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Zn^{2+} inhibition of [^{3}H]MK-801 binding is different in mouse brain and spinal cord: effect of glycine and glutamate

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Abstract

Zn^{2+} inhibits NMDA-type excitatory amino acid activity by a non-competitive action. Based on regional differences in the central nervous system (CNS) in binding characteristics of [^{3}H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ([^{3}H]MK-801) and other non-competitive antagonists of NMDA used to label open channels in the receptor complex, we compared the inhibitory influence of Zn^{2+} on [^{3}H]MK-801 binding in whole mouse brain and spinal cord membranes. Radioligand binding techniques were used in the presence and absence of maximally effective concentrations of glycine and glutamate. Using extensively washed membranes without exogenous glycine and glutamate, Zn^{2+} was found to be a weaker inhibitor of the [^{3}H]MK-801-labeled site in the spinal cord than in the whole brain. In contrast, exogenous glycine and glutamate decreased the inhibitory effect of Zn^{2+} in the brain but dramatically increased the inhibitory effect of Zn^{2+} in the spinal cord. Thus the inhibitory effect of Zn^{2+} in the spinal cord appears to be magnified by glutamatergic and glycinergic activity while that in the brain is not. The different actions of Zn^{2+} may be attributable to the differential distribution of NMDA receptor subunits in the mouse brain and spinal cord.

Keywords: Zn^{2+} inhibition; NMDA receptor, binding; MK-801; σ Receptor; Spinal cord; (Mouse)

1. Introduction

A role for the divalent metal ion zinc (Zn^{2+}) in the modulation of NMDA receptor activity has been proposed based on the finding that micromolar concentrations of Zn^{2+} reversibly block the depolarizing actions of NMDA receptors. Zn^{2+} inhibits activity of the NMDA receptor-associated ion channels in cortical (Peters et al., 1987; Christine and Choi, 1990), hippocampal neurons (Westbrook and Mayer, 1987; Mayer et al., 1989; Legendre and Westbrook, 1990) and cerebellar granule cells (Traynelis and Cull, 1990; Fagni et al., 1995). There is a large population of Zn^{2+}-containing neurons in the mammalian central nervous system (CNS) located mostly in the glutamatergic pathways of the telencephalon (for review see Frederickson and Moncrieff, 1994). Here Zn^{2+} is localized in presynaptic vesicles and upon depolarization is released in a calcium- and impulse-dependent manner (Assaf and Chung, 1984; Howell et al., 1984). The actions of Zn^{2+} are largely independent of voltage (Peters et al., 1987; Westbrook and Mayer, 1987) suggesting that Zn^{2+} acts at a superficial site on the NMDA receptor complex (Reynolds and Miller, 1988b) that is not within the channel pore.

The inhibitory effect of Zn^{2+} on NMDA receptor activity described in electrophysiological studies has been confirmed by biochemical techniques. Zn^{2+} is able to inhibit channel binding at NMDA receptors labeled with [^{3}H]1-(1-phenylcyclohexyl)piperidine ([^{3}H]PCP) (Vignon et al., 1982), [^{3}H]1-[1-(2-thienyl)phenylcyclohexyl]piperidine ([^{3}H]TCP) (Yeh et al., 1990), [^{3}H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate ([^{3}H]MK-801) (Reynolds and Miller, 1988a,b; Wong et al., 1988), [^{125}I]MK-801 (Rajdev and Reynolds, 1992) or [^{3}H]dextrorphan (Franklin and Murray, 1992). In rat brain (Vignon et al., 1982; Reynolds and Miller, 1988a,b; Wong et al., 1988; Enomoto et al., 1992; Franklin and Murray, 1992), human cerebral cortex (Hubbard et al., 1989) and NG108-15 hybrid cells (Ohkuma et al., 1994) Zn^{2+} in-

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hibits [^{3}H]MK-801 binding in a concentration-dependent manner over the concentration range that has been proposed to occur in vivo (up to 300 μM) (Assaf and Chung, 1984). In the presence of saturating concentrations of glycine and glutamate, Zn^{2+} decreases the rates of association and dissociation of [^{3}H]MK-801 to rat brain membranes (Reynolds and Miller, 1988a,b) and of [^{3}H]TCP to rat hippocampus (Yeh et al., 1990). The inhibitory action of Zn^{2+} on the NMDA receptor has also been found to be modulated by polyamines such as spermidine (Reynolds, 1992).

Although much attention has been focused on the ability of Zn^{2+} to alter NMDA activity in certain brain sections, to our knowledge no studies have been performed to examine the effect of Zn^{2+} on NMDA activity in the spinal cord where high levels of NMDA receptor binding sites were revealed by receptor-ligand autoradiography (Monaghan and Cotman, 1985; Jansen et al., 1990; Mitchell and Anderson, 1991; Shaw et al., 1991; Kalb et al., 1992; Gonzalez et al., 1993). There are Zn^{2+}-containing boutons lightly scattered in the spinal cord (Schroder, 1979). Although the estimated amount of Zn^{2+} in the spinal cord is approximately 1/3 of that in the hippocampus (Frederickson et al., 1982), spinal cord may reflect a releasable pool of Zn^{2+} as the selenium staining technique revealed a dense Zn^{2+} localization in the substantia gelatinosa (Danscher, 1982).

The purpose of the present study was to compare the inhibition of [^{3}H]MK-801 binding by Zn^{2+} in whole mouse brain and spinal cord membranes using radioligand binding techniques. Because Zn^{2+} has been proposed to alter MK-801 binding by a mechanism that involves activation of the modulatory glycine binding site (Yeh et al., 1990), we examined the effect of Zn^{2+} both in the presence and absence of glycine and glutamate. When added, the concentration of amino acids used was one that maximally opens the channel in the NMDA receptor complex, optimizing MK-801 binding. As Zn^{2+} has been described as a potential and potent endogenous ligand for the σ_2 site (Connor and Chavkin, 1992), we also examined and compared the ability of Zn^{2+} to displace σ receptor ligands, [^{3}H](+)-pentazocine and [^{3}H](1,3-di(tolyl)guanidine) ([^{3}H]DTG), from their binding sites in mouse brain and spinal cord using the same technique.

2. Materials and methods

2.1. Materials

[^{3}H](+)-Pentazocine (35.3 Ci/mmol), [^{3}H]DTG (39.4 Ci/mmol) and [^{3}H]MK-801 (22.5 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA, USA). (+)-MK-801, haloperidol, glycine, glutamate and zinc chloride were obtained from Sigma (St. Louis, MO, USA).

2.2. Membrane preparation

Crude membranes were prepared for [^{3}H](+)-pentazocine and [^{3}H]DTG binding. Male Swiss-Webster mice (20–25 g, Sasco, Omaha, NE, USA) were decapitated. Brains and spinal cords were rapidly removed and homogenized in 40 volumes of 10 mM Tris-HCl buffer (pH 7.4) at 4°C with a Brinkmann Polytron (setting 8, for 5 s). The homogenate was centrifuged at $30\,000 \times g$ for 20 min at 4°C. The resulting pellet was resuspended in the same amount of buffer and incubated at 37°C for 30 min. The suspension was then centrifuged ($30\,000 \times g$, 20 min, 4°C) and the final pellet was resuspended in 15 volumes (350–400 μg/ml protein) of 50 mM Tris-HCl buffer (pH 7.7) at 37°C. The homogenate was used immediately for binding studies.

Previously frozen, osmotically shocked, well washed membranes were prepared (P_2 pellets) for [^{3}H]MK-801 binding studies. Homogenization of tissue in 10 volumes of 0.32 M sucrose at 4°C using a Brinkmann Polytron homogenizer (setting 8, for 5 s) was followed by centrifugation at $1000 \times g$ for 12 min. The supernatant was stored on ice and the pellet was resuspended and re-homogenized twice. The supernatants were pooled and centrifuged at $30\,000 \times g$ for 40 min at 4°C. The P_2 pellets were re-homogenized in ice-cold distilled water and centrifuged at $30\,000 \times g$ for 20 min at 4°C. This final wash procedure was repeated three times and the last pellets were frozen and stored at −20°C for 1–5 days. On the day of assay, membrane pellets were thawed and resuspended in ice-cold distilled water then centrifuged for 20 min at $30\,000 \times g$ at 4°C. The pellets were washed three times in 5 mM Tris-HCl buffer pH 7.4, 4°C and centrifuged at $30\,000 \times g$ for 20 min before immediate use in binding studies. The final pellet was resuspended in 15 volumes (approximately 200–250 μg/ml protein) of 5 mM Tris-HCl buffer, pH 7.4 at 26°C.

2.3. Radioligand binding assay

Saturation binding experiments were performed in 5 mM Tris-HCl buffer pH 7.4 at 25°C for 60 min in the presence of 10 μM glycine and 10 μM glutamate using P_2 pellets. For determination of equilibrium dissociation values (K_d) and the number of binding sites (B_{max}) experiments were conducted over a concentration range of 0.25–64 nM [^{3}H]MK-801. For inhibition studies 1 nM [^{3}H]MK-801 was incubated in the absence or presence of either glutamate (10 μM), glycine (10 μM) or both amino acids with 10 concentrations of Zn^{2+} (zinc chloride) ranging from 100 nM to 10 mM. Non-specific binding was defined by addition of a final concentration of 10 μM MK-801. For inhibition studies at the σ site 0.75 nM [^{3}H](+)-pentazocine or 3 nM [^{3}H]DTG was incubated with 10 concentrations of Zn^{2+} ranging from 100 nM to 10 mM. Non-specific binding was defined by addition of a final concen-

Table 1
Saturation binding data of [^{3}H]MK-801

Tissue	n	K_d (nM)	B_{max} (fmol/mg protein)
Whole brain	4	5.7 ± 1.0	2568 ± 34
Spinal cord	5	8.1 ± 1.6	713 ± 56 [a]

Saturation binding experiments were performed in the presence of glycine and glutamate, 10 μM each, using osmotically shocked, well washed P_2 pellets. The values are the means ± S.E.M. of independent determinations performed in duplicates.
[a] Significantly different from brain ($P < 0.05$, unpaired Student's t-test).

tration of 10 μM haloperidol. The assays were terminated by rapid filtration through Whatman GF/C glass fiber filter on a Brandel Cell Harvester using 3×4 ml ice-cold 5 mM Tris-HCl buffer, pH 7.4 at 4°C. Filters were presoaked in 0.1% polyethylenimine for 2 h at 4°C. The filter-bound radioactivity was determined by liquid scintillation spectrometry at a 50% efficiency. Membrane protein concentrations were measured using the method of Lowry et al. (1951) with bovine serum albumin as the standard. Equilibrium-saturation and inhibition binding data were analyzed with the EBDA (Munson and Rodbard, 1980) and LIGAND (McPherson, 1983) computer programs. In spinal cord, because of the partial inhibition, IC_{50} values were calculated based on the fraction of [^{3}H]MK-801 binding that was inhibited.

2.4. Statistical analysis

Statistical comparison of the IC_{50} mean values was performed using one-way analysis of variance (ANOVA) followed by Scheffe's F test with the level of significance set at $P < 0.05$. Student's two-tailed, unpaired t-test was used to determine the level of statistical difference between K_d and B_{max} mean values calculated from whole brain versus spinal cord membranes.

3. Results

In the first set of experiments, binding of [^{3}H]MK-801 to well washed membranes prepared from mouse whole brain and spinal cord homogenates was studied in the presence of added glycine and glutamate (10 μM). Saturation of binding was achieved by increasing concentrations of [^{3}H]MK-801 from 0.25 to 64 nM. The computer-assisted Scatchard analysis of [^{3}H]MK-801 binding indicated a one-site fit with an apparent K_d of 5.7 ± 1.0 nM and B_{max} of 2568 ± 34 fmol/mg of protein for the whole brain membranes. In spinal cord homogenates the equilibrium dissociation constant (K_d) of 8.1 ± 1.6 nM for [^{3}H]MK-801 binding did not differ significantly from that in the brain. The density of the [^{3}H]MK-801 binding sites, however, was significantly lower in the spinal cord (B_{max} of 713 ± 56 fmol/mg of protein) compared to the brain (Table 1).

The inhibitory effect of Zn^{2+} on [^{3}H]MK-801 binding was examined in well washed membranes of the brain and spinal cord. The inhibition of [^{3}H]MK-801 binding by Zn^{2+} was determined in the absence and presence of either exogenously added glycine, glutamate or both amino acids. In mouse brain membrane, Zn^{2+} was a potent inhibitor at the [^{3}H]MK-801-labeled site ($IC_{50} = 9.77 \pm 1.48$ μM) in the absence of added amino acids. Exogenously added glycine or glutamate alone did not affect the inhibition of [^{3}H]MK-801 binding by Zn^{2+} (Table 2). In the presence of maximally effective concentrations (10 μM) of both amino acids, however, the effect of Zn^{2+} was significantly reduced producing an IC_{50} of 42.6 ± 5.1 μM (Fig. 1, Table 2).

In contrast to the brain, Zn^{2+} was found to be a weak inhibitor at the [^{3}H]MK-801-labeled site in membranes of the spinal cord when either no amino acids were added or glycine and glutamate were added separately. Zn^{2+} inhibited [^{3}H]MK-801 binding only partially with an estimated IC_{50} value in the 50–100 μM concentration range. Because of the low specific binding of [^{3}H]MK-801 in the

Table 2
Inhibition of [^{3}H]MK-801 binding by Zn^{2+} (IC_{50} values in μM) in the absence of exogenously added amino acids and in the presence of either glycine, glutamate, or both amino acids

Tissue	[^{3}H]MK-801			
	Without Gly and Glu ($n = 6$)	+ Gly (10 μM) ($n = 3$)	+ Glu (10 μM) ($n = 3$)	+ Gly and Glu (10 μM) ($n = 6$)
Whole brain	9.8 ± 1.5	18.6 ± 1.7	16.7 ± 0.3	42.6 ± 5.1 [a]
Spinal cord	61.4 ± 3.2 [b]	20.2 ± 2.99	25.8 ± 2.6	4.1 ± 0.68 [a]

Binding of [^{3}H]MK-801 (1 nM) was determined using osmotically shocked, well washed P_2 pellets. The values are the means ± S.E.M. of independent determinations performed in duplicates.
[a] Significantly different from corresponding value obtained from homogenates when no exogenously added glysine and glutamate were present ($P < 0.05$, ANOVA followed by Scheffe's F-test).
[b] Significantly different from brain ($P < 0.05$, ANOVA followed Scheffe's F-test). Spinal cord values represent partial inhibition and, therefore, IC_{50} values were calculated based on the fraction of [^{3}H]MK-801 binding that was inhibited.

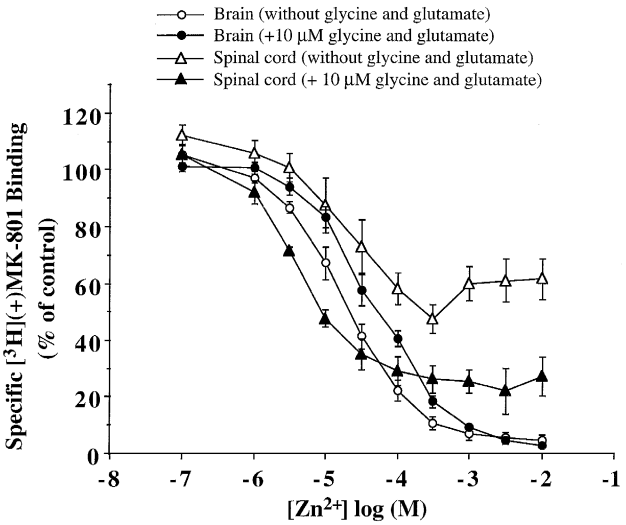

Fig. 1. Concentration-dependent inhibition of [^{3}H]MK-801 binding by Zn^{2+} in mouse whole brain and spinal cord. Experiments were performed in osmotically shocked, well washed P_2 pellets in the absence or presence of glycine and glutamate, 10 μM each. Each point represents the mean ± S.E.M. of three independent determinations performed in duplicates.

absence of glutamate and glycine together with the minimal inhibitory effect of Zn^{2+}, the IC_{50} could not be calculated using the same analysis as that used for brain. However, the inhibitory effect of Zn^{2+} on spinal [^{3}H]MK-801 binding was markedly enhanced ($IC_{50} = 4.14 \pm 0.68$ μM) by the maximally effective concentrations of glycine and glutamate, added together (Fig. 1, Table 2).

We also examined the ability of Zn^{2+} to compete with the selective σ_1 receptor ligand [^{3}H](+)-pentazocine and relatively non-selective σ_1 and σ_2 ligand [^{3}H]DTG in crude membranes of brain and spinal cord. No tissue-specific differences in Zn^{2+} inhibition of σ ligand binding were found (Table 3). The competition curve representing Zn^{2+} inhibition of [^{3}H]DTG was best fit by a one-site model with an estimated $IC_{50\,brain} = 16.2 \pm 1.6$ μM and $IC_{50\,spinal\,cord} = 23.0 \pm 1.2$ μM values. Although in mouse brain and spinal cord Zn^{2+} did not discriminate between [^{3}H]DTG-labeled σ_1 and σ_2 sites as previously described by Connor and Chavkin (1992), in both tissues it showed an approximately 100-fold higher affinity for [^{3}H]DTG binding compared to that for the [^{3}H](+)-pentazocine-labeled sites.

In addition, we investigated the possible involvement of σ receptors in the Zn^{2+} inhibition of [^{3}H]MK-801 binding. Inhibition experiments performed in the absence and presence of exogenous amino acids were repeated when 500 nM haloperidol was added to cover both σ_1 and σ_2 sites (Kovács and Larson, 1995). IC_{50} values for Zn^{2+} at the [^{3}H]MK-801-labeled sites, estimated in the absence and presence of haloperidol, did not differ significantly in either tissue.

Table 3
Inhibition of [^{3}H](+)-pentazocine and [^{3}H]DTG binding (IC_{50} values in μM) by Zn^{2+}

Tissue	*n*	[^{3}H(+)-Pentazocine	[^{3}H]DTG
Whole brain	3	1.7 ± 0.8 nM	16.2 ± 1.6 μM
Spinal cord	3	2.4 ± 0.7 nM	23.0 ± 1.2 μM [a]

Sigma ligand binding was studied in crude membrane preparations using [^{3}H](+)-pentazocine (0.75 nM) or [^{3}H]DTG (3 nM). The values are the means ± S.E.M. of independent determinations performed in duplicates.
[a] Significantly different from brain ($P < 0.05$, ANOVA followed by Scheffe's *F*-test).

4. Discussion

Our finding that exogenous glycine and glutamate, added together, decrease the potency of Zn^{2+} to inhibit [^{3}H]MK-801 binding to homogenates of mouse whole brain is consistent with earlier work in rat brain (Reynolds and Miller, 1988b; Wong et al., 1988) or in human cerebral cortex (Hubbard et al., 1989). In other reports, the inhibitory effect of Zn^{2+} seems to be independent of the addition of these amino acids in rat brain (Enomoto et al., 1992; Franklin and Murray, 1992). Our findings suggest that simultaneous application of glycine and glutamate is required to produce a significant effect on Zn^{2+} inhibition of [^{3}H]MK-801 binding in mouse whole brain and spinal cord.

The present report describes a marked tissue-specific difference in the inhibition of [^{3}H]MK-801 binding by Zn^{2+}. In contrast to the brain, Zn^{2+} was found to be a weak and only partial inhibitor of [^{3}H]MK-801 binding in the osmotically shocked, well washed spinal cord tissue homogenates when no exogenous amino acids were used or glycine and glutamate were added separately. This was dramatically changed upon addition of exogenous glycine and glutamate which caused a several-fold increase in the inhibitory effect of Zn^{2+}. These findings raise the possibility of a different mechanism underlying the Zn^{2+} inhibition of NMDA receptor activity in the spinal cord than in the brain.

NMDA receptor complexes are heteromeric structures and can be reconstituted from two subunit types (for review see Sprengel and Seeburg, 1993): the NR1 (termed ζ1 for mouse) subunit, which is found in all NMDA receptor complexes and one of four NR2 subunits (NR2A–NR2D) (termed ε1–ε4 for mouse). NR2 subunits appear to be modulatory as NMDA receptors display different anatomical distribution and functional properties, different affinity for agonists and antagonists acting at the glycine and glutamate site and different affinities for Mg^{2+} depending on which of the four NR2 subunits assemble with NR1 (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993).

One might speculate that a distinct subunit formation of the NMDA receptor in the spinal cord may account for the

different sensitivities to Zn^{2+} inhibition. In support of this, subtype-specific modulation of stably expressed recombinant human NMDA receptors by Zn^{2+} was reported (Grimwood et al., 1996). Additionally, a subset of NR1 splice variants were potentiated rather than inhibited by Zn^{2+} (Hollmann et al., 1993). While predominantly NR2A and NR2B mRNAs are expressed in the adult rodent brain, the cerebellum contains a large amount of NR2C subunit mRNA (Kutsuwada et al., 1992; Monyer et al., 1992; Watanabe et al., 1992). In the presence of maximally effective concentrations of glycine and glutamate, Zn^{2+} was a less potent inhibitor of [^{3}H]MK-801 binding in rat cerebellum, compared to cortical homogenates (Reynolds and Palmer, 1991)

The molecular heterogeneity of the NMDA receptor channel in the spinal cord has recently been described. In spite of some discrepancies in the reported distribution of NR2 subunits, there is a consensus that the NR1 subunit is distributed throughout the gray matter of the spinal cord (Furuyama et al., 1993; Tölle et al., 1993; Watanabe et al., 1994). An absence of NR2A and NR2B subunit mRNA and low levels of NR2C and NR2D subunit mRNA in the substantia gelatinosa of the rat lumbar spinal cord were demonstrated by Tölle et al. (1993). Watanabe et al. (1994) demonstrated ζ1-ϵ1, ζ1-ϵ2 subunit mRNA localized in the dorsal horn of the mouse cervical cord. The presence of the NMDA receptors in the ventral horn of the spinal cord was confirmed by both groups, suggesting NR1-NR2D (Tölle et al., 1993) or ζ1-ϵ1 (Watanabe et al., 1994) configurations. Thus the heteromer configuration of the NMDA receptor channel in the rodent spinal cord seems to be species and site specific.

Another possible source of variation in Zn^{2+} inhibition of the [^{3}H]MK-801 binding between brain and spinal cord could be the recently demonstrated presence of presynaptic NMDA autoreceptors on rat primary afferent fibers in the spinal cord (Liu et al., 1994). Interestingly, presynaptically localized NMDA receptors in 35–37% of immunoreactively labeled synapses were observed in the superficial dorsal horn on terminals of glutamatergic primary afferent fibers. Similar presynaptic NMDA receptor labeling was also observed on mossy fiber axons of the primate hippocampus (Siegel et al., 1994). The spinal NMDA autoreceptors are hypothesized to regulate and presumably increase the release of neurotransmitters involved in sensory processing (Liu et al., 1994). Although the concentration of Zn^{2+} is highest in the dorsal horn of the spinal cord, there are no available data regarding the heteromer subunit composition, the agonist, antagonist or cation modulation of that NMDA autoreceptor, which in the presence of maximally effective glycine and glutamate could serve as a facilitating mechanism for Zn^{2+} inhibition in the spinal cord.

Zn^{2+} released upon stimulation from hippocampal mossy fibers was identified as a potential endogenous ligand for σ receptors in rat brain. In binding studies, Zn^{2+} showed 50-fold greater selectivity for the [^{3}H]DTG-labeled σ_2 site compared to [^{3}H]DTG-labeled σ_1 site in the rat (Connor and Chavkin, 1992). In the present experiments, Zn^{2+} did not discriminate between [^{3}H]DTG-labeled σ_1 and σ_2 sites in whole mouse brain or spinal cord homogenates, but competed for [^{3}H]DTG binding with an approximately 100-fold higher affinity than for [^{3}H](+)-pentazocine-labeled sites in both tissues. Our findings support the view that Zn^{2+} may act as an endogenous ligand at the σ_2 site in mouse spinal cord as well. We have previously shown that σ_2 sites located in the mouse spinal cord may not be identical to those found in brain (Kovács and Larson, 1995). The present study, however, did not reveal significant differences in IC_{50} values of Zn^{2+} at σ sites in spinal cord compared to those of the brain. In addition, covering of σ sites with haloperidol had no effect on the inhibition of [^{3}H]MK-801 binding by Zn^{2+} in either tissue. Enhanced inhibition of the spinal [^{3}H]MK-801 binding by Zn^{2+} in the presence of glycine and glutamate is thus not due to an indirect modulation of the NMDA receptor complex by σ receptors as the possible link was postulated by other investigators (Yoneda and Ogita, 1991; Yamamoto et al., 1995).

In summary, we have confirmed the inhibitory effect of Zn^{2+} on [^{3}H]MK-801 binding as a reflection of NMDA receptor activity in the mouse brain homogenates. Our data further suggest that Zn^{2+} is an even more potent inhibitor of NMDA activity in the mouse spinal cord. This effect appears to be important as the inhibitory effect of Zn^{2+} in the spinal cord is linked to the level of activity at the glutamate and glycine sites on the receptor complex whereas that in the brain is not. Thus, in spite of the lower concentration of Zn^{2+} in the dorsal spinal cord than, for example, in the hippocampus, Zn^{2+} may play a relatively greater role as an inhibitor at NMDA receptors in the spinal cord than in the brain.

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