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Pharmacological characterization of [^{3}H]MK-801 binding in the rat spinal cord

Frédéric Souverbie [a], Li-Li Mo [a], Yun Liu [b], Gabriel von Euler [b], Erik Sundström [a,*]

[a] *Division of Geriatric Medicine, Karolinska Institutet, Huddinge University Hospital, KFC Novum, S-141 86 Huddinge, Sweden*
[b] *Department of Neuroscience, Karolinska Institutet, S-171 77 Stockholm, Sweden*

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Abstract

Using a receptor binding assay for [^{3}H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5-10-imine (MK-801) the pharmacology of spinal cord NMDA receptors was compared to that of NMDA receptors in the cerebral cortex. The affinities of glutamate site agonists L-glutamate, L-aspartate, ibotenic acid, NMDA and quinolinic acid for stimulation of [^{3}H]MK-801 binding were 6–10 times lower in the spinal cord and the efficacy of quinolinic acid was 50% of that of the other agonists in this region. Also the affinities of glycine site agonists glycine, D-serine, D-alanine and L-serine were lower in the spinal cord as were the affinities of the non-competitive antagonists phencyclidine, (±)-cyclazocine and dextromethorphan. The divalent cations Zn^{2+}, Mg^{2+} and Ca^{2+} had 4–8 times lower affinity for spinal NMDA receptors while the affinity of Co^{2+} was 50 times lower. The affinity of [^{3}H]MK-801 was 2.5-fold lower in the spinal cord. These data show that spinal cord NMDA receptors show qualitative and quantitative differences compared to those in the cerebral cortex.

Keywords: Excitatory amino acid; NMDA (*N*-methyl-D-aspartate); MK-801 binding; Spinal cord

1. Introduction

As one of the most abundant neurotransmitters in the central nervous system, glutamate, as well as other excitatory amino acids, plays an essential role in the normal function of the spinal cord (Headley and Grillner, 1990). Excitatory amino acids have also been postulated to be involved in pathological processes in the spinal cord during trauma and ischemia (Gómez-Pinilla et al., 1989; Faden et al., 1990; Hao et al., 1992).

Pre- and post-synaptic glutamate responses are mediated by ionotropic as well as metabotropic receptors of which the former are divided into three types, NMDA, AMPA and kainic acid receptors (see Mayer and Westbrook, 1987). An abundance of studies have shown that the NMDA receptor, in addition to its glutamate site, has a separate site for the endogenous agonist glycine (Johnson and Ascher, 1987) as well as modulatory sites for polyamines (Ransom and Stec, 1988) and Zn^{2+} (Peters et al., 1987). A most important feature of the receptor complex is the voltage-dependent block of the ion channel by Mg^{2+} (Nowak et al., 1984). Moreover, non-competitive NMDA receptor antagonists, like phencyclidine and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5-10-imine (MK-801), bind to sites located inside the NMDA receptor ion channel (Kemp et al., 1986; Fagg, 1987). Binding assays of [^{3}H]MK-801 or other substances which bind within the receptor ion channel can be used to monitor activation of the NMDA receptor by agonists as well as inhibition of stimulated [^{3}H]MK-801 binding (Reynolds et al., 1987; Bonhaus and McNamara, 1988; Wong et al., 1988).

Several studies on the pharmacology of the NMDA receptor have indicated that the NMDA receptor population is not homogenous but that there is a regional heterogeneity. The first indication of NMDA receptor subtypes was the observation that quinolinic acid, an endogenous tryptophan metabolite, excites rat spinal cord neurons much less efficiently than cortical neurons when compared to glutamate (Perkins and Stone, 1983a). Anatomically, a subpopulation of pre-synaptic NMDA autoreceptors has been identified in the spinal cord (Liu et al., 1994).

* Corresponding author. Division of Geriatric Medicine, KFC Novum, S-141 86 Huddinge, Sweden. Tel.: 46 8 746 57 67; fax: 46 8 746 52 35; e-mail: erik.sundstrom@kfcm13.hs.sll.se

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Several studies have demonstrated regional receptor heterogeneity (Monaghan et al., 1988; Ebert et al., 1991; Monaghan and Beaton, 1991; Beaton et al., 1992) and on the framework provided by the cloning of the NMDA receptor subunits (Moriyoshi et al., 1991; Ishii et al., 1993), Buller et al. (1994) have suggested a scheme of four subtypes of NMDA receptors. However, in this scheme they did not include spinal cord NMDA receptors. An obvious reason for this is the lack of information on the pharmacology of spinal NMDA receptors. Thus, although a few studies have been published utilizing receptor binding techniques in the spinal cord (Sharif et al., 1991; Gudehithlu et al., 1994; Sandberg et al., 1994), none have investigated differences in the agonist, antagonist and modulatory sites of the NMDA receptor complex between the spinal cord and supraspinal regions. In the present study, we have therefore characterized [^{3}H]MK-801 binding in the rat spinal cord, and for comparison in the cerebral cortex, to provide a pharmacological profile of NMDA receptors in these two regions. Preliminary results from this study have been presented previously (Von Euler et al., 1994)

2. Materials and methods

2.1. Membrane preparation

Male Sprague-Dawley rats (200–250 g, B & K Universal, Sollentuna, Sweden) were decapitated. The spinal cord and cerebral cortex were quickly removed, frozen and kept at −50°C for up to 48 h prior to membrane preparation. The tissue (two spinal cords and one half of a cerebral cortex per assay) was homogenized in 30 ml ice-cold 0.32 M sucrose with a glass-Teflon homogenizer and then centrifuged at 4°C at 1000 × *g* for 5 min. The supernatant was then centrifuged at 48 000 × *g* for 15 min, and the pellet was resuspended in 30 ml ice-cold 5 mM Tris-HCl containing 1 mM EDTA (pH 7.4) and centrifuged again. This washing was repeated three times after which the homogenate was frozen at −50°C for at least 24 h. On the day of the assay, the membrane preparation was thawed at room temperature and washed again three times.

2.2. [^{3}H]MK-801 binding

Studies on the stimulation and inhibition of [^{3}H]MK-801 binding were performed in the presence of 2.5 nM [^{3}H]MK-801, 10 μM L-glutamate and/or 30 μM glycine plus increasing concentrations of the drugs tested as described for each experiment. Saturation binding of [^{3}H]MK-801 to well-washed cortical or spinal cord membranes was performed with 12 concentrations of the ligand from 0.2 to 51.2 nM in the presence of 10 μM L-glutamate, 30 μM glycine and 10 μM (cerebral cortex) or 50 μM (spinal cord) spermidine. In initial experiments, these concentrations of spermidine were found to cause maximal stimulation of [^{3}H]MK-801 binding in the two regions studied (data not shown). Membranes were incubated in a final volume of 0.5 ml (saturation experiments) or 1 ml of 5 mM Tris-HCl buffer with 1 mM EDTA, pH 7.4, at 20°C for 3 h, which is long enough to reach steady state (Liu et al., submitted). Non-specific binding was defined as binding in the presence of 10 μM MK-801 or, for saturation binding, 100 μM phencyclidine. All cations decreased non-specific binding slightly at the highest concentrations, which was adjusted for.

Protein concentrations were determined using bovine serum albumin as standard (Markwell et al., 1978). The protein content of the assays was 800–1200 μg/ml for the spinal cord and 40–60 μg/ml for the cerebral cortex.

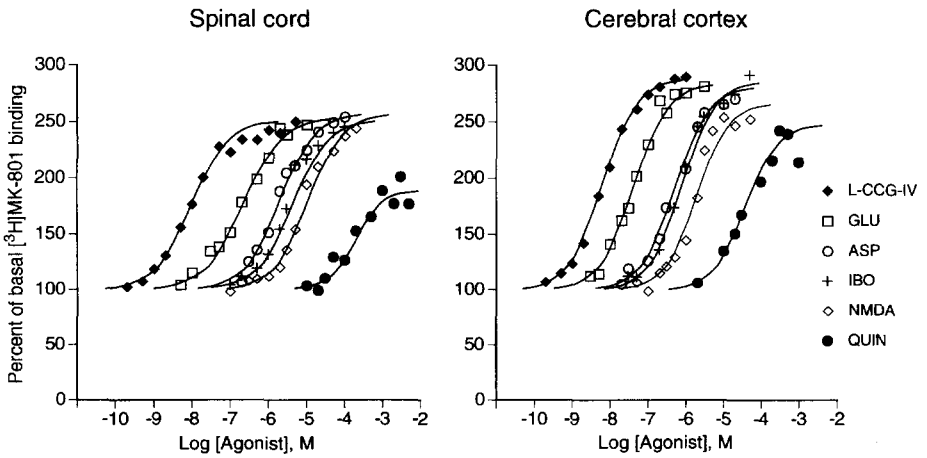

Fig. 1. Effects of increasing concentrations of the glutamate site agonists (2*S*,3*R*,4*S*)-α-(carboxycyclopropyl)glycine (L-CCG-IV), L-glutamate (GLU), L-aspartate (ASP), ibotenic acid (IBO), *N*-methyl-D-aspartate (NMDA) and quinolinic acid (QUIN) on the binding of 2.5 nM [^{3}H]MK-801 to well-washed membranes from spinal cord (left panel) and cerebral cortex (right panel). Binding was performed in the presence of 30 μM glycine. Data are expressed as percentage of binding without addition of the glutamate site agonists. Each point represents the mean of 4–7 experiments. S.D. was 15–30% for the spinal cord and 5–20% for the cerebral cortex.

Incubation was terminated by vacuum filtration using a 48-well Brandel cell harvester with Whatman GF/B filters, pre-soaked for 30 min in 0.05% polyethyleneimine. Radioactivity was determined by liquid scintillation counting using Ready Safe scintillation cocktail (Beckman Instruments, Fullerton, CA, USA) and a Beckman LS 1801 scintillation counter.

2.3. Data analysis

All data from saturation binding experiments and inhibition experiments were analyzed using Ligand for Macintosh software (Elsevier Biosoft). K_i values were calculated according to Cheng and Prusoff (1973). Stimulation curves were analyzed using the program GraphPad Prism (GraphPad Software).

2.4. Chemicals

[^{3}H]MK-801 (22.5 Ci/mmol) was obtained from Du Pont NEN. (+)-MK-801, (±)-cyclazocine and dextromethorphan were purchased from RBI (Natick, MA, USA). L-Glutamate, L-aspartate, glycine, spermidine and L-serine were from Sigma (St. Louis, MO, USA) and NMDA, ibotenic acid, quinolinic acid, D-serine and D-alanine were from Tocris Cookson (Bristol, UK). Phencyclidine was a kind gift from Dr. Mats Garle. The pH of all stock solutions was adjusted to 7.4 by NaOH. All other chemicals used were purchased from a local supplier.

3. Results

3.1. Stimulation by agonists at the glutamate site

Five presumed agonists at the glutamate binding site of the NMDA receptor were tested for their ability to stimulate binding of [^{3}H]MK-801 in the presence of 30 μM glycine (Fig. 1). The rank order of EC_{50} values for the glutamate site agonists was found to be (2*S*,3*R*,4*S*)-α-(carboxycyclopropyl)glycine < L-glutamate < L-aspartate < ibotenic acid < NMDA ≪ quinolinic acid in both the spinal cord and the cerebral cortex. The EC_{50} of each agonist was 2–10 times higher in the spinal cord compared to the cerebral cortex (Table 1).

Table 1
Effects of agonists at the glutamate and at the glycine site on [^{3}H]MK-801 binding

	Spinal cord EC_{50}	Cerebral cortex EC_{50}
L-CCG-IV	0.0103 (0.0051–0.0208)	0.0072 (0.0063–0.0083)
L-Glutamate	0.293 (0.174–0.494)	0.051 (0.037–0.070)
L-Aspartate	1.62 (0.833–3.14)	0.294 (0.227–0.382)
Ibotenic acid	2.62 (1.20–5.74)	0.486 (0.306–0.771)
NMDA	4.57 (3.15–6.63)	0.707 (0.298–1.68)
Quinolinic acid	120 (55.8–257)	17.8 (5.12–61.8)
Glycine	0.138 (0.071–0.271)	0.052 (0.016–0.175)
D-Serine	0.154 (0.085–0.280)	0.068 (0.030–0.157)
D-Alanine	0.561 (0.229–1.37)	0.182 (0.118–0.279)
L-Serine	2.96 (0.459–19.1)	2.11 (1.11–4.00)

The binding experiments with glutamate site agonists were performed in the presence of 30 μM glycine and the experiments with glycine site agonists were performed in the presence of 10 μM glutamate. EC_{50} values are given in μM. Results represent the geometric mean of 4–7 determinations with the 95% confidence interval within parentheses.

Binding of [^{3}H]MK-801 to spinal cord membranes was increased to approximately 250% of the basal binding by adding increasing concentrations of glutamate site agonists while the increase in binding to cerebrocortical membranes was slightly larger. The only exception to this was the increase in binding to the spinal cord membranes induced by quinolinic acid, which was only half of that induced by the other agonists ($P < 0.05$). In cortical membranes, the

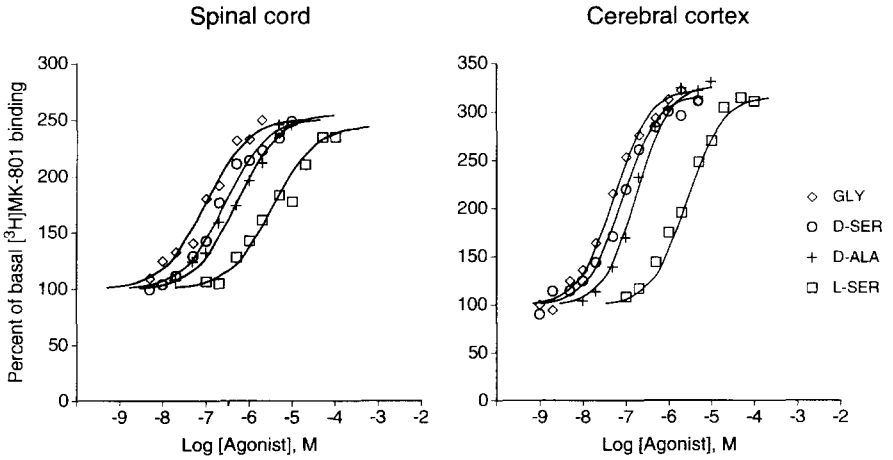

Fig. 2. Effects of increasing concentrations of the glycine site agonists glycine (GLY), D-serine (D-SER), D-alanine (D-ALA) and L-serine (L-SER) on the binding of 2.5 nM [^{3}H]MK-801 to well-washed membranes from spinal cord (left panel) and cerebral cortex (right panel) in the presence of 10 μM glutamate. Data are expressed as percentage of binding without addition of the glycine site agonists. Each point represents the mean of 4–6 experiments. S.D. was 10–35% for the spinal cord and 15–35% for the cerebral cortex.

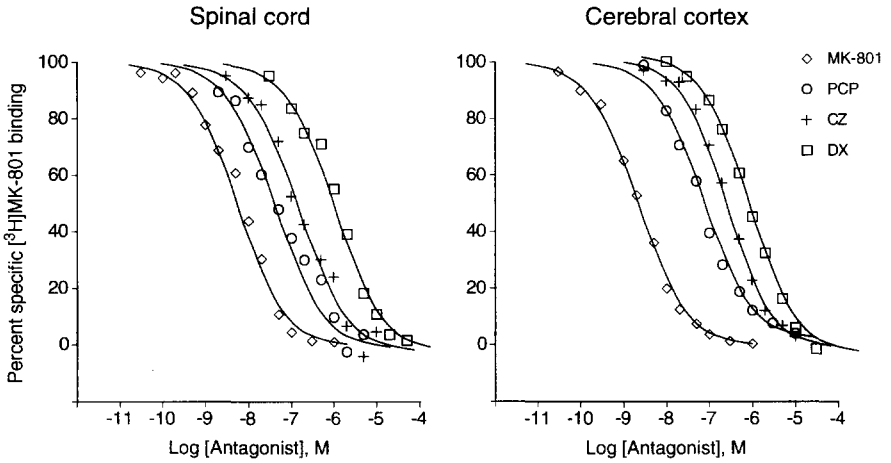

Fig. 3. Displacement of [^{3}H]MK-801 binding to well-washed membranes from the spinal cord (left panel) and cerebral cortex (right panel) by non-competitive NMDA antagonists. Binding was performed with 2.5 nM of the ligand in the presence of 10 μM glutamate and 30 μM glycine and increasing concentrations of MK-801, phencyclidine (PCP), (+)-cyclazocine (CZ) and dextrometorphan (DX). Data are expressed as percentage of binding in the absence of the NMDA antagonists. Each point represents the mean of 4–7 experiments. S.D. was 5–28% for the spinal cord and 2–20% for the cerebral cortex.

average maximal stimulation of [^{3}H]MK-801 binding by both NMDA and quinolinic acid was 25% lower than for the other glutamate site agonists, although this difference was non-significant.

3.2. Stimulation by agonists at the glycine site

We examined the stimulation of [^{3}H]MK-801 binding by four agonists at the glycine site of the NMDA receptor with the glutamate site saturated by the addition of 10 μM L-glutamate. The various glycine site agonists showed a similar pattern of affinity with the rank order EC_{50} being glycine < D-serine < D-alanine ≪ L-serine (Fig. 2). The EC_{50} values were approximately twice as high in the spinal cord as in the cerebral cortex (Table 1). All agonists increased the binding of [^{3}H]MK-801 to approximately 250% of basal binding for spinal cord membranes and to 325% in cortical membranes.

3.3. Inhibition by antagonists at the MK-801 binding site

Four non-competitive NMDA receptor antagonists, MK-801, phencyclidine, (+)-cyclazocine and dextromethorphan were studied for their ability to displace binding of [^{3}H]MK-801. The displacement curves were monophasic and the Hill constants were not significantly different from unity (data not shown). The rank order of K_i values was MK-801 < phencyclidine < (+)-

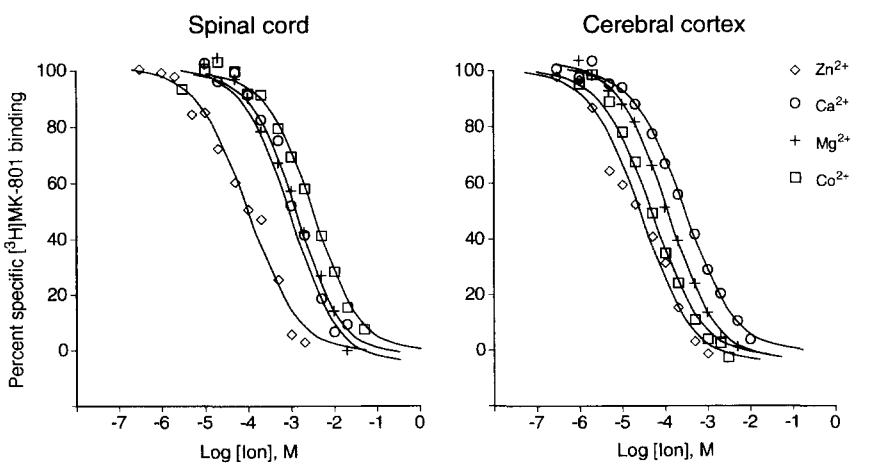

Fig. 4. Effects of the divalent cations Zn^{2+}, Co^{2+}, Mg^{2+} and Ca^{2+} on the binding of 2.5 nM [^{3}H]MK-801 to well-washed membranes from the spinal cord (left panel) and cerebral cortex (right panel). Binding was performed in the presence of 10 μM glutamate and 30 μM glycine and increasing concentrations of the cations. Results are expressed as percentage of binding in the absence of the cations. Each point represents the mean of 5–6 experiments. S.D. was 4–22% for the spinal cord and 3–18% for the cerebral cortex.

Table 2
Inhibition of [^{3}H]MK-801 binding performed in the presence of 30 μM glycine and 10 μM glutamate

	Spinal cord K_i	Cerebral cortex K_i
MK-801	3.03 (1.19–7.67)	0.499 (0.450–0.553)
Phencyclidine	15.9 (8.45–29.7)	12.2 (10.2–14.5)
(+)-Cyclazocine	63.3 (38.6–103.9)	54.4 (47.2–62.7)
Dextromethorphan	412 (302–562)	194 (179–211)
	IC_{50}	IC_{50}
Zn^{2+}	48.4 (10.0–234)	25.7 (13.1–50.5)
Ca^{2+}	893 (550–1449)	234 (121–454)
Mg^{2+}	914 (597–1397)	115 (87.1–152)
Co^{2+}	2583 (1213–5501)	52.1 (30.3–89.5)

K_i values are presented in nM and IC_{50} values in μM. Results represent the geometric mean of 4–7 determinations with the 95% confidence interval within parentheses.

cyclazocine < dextromethorphan (Fig. 3). The K_i values for these antagonists were all higher in the spinal cord than in the cerebral cortex (Table 2).

3.4. Inhibition by divalent cations

We studied the effects of increasing concentrations of the divalent cations Zn^{2+}, Mg^{2+}, Ca^{2+} and Co^{2+} on binding of [^{3}H]MK-801 at saturating concentrations of L-glutamate and glycine (Fig. 4). The IC_{50} values for the examined cations were 4–8 times higher in the spinal cord than in the cerebral cortex except for Co^{2+}, which had an IC_{50} value that was more than 50 times higher in the spinal cord. This led to the rank order of IC_{50} values being $Zn^{2+} < Ca^{2+} \approx Mg^{2+} < Co^{2+}$ in the spinal cord and $Zn^{2+} < Co^{2+} < Mg^{2+} < Ca^{2+}$ in the cerebral cortex (Table 2). None of the cations potentiated binding of [^{3}H]MK-801 to cortical or spinal cord membranes under these conditions.

3.5. Saturation parameters for [^{3}H]MK-801 binding

In the presence of L-glutamate, glycine and spermidine to maximally stimulate binding in both tissues in order to ensure equal conditions, [^{3}H]MK-801 appeared to bind to a single site in synaptic membranes prepared both from cortical and from spinal cord membranes. The number of binding sites in the spinal cord was approximately 40 times lower than in the cerebral cortex (Table 3). [^{3}H]MK-801 bound with lower affinity to spinal cord membranes with a K_D 2.5 times higher than that in the cerebral cortex (Table 3).

Table 3
Saturation binding parameters for [^{3}H]MK-801 binding performed in the presence of 10 μM glutamate, 30 μM glycine and 50 μM (spinal cord) or 10 μM (cerebral cortex) spermidine

	K_D (nM)	B_{max} (pmol/mg protein)
Spinal cord	1.27 (0.90–1.64)[a]	0.161 (0.031–0.291)[a]
Cerebral cortex	0.532 (0.194–0.870)	6.53 (1.66–11.4)

Results represent the geometric mean of 6 determinations with the 95% confidence interval within parentheses. [a] $P < 0.01$.

4. Discussion

The data presented here indicate that NMDA receptors in the rat spinal cord are pharmacologically distinct from cortical NMDA receptors. One of the major findings was that the affinity of [^{3}H]MK-801 binding in the spinal cord was less than half of the affinity in the cerebral cortex. This finding is supported by a recent study by Gudehithlu et al. (1994) in which the K_D for [^{3}H]MK-801 in spinal cord seems to be 1.5–2.5 times higher than in the cerebral cortex although statistical comparisons were not provided. The K_D values of [^{3}H]MK-801 binding in the latter study and in the study by Sharif et al. (1991) are higher than in the present study. It should be noted that these previous studies were performed in the absence of polyamines. This may explain the overall higher affinity of [^{3}H]MK-801 in our study as polyamines have been shown to increase the affinity of [^{3}H]MK-801 (Ransom and Stec, 1988). Sun and Faden (1994) recently found that [^{3}H]MK-801 binds to two sites in the spinal cord with the high-affinity site having six times higher affinity than the 'low'-affinity site but only representing 10% of the total receptor population. However, neither we nor Gudehithlu et al. (1994) were able to detect two binding sites for [^{3}H]MK-801 in the spinal cord.

The affinities of the examined glutamate site agonists as determined by their stimulation of [^{3}H]MK-801 binding were up to an order of magnitude lower in the spinal cord than in the cerebral cortex. Furthermore, the efficacy of quinolinic acid was considerably lower than the other glutamate site agonists in the spinal cord. We also noted a tendency for lower efficacy of both quinolinic acid and NMDA than the other agonists in the cerebral cortex, but this difference was not statistically significant. To our knowledge there are no previous studies published on stimulation of [^{3}H]MK-801 binding in the spinal cord. Neither are we aware of studies utilizing [^{3}H]glutamate binding in spinal cord tissue. We have so far been unsuccessful in achieving [^{3}H]glutamate binding to spinal cord membranes with a specific binding higher than 10–20%, even with solubilization of the receptors or in membrane preparations involving gradient centrifugation to remove myelin. NMDA receptors in the brain stem and cerebellum show a lower affinity for glutamate site agonists than those in forebrain regions, but the EC_{50} values are only 3–6 times higher (Reynolds and Palmer, 1991), a somewhat smaller difference than we found between the spinal cord and cerebral cortex. An interesting parallel is that Reynolds and Palmer (1991) observed that also in the cerebellum, quinolinic acid has much lower efficacy. On the basis of

studies on regional receptor heterogeneity (Monaghan et al., 1988; Ebert et al., 1991; Monaghan and Beaton, 1991; Beaton et al., 1992), Buller et al. (1994) suggested four pharmacologically identified NMDA receptor subtypes, (1) agonist-preferring, (2) antagonist-preferring, (3) cerebellar, and (4) midline thalamus NMDA receptors which closely match the expression of NR2B, NR2A, NR2C and NR2D respectively. Of the two binding sites for homoquinolinate in brain, the low-affinity binding site has 10 times lower affinity in the cerebellum than in the forebrain. Similarly, the single low-affinity site for homoquinolinate on the NR1-NR2C receptor expressed in cell lines has an affinity that is 10 times lower than that on NR1-NR2A and NR1-NR2B receptors. Interestingly, Buller et al. (1994) noted that, although homoquinolinate binds to only one site on recombinant NR1-NR2C receptors, it was less efficacious than NMDA. This indicates that the low efficacy of homoquinolinate is probably not due to there being several populations of NMDA receptors, but rather to homoquinolinate, and possibly also quinolinic acid, being a partial agonist at a low-affinity site of certain NMDA receptor subtypes. Quinolinic acid has previously been shown to inhibit cerebellar [^{3}H]glutamate binding at a high-affinity (K_i = 24.2 μM) and a low-affinity (K_i = 275 μM) receptor population (Monaghan and Beaton, 1991) and the efficacy of quinolinic acid is considerably lower than of other glutamate site agonists in the cerebellum (Reynolds and Palmer, 1991). Since the efficacy is equally low in the spinal cord, it is probable that in both these regions quinolinic acid acts as a partial, low-affinity agonist. However, the large proportion of glutamate- and NMDA-responding spinal neurons that in electrophysiological studies does not respond at all to quinolinic acid (Perkins and Stone, 1983a,b) is more in line with the existence of two pharmacologically different receptor populations.

Recently, Moroni et al. (1995) showed that NMDA receptors in the guinea pig myenteric plexus can be distinguished pharmacologically from rat cortical NMDA receptors by their high affinity for the NMDA receptor agonist (2*S*,3*R*,4*S*)-α-(carboxycyclopropyl)glycine (L-CCG-IV) relative to their affinity for NMDA. We did not, however, find such a difference when comparing the spinal cord to the cerebral cortex.

Reynolds and Palmer (1991) also showed that the affinity of glycine in the brain stem but not in cerebellum was about half that in the forebrain, a difference similar to what we have seen for all glycine site agonists studied. Our results do not show any qualitative differences for glycine site agonists in so far that both the rank order of these agonists and their efficacy were the same in the spinal cord and cortex.

Non-competitive NMDA antagonists also showed lower affinities for their binding site within the receptor ion channel in the spinal cord than in the cerebral cortex. In the cerebellum, MK-801 has relatively lower affinity while dextromethorphan and cyclazocine have higher affinity in both the cerebellum and medial thalamus (Beaton et al., 1992). Our results show that neither dextromethorphan nor cyclazocine has higher affinity than MK-801 in the spinal cord.

The mechanism(s) of inhibition of [^{3}H]MK-801 binding by divalent cations is somewhat unclear. Mg^{2+} causes a characteristic voltage-dependent block of the NMDA receptor ionophore (Nowak et al., 1984). Mg^{2+} inhibits glutamate- and glycine-stimulated binding of [^{3}H]MK-801 (Reynolds et al., 1987, Von Euler and Liu, 1993) as do other divalent cations, such as Co^{2+} and Ca^{2+} (Hollman et al., 1993; Liu and Von Euler, 1996). Zn^{2+}, in contrast, inhibits NMDA receptor function in a voltage-independent manner by interacting at a separate site (Peters et al., 1987). Nevertheless, as for other substances affecting [^{3}H]MK-801 binding, inhibition of [^{3}H]MK-801 binding by divalent cations forms a pattern that can be useful for identifying certain receptor subgroups. We have found a clear difference in the rank order of the investigated cations, with Co^{2+} showing much lower affinity in the spinal cord than in the cerebral cortex, with affinity in the latter being similar to previously published data (Reynolds and Miller, 1988). Previous studies have consistently shown a higher IC_{50} value for Ca^{2+} than for Mg^{2+} (Reynolds and Miller, 1988; Liu and Von Euler, 1996). We could not show a similar difference in the spinal cord.

In conclusion, we have demonstrated regionally unique pharmacological features of rat spinal cord NMDA receptors. Knowledge of these receptor-specific features should prove valuable when comparing the native receptor to recombinant NMDA receptors in establishing the molecular structure of native NMDA receptors. Moreover, it opens up the possibility to develop antagonists that selectively can interfere with pathological processes in the spinal cord. Whether the differences between NMDA receptors in the spinal cord and the cerebral cortex are due to the stoichiometry of receptor subunits, chemical modification of the receptors, or both, is at present under investigation.

Acknowledgements

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