text{(Maximal binding induced by Pb}^{2+} + \text{or Mg}^{2+} + \text{)} \times 100
\]

This transformation was used because as the amount of added Pb\textsuperscript{2+} or Zn\textsuperscript{2+} increases, the basal level of \[^{3}H\]MK-801 binding decreases, giving the appearance that maximal enhancement is reduced.

Statistical analysis was performed using one-way ANOVA followed by Duncan’s New Multiple Range Test for comparisons of means.

Results

Biphasic Effect of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} on \[^{3}H\]MK-801 Binding to NMDARs in Extensively Washed Adult Rat Brain Membranes. The addition of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (1–10,000 μM) to extensively washed adult rat brain membranes containing nominal subsaturating concentrations of glutamate and glycine (low mM range) resulted in a biphasic effect on \[^{3}H\]MK-801 binding to NMDARs (Fig. 1). A potentiation of \[^{3}H\]MK-801 binding was measured from approximately 1 to 600 μM concentrations. The mean ± S.E.M. Ca\textsuperscript{2+} EC\textsubscript{50} value (concentration that enhances binding to 50% of maximal) was 41.9 ± 6.3 μM (n = 14) with a maximal enhancement of 177.0 ± 7.8% of basal binding (no Ca\textsuperscript{2+} added). Concentrations of Ca\textsuperscript{2+} higher than 800 μM inhibited \[^{3}H\]MK-801 binding with a mean ± S.E.M. IC\textsubscript{50} value of 34.6 ± 1.33 mM. The mean ± S.E.M. Mg\textsuperscript{2+} EC\textsubscript{50} value was 50.0 ± 7.0 μM (n = 6) with a maximal enhancement of 144.3 ± 6.5% of basal binding (no Mg\textsuperscript{2+} added). Similar to Ca\textsuperscript{2+}, concentrations of Mg\textsuperscript{2+} higher than 800 μM inhibited \[^{3}H\]MK-801 binding with a mean ± S.E.M. IC\textsubscript{50} value of 4.36 ± 1.78 mM. On a molar basis both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} were equally potent in stimulating \[^{3}H\]MK-801 binding, but Ca\textsuperscript{2+} appeared to be more efficacious.

To determine the nature of the Ca\textsuperscript{2+} and Mg\textsuperscript{2+} potentiation of \[^{3}H\]MK-801 binding, saturation isotherms and Scatchard analysis were performed. Low concentrations of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (600 μM) increased the apparent number of
Increased basal levels of binding in the presence of glutamate so that the additional effect of Ca\(^{2+}\) on binding was markedly reduced (data not shown). Saturation of the glutamate site (50 \(\mu\)M) with the addition of a near saturating concentration of glycine (2 \(\mu\)M), increased the potency and reduced efficacy of Ca\(^{2+}\) and Mg\(^{2+}\) potentiation of the NMDAR beyond that obtained with glutamate alone. Again, this was the result of an agonist-induced increase in basal binding. When both the glutamate (50 \(\mu\)M) and glycine (20 \(\mu\)M) sites were maximally saturated, Ca\(^{2+}\) and Mg\(^{2+}\) were not effective in potentiating \(^{[3]}\)MK-801 binding (Table 2).

The glycine site-competitive antagonist dichlorokynurenic acid (DCKA) was used to further elucidate the mechanism by which Ca\(^{2+}\) and Mg\(^{2+}\) potentiate \(^{[3]}\)MK-801 binding. DCKA at a 100-\(\mu\)M concentration reversed the effect of saturating agonist concentrations on the potentiation of \(^{[3]}\)MK-801 binding by divalent cations (Table 2 and Fig. 2). These data are consistent with the hypothesis that low \(\mu\)M concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) potentiate the NMDAR at the glycine site. The effect of glutamate and glycine on the inhibition of the receptor by high concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) was also measured. Saturating concentrations of glutamate and glycine increased the potency of NMDAR inhibition by these cations (Table 2).

**Ca\(^{2+}\) and Mg\(^{2+}\) Increase the Affinity of the NMDAR for Glycine.** The previous experiments suggested that \(\mu\)M concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) potentiate NMDAR function by modulating the glycine site. To study this possibility, glycine/\(^{[3]}\)MK-801 dose-effect curves were performed in the presence of saturating concentrations of glutamate (50 \(\mu\)M) and increasing cation concentrations. In the presence of saturating concentrations of glutamate, Ca\(^{2+}\) and Mg\(^{2+}\) increased glycine affinity (decreased glycine EC\(_{50}\) value) in a dose-dependent manner (Table 3 and Fig. 3). No significant cation effect was measured for the glycine-induced maximal enhancement (efficacy) of \(^{[3]}\)MK-801 binding (Table 3).

To assess the possibility that Ca\(^{2+}\) and Mg\(^{2+}\) potentiate \(^{[3]}\)MK-801 binding by altering the affinity of the NMDAR for glutamate, we performed glutamate/\(^{[3]}\)MK-801 dose-effect curves in the presence of a saturating concentration of glycine and increasing concentrations of cations. We postulated that if cation-induced NMDAR potentiation is mediated at the glycine site, cations should not alter to an appreciable degree the glutamate affinity when the glycine site is saturated. The results indicate that Ca\(^{2+}\) has a significant effect on the glutamate EC\(_{50}\) as an absolute value (Ca\(^{2+}\): \(F_{2,9} = 5.08; p = .033\)) and as a percent change from control values (Ca\(^{2+}\): \(F_{2,9} = 16.3; p = .001\); Table 4). However, the effect of Ca\(^{2+}\) on the glutamate potency was 2-fold less than the effect of Ca\(^{2+}\) on the glycine potency. Mg\(^{2+}\) had no significant effect on the potentiation of glutamate (EC\(_{50}\) value) to enhance \(^{[3]}\)MK-801 binding. Neither cation affected maximal potentiation of \(^{[3]}\)MK-801 binding by glutamate (Table 4).

**Developmental Profile of NMDAR Potentiation by Ca\(^{2+}\) and Mg\(^{2+}\).** We determined the Ca\(^{2+}\) and Mg\(^{2+}\) potentiation of \(^{[3]}\)MK-801 binding to extensively washed neuronal membranes from rats at different postnatal ages (Fig. 4). In these experiments the Ca\(^{2+}\) and Mg\(^{2+}\) potentiation of \(^{[3]}\)MK-801 binding was also performed in the absence of exogenously added glutamate and glycine. An age-dependent effect was measured for the amount of \(^{[3]}\)MK-801 binding associated with Ca\(^{2+}\) potentiating effects (\(F_{6,30} = 15.0; p = .0001\); \(^{[3]}\)MK-801 binding to neuronal membranes from...
potentiation (Table 5). However, Zn2+ receptor channel. Each value is the mean of [3H]MK-801 binding to extensively washed adult rat brain membranes.

The effects of age on the potency (EC50) of Ca2+ development and increased as a function of age peaking at 17 days 2-day-old rats were not potentiated by Ca2+ or Mg2+. The amount of [3H]MK-801 binding associated with the Ca2+ enhancement was present at low levels during early development and increased as a function of age peaking at 17 days before decreasing to adult levels (Fig. 4). These data indicate that developmental changes occur at the divergent cation-binding site associated with NMDAR potentiation. A similar developmental pattern to that of Ca2+ was present for Mg2+ (Fig. 4). The effects of age on the potency (EC50) of Ca2+ or Mg2+ to enhance [3H]MK-801 binding are also presented in Fig. 4. Age did not alter the potency of these divergent cations to enhance [3H]MK-801 binding (P = 0.05).

Pb2+ and Zn2+ Antagonize the Ca2+ and Mg2+ Potentiation of the NMDAR. Pb2+ and Zn2+ decreased (increased the EC50 value) the Ca2+ and Mg2+ potentiation of [3H]MK-801 binding to extensively washed adult rat brain membranes in a dose-dependent fashion (Table 5). Both cations also reduced the maximal magnitude of [3H]MK-801 potentiation (Table 5). However, Zn2+ appeared to be more potent than Pb2+ in altering the Ca2+ and Mg2+ EC50 value and reducing the maximal potentiation of [3H]MK-801 binding to adult rat brain membranes.

**Discussion**

NMDAR activity is exquisitely sensitive to regulation by physiologically relevant divalent cations such as Ca2+, Mg2+, and Zn2+ as well as divalent cations of toxicological importance such as Pb2+. We demonstrate that the glycine site of the NMDAR is susceptible to modulation by divalent cations and the modulation is most pronounced when the NMDAR is susceptible to potentiation by divalent cations. Moreover, the NMDAR activity is exquisitely sensitive to regulation by Pb2+ and Zn2+.

![Image](https://image.pollinations.ai/prompt/a graph showing the effect of Pb2+ and Zn2+ on [3H]MK-801 binding.)

**Tables**

**Table 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cation</th>
<th>EC50</th>
<th>% Enhancement</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no Glu or Gly)</td>
<td>Ca2+</td>
<td>55.3 ± 9.9a</td>
<td>211.9 ± 15.4a</td>
<td>7.53 ± 1.50a</td>
</tr>
<tr>
<td>50 μM Glu</td>
<td>Ca2+</td>
<td>16.3 ± 1.8b</td>
<td>80.2 ± 4.5b</td>
<td>1.13 ± 0.04b</td>
</tr>
<tr>
<td>50 μM Glu + 2 μM Gly</td>
<td>Ca2+</td>
<td>6.3 ± 2.7c</td>
<td>15.2 ± 4.1c</td>
<td>0.92 ± 0.08c</td>
</tr>
<tr>
<td>50 μM Glu + 20 μM Gly</td>
<td>Ca2+</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.88 ± 0.04c</td>
</tr>
<tr>
<td>50 μM Glu + 2 μM Gly + 100 μM DCKA</td>
<td>Ca2+</td>
<td>40.6 ± 3.2d</td>
<td>209.9 ± 24.2d</td>
<td>2.12 ± 0.18d</td>
</tr>
<tr>
<td>Control (no Glu or Gly)</td>
<td>Mg2+</td>
<td>55.0 ± 8.0e</td>
<td>207.7 ± 25.8e</td>
<td>6.89 ± 1.77e</td>
</tr>
<tr>
<td>50 μM Glu</td>
<td>Mg2+</td>
<td>8.0 ± 1.0f</td>
<td>51.8 ± 3.8f</td>
<td>0.33 ± 0.01f</td>
</tr>
<tr>
<td>50 μM Glu + 2 μM Gly</td>
<td>Mg2+</td>
<td>7.0 ± 3.0gf</td>
<td>16.8 ± 2.9f</td>
<td>0.27 ± 0.02f</td>
</tr>
<tr>
<td>50 μM Glu + 20 μM Gly</td>
<td>Mg2+</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.31 ± 0.05f</td>
</tr>
<tr>
<td>50 μM Glu + 2 μM Gly + 100 μM DCKA</td>
<td>Mg2+</td>
<td>40.0 ± 3.0hf</td>
<td>219.3 ± 25.8f</td>
<td>1.70 ± 0.13f</td>
</tr>
</tbody>
</table>

N.D., stimulation not detectable.

* Within a treatment group, means with different letter superscripts are significantly different (p < .05).

**Table 3**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Gly EC50</th>
<th>% change</th>
<th>Maximal Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μM Glu (control)</td>
<td>133.3 ± 34.3</td>
<td>0</td>
<td>1188 ± 84</td>
</tr>
<tr>
<td>50 μM Glu + 1 μM Ca2+</td>
<td>85.3 ± 14.0</td>
<td>−31.9 ± 6.4a</td>
<td>1109 ± 21</td>
</tr>
<tr>
<td>50 μM Glu + 10 μM Ca2+</td>
<td>60.0 ± 12.6</td>
<td>−59.8 ± 4.1b</td>
<td>1203 ± 43</td>
</tr>
<tr>
<td>50 μM Glu (control)</td>
<td>101.0 ± 6.0</td>
<td>0</td>
<td>807 ± 45</td>
</tr>
<tr>
<td>50 μM Glu + 1 μM Mg2+</td>
<td>94.0 ± 8.5</td>
<td>−6.8 ± 6.2</td>
<td>819 ± 48</td>
</tr>
<tr>
<td>50 μM Glu + 10 μM Mg2+</td>
<td>62.0 ± 10.9c</td>
<td>−38.8 ± 9.9c</td>
<td>864 ± 35</td>
</tr>
</tbody>
</table>

* Maximal binding associated with a single [3H]MK-801 concentration (2.5 nM).

**Fig. 2.** Effect of glutamate, glycine, and DCKA on the Ca2+ potentiation of [3H]MK-801 to the NMDA receptor channel. Each value is the mean ± S.E.M. of four different determinations. Similar results were obtained with Mg2+.
Mg$^{2+}$ site located deep within the NMDAR ion channel (Mayer et al., 1984; Coan and Collingridge, 1985). Concentrations of Mg$^{2+}$ that inhibit NMDA-evoked currents also inhibit $[^3H]$MK-801 binding (Fig. 1). Calcium concentrations in the mM range are also known to block channel conductance possibly by binding to a site located near the entrance of the channel (Jahr and Stevens, 1993; Premkumar and Auerbach, 1996; Sharma and Stevens, 1996). These findings suggest that fluctuations in synaptic concentrations of Ca$^{2+}$ and Mg$^{2+}$ that result from changes in synaptic activity may produce different functional effects on native NMDARs.

**Mg$^{2+}$ Potentiate NMDAR Function by Modulation of the Glycine Site.** The potentiating effects of Ca$^{2+}$ and Mg$^{2+}$ on the NMDAR are masked if saturating concentrations of glutamate and glycine are present (Table 2 and Fig. 2). This suggests that the potentiating divalent cation site is not independent from the glutamate and glycine sites as has been shown with other modulatory sites such as the polyamine site (Williams et al., 1989). The polyamine site is independent from the glutamate and glycine sites because it potentiates $[^3H]$MK-801 binding beyond the enhancement obtained in the presence of saturating concentrations of glutamate and glycine. Because NMDAR potentiation by Ca$^{2+}$ and Mg$^{2+}$ is not measurable at saturating glutamate and glycine concentrations, the Ca$^{2+}$ and Mg$^{2+}$ site must be associated to one of the agonist sites. Evidence linking the divalent cation site with the NMDAR glycine site is shown by cation potentiation of NMDAR with the competitive glycine site antagonist DCKA in the presence of saturating agonists concentrations (Table 2 and Fig. 2). Our data are consistent with those of Gu and Huang (1994) and Wang and MacDonald (1995) in which the addition of potentiating concentrations of Ca$^{2+}$ or Mg$^{2+}$ decreased the inhibitory potency of the NMDAR glycine site antagonist 7-chlorokynurenic acid on NMDAR-evoked currents.

Additional evidence for cation modulation of the glycine site is shown in Fig. 3. The addition of Ca$^{2+}$ produced a left shift in the glycine concentration effect curve, indicative of an increase in the potency of glycine to stimulate $[^3H]$MK-801 binding in the presence of Ca$^{2+}$. Each value is the mean ± S.E.M. of four different determinations. Similar results were obtained with Mg$^{2+}$.

**TABLE 4**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glutamate EC$_{50}$</th>
<th>Glutamate EC$_{50}$</th>
<th>Maximal Binding*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM Gly (control)</td>
<td>88.5 ± 4.3</td>
<td>0</td>
<td>988 ± 37</td>
</tr>
<tr>
<td>20 μM Gly + 1 μM Ca$^{2+}$</td>
<td>79.3 ± 8.2</td>
<td>−11.3 ± 5.6</td>
<td>1001 ± 39</td>
</tr>
<tr>
<td>20 μM Gly + 10 μM Ca$^{2+}$</td>
<td>60.9 ± 5.6</td>
<td>−31.5 ± 3.7</td>
<td>1077 ± 38</td>
</tr>
<tr>
<td>20 μM Gly (control)</td>
<td>109.0 ± 4.1</td>
<td>0</td>
<td>1038 ± 43</td>
</tr>
<tr>
<td>20 μM Gly + 1 μM Mg$^{2+}$</td>
<td>94.6 ± 7.8</td>
<td>−12.9 ± 7.5</td>
<td>1055 ± 40</td>
</tr>
<tr>
<td>20 μM Gly + 10 μM Mg$^{2+}$</td>
<td>97.6 ± 7.9</td>
<td>−11.9 ± 7.4</td>
<td>1061 ± 33</td>
</tr>
</tbody>
</table>

* Maximal binding associated with a single $[^3H]$MK-801 concentration (2.5 nM).

a Significantly different from control (p < .05).

b Significantly different from control and 1 μM Ca$^{2+}$ (p < .05).

Fig. 3. Effect of Ca$^{2+}$ on the affinity of the NMDA receptor for glycine. The addition of 10 μM Ca$^{2+}$ produced a left shift in the glycine concentration effect curve, indicative of an increase in the potency of glycine to stimulate $[^3H]$MK-801 binding in the presence of Ca$^{2+}$. Each value is the mean ± S.E.M. of four different determinations. Similar results were obtained with Mg$^{2+}$.

Fig. 4. Postnatal ontogeny of Ca$^{2+}$ and Mg$^{2+}$ potentiation of $[^3H]$MK-801 binding to extensively washed rat brain membranes. A, $[^3H]$MK-801 binding in fmol/mg protein associated with divalent cation potentiation. B, potency (EC$_{50}$) of divalent cation stimulation of $[^3H]$MK-801 binding. Each value is the mean of three to six different preparations.
site is shown in the glycine/[³H]MK-801 dose-effect curves carried out in the presence of divalent cations. Glycine affinity is increased by either Ca²⁺ or Mg²⁺, demonstrated by a left shift in the glycine/[³H]MK-801 dose-effect curves (Table 3 and Fig. 3). The glycine EC₅₀ data in Table 3 are expressed as absolute values and as a percentage of change from control. The data are presented two different ways because glycine EC₅₀ values expressed as absolute numbers are variable between experiments masking the cation effect. When the data in each experiment are analyzed as a percentage of control, the cation effect on glycine affinity is consistent and robust. The mean glycine EC₅₀ value in the absence of cations was 100 to 133 nM in the control condition, and changed to 60 to 62 nM in the presence of 10 μM Ca²⁺ or Mg²⁺ (Table 3). These findings indicate a divalent cation potentiation of NMDAR function mediated at the glycine site.

Ontogeny of Divalent Cation-Induced Potentiation of the NMDAR. The effect of neuronal development on the NMDAR potentiation by divalent cations is undefined and was investigated in the present study. The potency of Ca²⁺ or Mg²⁺ to stimulate [³H]MK-801 binding in developing animals did not change during development (Fig. 4). Brain tissue from 2-day-old rats did not exhibit Ca²⁺ or Mg²⁺ potentiation of [³H]MK-801 binding. The earliest measurable potentiation of the NMDAR by Ca²⁺ and Mg²⁺ was observed in 5- to 6-day-old rat brain. The amount of [³H]MK-801 binding associated with the Ca²⁺ and Mg²⁺ potentiation increased from low levels shortly after birth, to reach peak levels at 17 days of age before declining to adult levels (Fig. 4). This developmental pattern of [³H]MK-801 binding associated with NMDAR potentiation by Ca²⁺ and Mg²⁺ is similar to the developmental profile of NR1 and NR2A subunit mRNA and protein (Monyer et al., 1994; Riva et al., 1994; Luo et al., 1996; Wenzel et al., 1997). In rat forebrain, the NR2A subunit mRNA or protein is almost undetectable shortly after birth and increases after the first week of life (Monyer et al., 1994; Riva et al., 1994). Thus, the NR1 and/or NR2A subunits may contribute to the potentiating effect of Ca²⁺ and Mg²⁺. It is unlikely that the NR2B subunit would be responsible for the potentiation of the NMDAR by Ca²⁺ and Mg²⁺ as this subunit is abundantly expressed at 2 days of age, a time when divalent cation potentiation of [³H]MK-801 binding is not measurable.

A significant increase in the Ca²⁺ efficacy was also measured at 21 and 28 days of age relative to all other ages (data not shown). This age-dependent change suggests that at these ages, Ca²⁺-induced potentiation may be more easily accomplished and may be associated with enhanced glutamatergic neurotransmission. Ca²⁺ or Mg²⁺ facilitation of NMDAR activation during early development may be important in activity-dependent forms of synaptic plasticity such as long-term potentiation.

Divalent Cation Regulation at the NMDAR Glycine Site. Our studies suggest that divalent cations interact at a site that modulates the NMDAR glycine-binding site. Low concentrations of Ca²⁺ and Mg²⁺ potentiate [³H]MK-801 binding by increasing NMDAR affinity for glycine (Table 3, Fig. 3). Increasing concentrations of Ca²⁺ diminish the inhibition of NMDAR currents by Zn²⁺ (Mayer et al., 1989) or Pb²⁺ (Marchioro et al., 1996). These studies imply that Ca²⁺, Zn²⁺, and Pb²⁺ interact at the same site on the NMDAR. NMDAR currents and [³H]MK-801 binding are inhibited by Pb²⁺ and Zn²⁺ at high- and low-affinity sites (Christine and Choi, 1990; Guillarte, 1997). Concentrations of Pb²⁺ and Zn²⁺ associated with the low-affinity site (>10 μM) inhibit the NMDAR potentiation by Ca²⁺ and Mg²⁺ (Table 5). Other data show that Pb²⁺ competitively interacts with Ca²⁺ (Marchioro et al., 1996), and that Mg²⁺ potentiates [³H]glycine binding (Marvizon and Skolnick, 1988). Together, these studies suggest that Ca²⁺ and Mg²⁺ have opposing effects on glycine binding from Pb²⁺ and Zn²⁺, but that all the cations may interact at the same site. The cation site could exist independently from the glycine site where cation binding causes stoichiometric changes in the conformation of the glycine binding pocket. Alternatively, the cation site may be located within the glycine site. Cations could then directly interact with glycine to influence the affinity of glycine inside the pocket. The latter is less likely because Zn²⁺ has been shown to be a noncompetitive antagonist of [³H]glycine binding (Yeh et al., 1990), suggesting an allosteric rather than a direct interaction.

In summary, divalent cations appear to modulate the NMDAR glycine site with “agonist”- and “antagonist”-like properties. We suggest that in the presence of subsaturating concentrations of glycine, Ca²⁺ and Mg²⁺ act as agonists at a cationic site associated with the glycine site that “positively”

<table>
<thead>
<tr>
<th>Condition</th>
<th>N</th>
<th>EC₂⁰⁺</th>
<th>% Max. Enh.</th>
<th>N</th>
<th>EC₂⁰⁺</th>
<th>% Max. Enh.</th>
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</thead>
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<tr>
<td>No Pb²⁺</td>
<td>10</td>
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<td>100</td>
<td>7</td>
<td>20.7 ± 3.5</td>
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<td>31.9 ± 0.8</td>
<td>95.2 ± 5.9</td>
<td>4</td>
<td>22.4 ± 3.3</td>
<td>104.5 ± 4.0</td>
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<tr>
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<td>41.2 ± 7.6</td>
<td>95.1 ± 4.6</td>
<td>4</td>
<td>24.2 ± 4.7</td>
<td>116.6 ± 7.5</td>
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<tr>
<td>25 μM Pb²⁺</td>
<td>4</td>
<td>95.5 ± 6.1</td>
<td>101.1 ± 2.8</td>
<td>3</td>
<td>118.1 ± 26</td>
<td>106.2 ± 6.4</td>
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<tr>
<td>50 μM Pb²⁺</td>
<td>7</td>
<td>160.8 ± 25.4</td>
<td>101.2 ± 2.6</td>
<td>3</td>
<td>160.5 ± 9.7</td>
<td>99.2 ± 7.4</td>
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<tr>
<td>100 μM Pb²⁺</td>
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<td>88.0 ± 2.2</td>
<td>3</td>
<td>380.0 ± 101</td>
<td>73.1 ± 7.1</td>
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<td>100</td>
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<tr>
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<td>102.8 ± 6.4</td>
<td>4</td>
<td>29.2 ± 4.4</td>
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</table>

*See Materials and Methods for explanation of this measurement.

Within a treatment group, means with different superscript letters are significantly different at p < .05.

TABLE 5
Effects of Pb²⁺ and Zn²⁺ on the Ca²⁺ and Mg²⁺ enhancement of [³H]MK-801 binding to rat brain membranes in the absence of added glutamate and glycine

Each value is the mean ± S.E.M. of number of determinations (N).
modulates NMDAR function by increasing receptor affinity for glycine. Ca\(^{2+}\) and Mg\(^{2+}\) may produce this effect by preventing receptor desensitization or inactivation by decreasing the dissociation rate of glycine. Pb\(^{2+}\) and Zn\(^{2+}\), on the other hand, may act as antagonists at this site to "negatively" modulate NMDAR function by antagonizing the Ca\(^{2+}\) and Mg\(^{2+}\) effect. Ultimately, Pb\(^{2+}\) and Zn\(^{2+}\) may interfere with glycine binding, maintaining the receptor in a desensitized or inactive state. These findings identify mechanisms for the modulation of NMDAR function by the interaction of divalent cations at the glycine site.

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References
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