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Visualization of the NMDA recognition site in rat and mouse spinal cord by [^{3}H]CGS 19755 *in vitro* autoradiography

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Summary. The possibility to visualize the NMDA recognition site with [^{3}H]CGS 19755 *in vitro* autoradiography was evaluated in rat brain and spinal cord sections and thereafter used to study the distribution of the NMDA recognition site in rat and mouse spinal cord. The [^{3}H]CGS 19755 binding was concentrated to the dorsal horn, in particular to the substantia gelatinosa. Notable binding was also seen in the intermediate area and ventral horn, while some binding was observed in the funiculi. No major differences were seen in [^{3}H]CGS 19755 binding at various levels of the rat or mouse spinal cord, although a more dense binding was seen in the mouse. A similar distribution of the [^{3}H]CGS 19755 specific binding and the NMDA receptor associated ion-channel site, labeled with [^{3}H]TCP, was found in the mouse spinal cord. Taken together, our data indicate that the NMDA recognition site can be visualized in rat and mouse spinal cord by *in vitro* [^{3}H]CGS 19755 autoradiography.

Keywords: Amino acids – NMDA receptors – CGS 19755 – TCP – Spinal cord – Rat – Mouse

Abbreviations: NMDA: N-methyl-D-aspartate; CGS 19755: Cis-4-phosphonomethyl-2-piperidine carboxylic acid; D-AP5: D(−)-2-Amino-5-phosphonopentanoic acid; TCP: N-(1-2-thienylcyclohexyl)-3,4-piperidine; MK-801: (±)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; AMPA: α-Amino-3-hydroxy-5-methyl-isoxazolepropionic acid; kainate: 2-Carboxy-3-carboxymethyl-4-isopropenyl pyrrolidine; CGP 39653: D,L-(E)-2-amino-4-propyl-5-phosphonopentenoic acid.

Introduction

A number of autoradiographic, electrophysiological and immunocytochemical reports suggest that the primary afferent nociceptive input to the spinal cord is mediated, at least in part, by excitatory amino acids (EAAs),

such as L-glutamate (see De Biasi and Rustioni, 1988; Aanonsen et al., 1990; Jansen et al., 1990). The EAAs activate heteromeric ionotropic receptors that can be divided into three subtypes depending on which agonist they selectively bind; the 2-carboxy-3-carboxymethyl-4-isopropenyl pyrrolidine (kainate) receptor, the α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA) receptor and the N-methyl-D-aspartate (NMDA) receptor (Watkins and Evans, 1981; Monaghan et al., 1989; Young and Fagg, 1990).

Cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755), a rigid analog of 2-amino-5-phosphonopentanoic acid (AP5), is a selective and high-affinity competitive antagonist at the NMDA recognition site of the NMDA receptor complex (Lehmann et al., 1988; Murphy et al., 1988). In its tritiated form, CGS 19755 has been used successfully in homogenate binding assays to characterize the NMDA recognition site in the rodent brain (Murphy et al., 1988; Kaplita and Ferkany, 1990) and recently in the spinal cord (Sandberg et al., 1994).

Few attempts have been made to visualize NMDA recognition sites with selective competitive NMDA antagonists using autoradiographic methods, particularly in the spinal cord. This is primarily due to that the available competitive NMDA ligands have rapid association and dissociation kinetics. Furthermore, the relatively high levels of endogenous amino acids in the brain might interfere with the binding. To our knowledge, no comparative autoradiographic study with both competitive and non-competitive NMDA receptor antagonists has been used in mouse spinal cord. This is of particular interest, since we have earlier showed that competitive and non-competitive NMDA receptor antagonists displayed different effects in mouse nociceptive tests after spinal intrathecal administration (Näsström et al., 1992, 1993).

The present investigation was therefore undertaken to examine the distribution of NMDA recognition sites in the rat and mouse spinal cord by the use of [^{3}H]CGS 19755 *in vitro* autoradiography. The autoradiographic procedures were first studied in rat brain sections, since much more is known about the distribution and density of NMDA receptors in the brain than in the spinal cord (e.g. Jarvis et al., 1987; Monaghan et al., 1988; Jaarsma et al., 1993). Binding of [^{3}H]CGS 19755 was thereafter evaluated using different incubation protocols and finally used to study the distribution of NMDA recognition sites at various levels of the rat and mouse spinal cord. In comparison, autoradiography with the NMDA receptor associated ion-channel site ligand [^{3}H]N-(1-2-thienylcyclohexyl)-3,4-piperidine ([^{3}H]TCP) was used in the mouse spinal cord.

Materials and methods

In vitro autoradiography of [^{3}H]CGS 19755

Male Sprague-Dawley rats (approx. 200 g, B&K Universal AB, Sollentuna, Sweden) and male Swiss-Webster mice (approx. 25 g, B&K Universal AB, Sollentuna, Sweden) were decapitated and their spinal cords dissected out and frozen on dry ice. The spinal cords were stored at -70°C before sectioning. The spinal cords were cut in 20 μm thick horizon-

tal sections at cervical, thoracic and lumbar levels in a cryostat (Dittes, Heidelberg, Germany) at −15°C. The brains from Sprague-Dawley rats were cut in 20 μm thick horizontal sections. The sections were collected on gelatin coated glass slides, dried at room temperature, and stored in −70°C, until used. The animal experimental protocols performed in the present study were approved by the Swedish Committee for Ethical Experiments on Laboratory Animals (license S52/93 and S116/92, Stockholm, Sweden).

The sections were dried in room temperature for at least 120 minutes before incubations were performed. First a protocol (Protocol I) was used where the sections were preincubated at 4°C for 60 minutes in Tris-HCl buffer (50 mM, pH 8.0), followed by another preincubation at 37°C for 30 minutes. Thereafter, the sections were incubated for 30 minutes at room temperature in Tris-HCl buffer containing [^{3}H]CGS 19755 at concentrations ranging from 1 to 500 nM (brain sections) or at 30 nM (spinal cord). Non-specific binding was determined by adding 10 μM L-glutamate. Following the incubation, slides were washed different time periods in Tris-HCl buffer, followed by a rinse in 3 ml

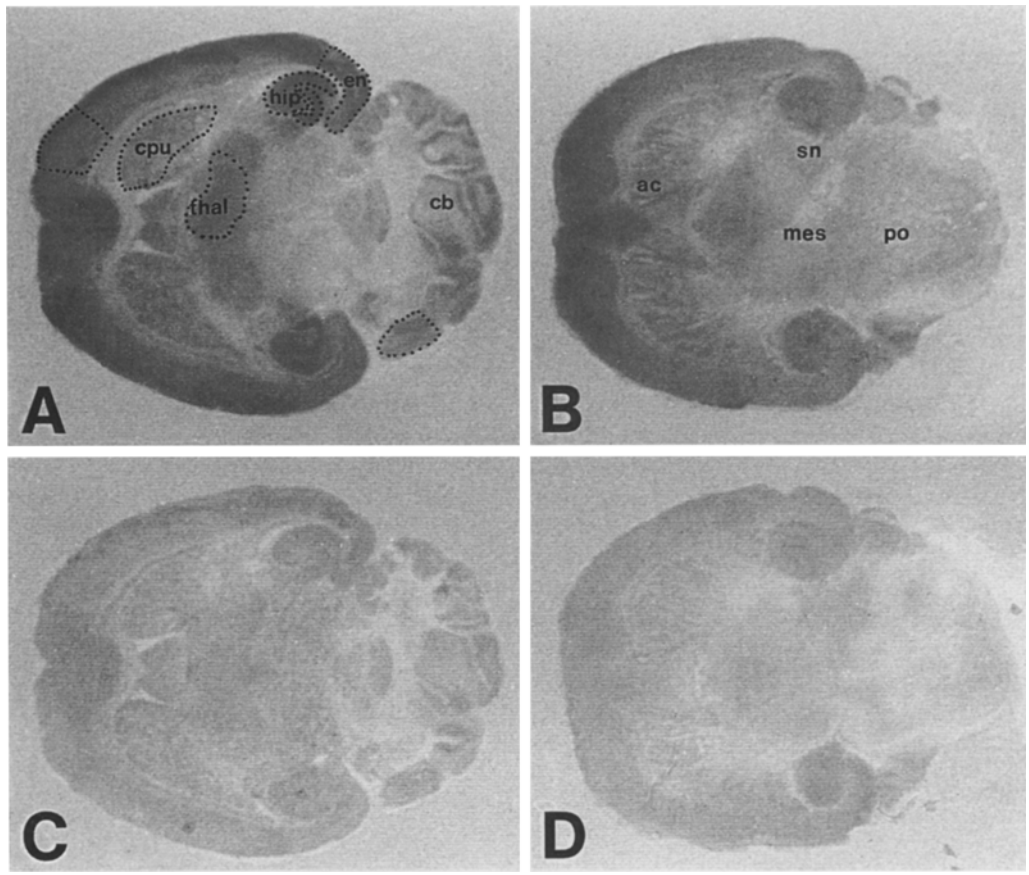

Fig. 1. Autoradiographs of *in vitro* binding with the competitive NMDA receptor antagonist [^{3}H]CGS 19755 in horizontal sections from two different levels of the rat brain (**A** and **B**) and autoradiographs of sections incubated with [^{3}H]CGS 19755 in the presence of 10 μM L-glutamate to define non-specific binding (**C** and **D**). *cpu* caudate-putamen; *thal* thalamic nuclei; *hip* hippocampus; *en* entorhinal cortex; *cb* cerebellum; *ac* nucleus accumbens; *sn* substantia nigra; *mes* mesencephalon; *po* pons. The dotted lines show the areas used for density measurements

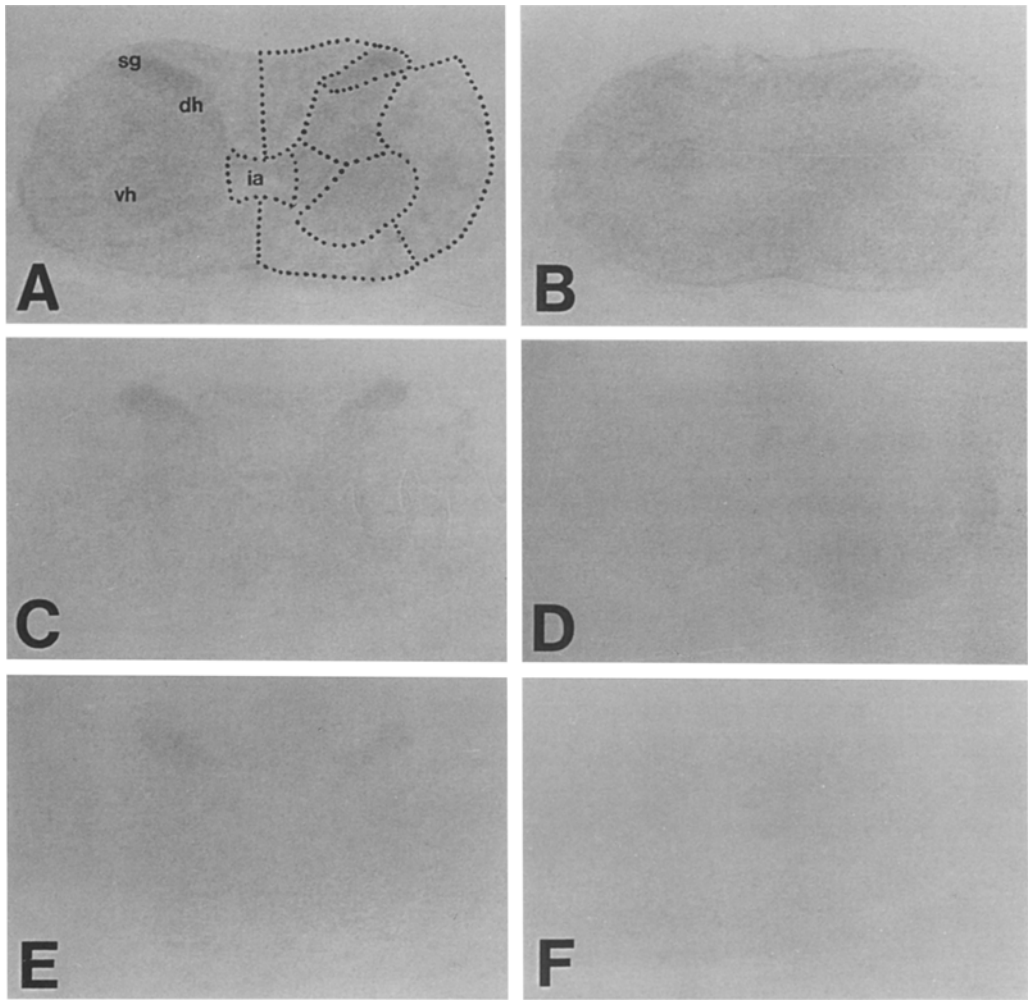

Fig. 2. Autoradiographs of *in vitro* binding in sections of rat spinal cord from the cervical part at approximately level C5 that were incubated with 30 nM [^{3}H]CGS 19755 (A, C, E) or 30 nM [^{3}H]CGS 19755 in the presence of 100 μM D-AP5 (B, D, F) and subsequently rinsed in 4°C buffer for different time periods. In (**A**) and (**B**) rinsing was performed for 15 sec, in (**C**) and (**D**) rinsing was performed for 30 sec and in (**E**) and (**F**) rinsing was performed for 1 min. *sg* substantia gelatinosa; *dh* dorsal horn; *ia* intermediate area; *vh* ventral horn. The dotted lines show the areas used for density measurements.

acetone-glutaraldehyde (19:1, v/v). The protocol was thereafter revised (Protocol II) and the sections were preincubated in the Tris-HCl buffer at 22°C for 3 × 20 minutes instead. The sections were then incubated for 60 minutes at 4°C in Tris-HCl buffer containing 30 nM [^{3}H]CGS 19755, 1.1 mM NAD^+ and 0.03% hydrazine, with or without glutamate dehydrogenase (30 U/ml) added. Non-specific binding was determined by adding 100 μM D-AP5 to the incubation solution. Following the incubation, slides were washed for 15 seconds, unless otherwise stated, in Tris-HCl buffer, followed by a rinse in 3 ml acetone-glutaraldehyde (19:1, v/v).

[^{3}H]TCP autoradiography was performed according to Maragos et al. (1986). The sections were preincubated for 30 minutes at 4°C in Tris-acetate buffer (50 mM, pH 7.4)

containing 4 mM $CaCl_2$. The sections were dried again followed by an incubation at room temperature for 45 minutes in the Tris-acetate buffer containing 1 mM magnesium acetate and 30 nM [^{3}H]TCP in the presence or absence of 20 μM phencyclidine (PCP; 1-[1-phenylcyclohexyl]piperidine) to define non-specific binding. The incubation was terminated by washing the sections in ice-cold Tris-acetate buffer for 3 × 1 min. The sections were then dried under a stream of cool air and subsequently apposed to ^{3}H-sensitive film (Amersham, England) and stored at −20°C in X-ray cassettes for 8 weeks.

Analysis of the autoradiographs were performed by quantitative microdensitometry using an IBAS Kontron image analysis system (IBAS 2000, Zeiss/Kontron, Germany), which converted the density in the film to estimated tissue equivalent binding in fmol/mg tissue (wet weight) by the use of calibrated ^{3}H-plastic standards (Amersham, England) co-exposed with the tissue sections. Areas selected for measurements are presented in Fig. 1 and Fig. 2. The term substantia gelatinosa is used for a region that approximately covers layer 2, while the term dorsal horn includes the remaining part of the gray matter in dorsal region. Photographs of the autoradiographs were taken from the screen display using a Polaroid Quick Print Unit with Agfapan APX25 black and white negative film.

Compounds

L-Glutamate (lot: 28C-0172) was purchased from Sigma Chemical Co. (St Louis, MO, USA). D-AP5 (lot: 21) was obtained from Tocris Neuramin (Essex, England). Glutamate dehydrogenase (lot: 11649748-91) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). NAD^+ (lot: 13862328-19) was purchased from Boehringer Mannheim GmbH. Hydrazine solution (35%) was obtained from Aldrich-Chemie GmbH (Steinheim, Germany). PCP was synthesized at Astra Arcus AB (Batch: OA 748/19, Södertälje, Sweden). [^{3}H]CGS 19755 (lot: 2923-109), 65.2 Ci/mmol, and [^{3}H]TCP (lot: 2923-093), 50.4 Ci/mmol, were purchased from New England Nuclear (DuPont, Boston, MA, U.S.A.).

Results

[^{3}H]CGS 19755 binding to rat brain sections

The binding characteristics of [^{3}H]CGS 19755 were initially studied in horizontal sections of rat brain using Protocol I. It was found that the rinse time in Tris-HCl buffer after [^{3}H]CGS 19755 incubation was critical in order to retain a high specific labeling. After longer rinsing times (2 min) in 4°C buffer, no labeling was seen at all (data not shown). Thus, a rinsing time of approximately 15 seconds was found to balance the rapid dissociation of [^{3}H]CGS 19755 binding with an acceptable level of background staining. A saturable concentration dependency in the specific binding of [^{3}H]CGS 19755 was seen, although the data was not sufficiently robust to allow quantitation of B_{max} and K_D values. At a concentration of 30 nM [^{3}H]CGS 19755, a rather intense labeling was observed with reasonably low background staining. Thus, based on these preliminary experiments, the 30 nM concentration was used in all further experiments.

A regionally specific labeling with [^{3}H]CGS 19755 was observed in the horizontal brain sections, with a moderate to dense labeling in the hippocampal formation, the cerebral cortex, in particular the superficial layers, the entorhinal cortex, the thalamic nuclei, and the granular layer of the cerebellar cortex (Fig. 1A and B). Moderate binding was observed in the caudate-putamen (striatum), nucleus accumbens, substantia nigra and several

Table 1. Binding of [^{3}H]CGS 19755 (30 nM) measured in autoradiographs of the rat brain

Region	Specific binding	% of total binding
Cerebral cortex	120 ± 20	58
Caudate-putamen	65 ± 10	51
Thalamus	73 ± 7	52
CA regions of hippocampus	138 ± 9	64
Dentate gyrus	151 ± 8	63
Cerebellum	15 ± 6	21
Entorhinal cortex	127 ± 8	64

Data are expressed as mean ± SEM fmol/mg tissue (specific binding) or % of total binding, n = 4–5. Specific binding was defined as total binding subtracted by binding in the presence of 10 μM L-glutamate.

other regions of the brain. After incubation in the presence of 10 μM L-glutamate, a fairly homogenous labeling with a much lower intensity was seen throughout the gray matter of the brain. Slightly lower background labeling was seen in the white matter after [^{3}H]CGS 19755 incubation in the presence of 10 μM L-glutamate (Fig. 1C and D). The highest specific binding was found in regions which showed high total binding, e.g. the hippocampal formation and cortical regions, while regions with lower total binding showed relatively less specific binding, as defined by 10 μM L-glutamate, compared to the total binding (Table 1).

[^{3}H]CGS 19755 binding to rat spinal cord sections

In the rat spinal cord, sections incubated with 30 nM [^{3}H]CGS 19755 using the Protocol I showed low to moderate total labeling in the gray matter. However, notable labeling was also observed in the white matter and, although the border could be discerned, there was no sharp contrast between the gray and white matter. After incubation in the presence of 10 μM L-glutamate, a homogenous labeling of low intensity was seen throughout the spinal cord, with no major difference between gray and white matter. The specific binding in the gray matter was low, ranging from 10 to 44% of the total binding. In the white matter, the specific binding constituted 1 to 15% of the total binding. For example, the specific binding in the substantia gelatinosa of the cervical spinal cord was 30.1 ± 2.4 (SEM) fmol/mg tissue with specific binding constituting 35% of the total binding.

To improve the labeling in the rat spinal cord, the incubation procedures were modified according to Protocol II. First, different rinsing times were evaluated (Fig. 2). As previously experienced, the rinsing time had a major influence on the binding of [^{3}H]CGS 19755 (Table 2). Again, a brief rinsing time of approximately 15 seconds was found to balance the dissociation of [^{3}H]CGS 19755 binding with a relatively low level of background staining.

Table 2. Effect of increasing rinsing times after incubation of [^{3}H]CGS 19755 (30 nM). The binding was measured in autoradiographs of the rat spinal cord

Time and region	Specific binding	% of total binding
15 sec wash (from Table 3)		
Substantia gelatinosa	91.2 ± 5.9	62
Dorsal horn (excl. subst. gel.)	49.5 ± 3.7	50
Dorsal funiculus	10.4 ± 3.5	20
30 sec wash		
Substantia gelatinosa	44.5 ± 5.5	45
Dorsal horn (excl. subst. gel.)	50.6 ± 5.7	48
Dorsal funiculus	13.9 ± 4.2	25
1 min wash		
Substantia gelatinosa	29.8 ± 2.4	53
Dorsal horn (excl. subst. gel.)	19.1 ± 2.5	44
Dorsal funiculus	−4.2 ± 1.2	20
2 min wash		
Substantia gelatinosa	15.8 ± 0.9	39
Dorsal horn (excl. subst. gel.)	9.1 ± 2.2	27
Dorsal funiculus	−4.2 ± 1.4	−20

Data are expressed as mean ± SEM fmol/mg tissue (specific binding) or % of total binding, n = 3–6. Specific binding was defined as total binding subtracted by binding in the presence of 100 μM D-AP5.

Hence, with this incubation procedure, the border between the gray and white matter could easily be discerned (Fig. 2A and Fig. 3A–C). After incubation in the presence of 100 μM D-AP5, a homogenous labeling of low intensity was seen throughout the spinal cord, with no major difference between gray and white matter (Fig. 2B). Furthermore, a higher specific binding was seen in the gray matter when using Protocol II compared to Protocol I. The effect of preincubation and incubation in the presence of glutamate dehydrogenase was also studied. However, the binding did not improve following addition of glutamate dehydrogenase to the buffers. Indeed, a slightly reduced specific binding was observed (data not shown).

Hence, a more detailed characterization of the [^{3}H]CGS 19755 binding was performed at different levels of the rat spinal cord (Fig. 3A–C), when the modified incubation protocol II was used, without any glutamate dehydrogenase added, and a rinsing time of 15 sec. The specific binding in the gray matter ranged from 32 to 62% of the total binding. A much higher specific binding was seen in the substantia gelatinosa of the dorsal horn, while a similar magnitude of specific binding was seen in the rest of the dorsal horn, the intermediate area and the ventral horn. In the white matter, a low labeling was seen, that consisted primarily of non-specific binding (Table 3). No major differences were seen in the specific binding in the gray matter of the same region at various levels of the spinal cord (Table 3).

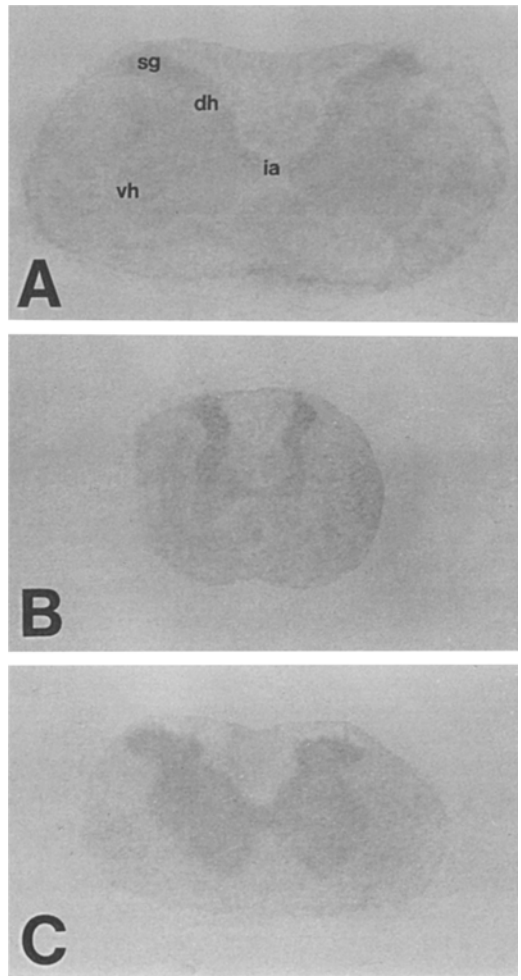

Fig. 3. Autoradiographs of *in vitro* binding with the competitive NMDA receptor antagonist [^{3}H]CGS 19755 in sections of rat spinal cord from the cervical part at approximately level C5 (**A**), thoracic part at approximately level Th10 (**B**), lumbar part at approximately level L5 (**C**). *sg* substantia gelatinosa; *dh* dorsal horn; *ia* intermediate area; *vh* ventral horn. The same magnification has been used for A through C

[^{3}H]CGS 19755 binding to mouse spinal cord sections

In the mouse spinal cord, sections incubated with 30 nM [^{3}H]CGS 19755 using Protocol I showed a moderate labeling in the gray matter, while the labeling in the white matter was clearly less. The border between the gray and white matter could be discerned, although it was not well identified in the ventral horn region (Fig. 4A). After incubation in the presence of 10 μM L-glutamate, a rather homogenous labeling of lower intensity was seen throughout the spinal cord, although the border between the white and gray matter could occasionally be seen (Fig. 4B). Since the labeling was found to be acceptable using the original incubation protocol, the [^{3}H]CGS 19755 binding was studied without any further modifications. The specific binding in the gray matter

Table 3. Binding of [^{3}H]CGS 19755 (30 nM) measured in autoradiographs of the rat spinal cord

Region	Specific binding	% of total binding
Cervical (C5)		
Substantia gelatinosa	91.2 ± 5.9	62
Dorsal horn (excl. subst. gel.)	49.5 ± 3.7	50
Ventral horn	30.2 ± 4.1	35
Intermediate area	52.5 ± 6.0	46
Dorsal funiculus	10.4± 3.5	20
Lateral funiculus	2.3± 3.7	4
Ventral funiculus	1.0± 3.1	2
Thoracic spinal cord (Th10)		
Substantia gelatinosa	72.3 ± 7.6	55
Dorsal horn (excl. subst. gel.)	49.5 ± 5.4	47
Ventral horn	39.1 ± 4.1	43
Intermediate area	31.5 ± 5.9	32
Dorsal funiculus	−2.5 ± 3.6	−5
Lateral funiculus	8.8 ± 2.5	15
Ventral funiculus	6.4 ± 3.2	11
Lumbar spinal cord (L5)		
Substantia gelatinosa	78.2 ± 6.8	54
Dorsal horn (excl. subst. gel.)	49.6 ± 4.3	47
Ventral horn	45.0 ± 5.0	51
Intermediate area	63.4 ± 10.6	50
Dorsal funiculus	2.9 ± 4.4	6
Lateral funiculus	4.9± 2.1	11
Ventral funiculus	5.7± 3.3	14

Data are expressed as mean ± SEM fmol/mg tissue (specific binding) or % of total binding, n = 5–6. Specific binding was defined as total binding subtracted by binding in the presence of 100 μM D-AP5. A 15 sec rinsing time was used after the incubation.

constituted 39 to 53% of the total binding. A similar level of specific binding was seen in the deeper layers of the dorsal horn and the intermediate area, while the binding in the superficial layers of the dorsal horn, i.e. substantia gelatinosa, showed the most intense specific labeling. The specific binding in the ventral horn was somewhat lower. No major differences were seen in the specific binding in the gray matter at the various levels of the spinal cord studied. In the white matter, the specific binding was about a third or less of that seen in the gray matter (Table 4).

[^{3}H]TCP binding to mouse spinal cord sections

The binding of [^{3}H]TCP in the mouse spinal cord was studied after incubation at a concentration of 30 nM. Moderate labeling was seen in the gray matter, while the labeling in the white matter was clearly lower. The substantia gelatinosa of the dorsal horn showed the most intense labeling and the border

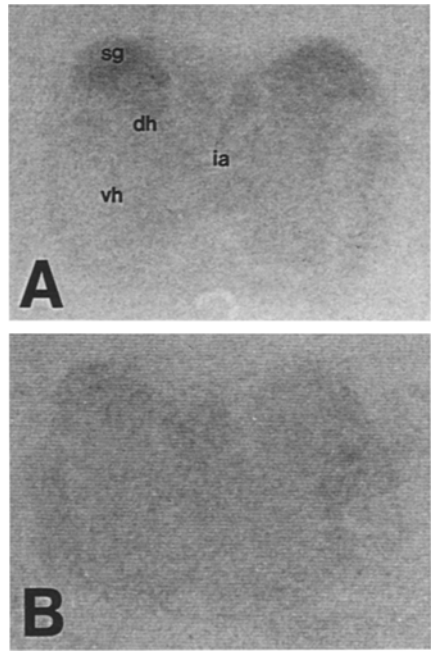

Fig. 4. Autoradiographs of *in vitro* binding with the competitive NMDA receptor antagonist [^{3}H]CGS 19755 in sections of mouse spinal cord from the lumbar part at approximately level L5 (**A**). Sections from the same levels were also incubated with [^{3}H]CGS 19755 in the presence of 10 μM L-glutamate to define non-specific binding (**B**). *sg* substantia gelatinosa; *dh* dorsal horn; *ia* intermediate area; *vh* ventral horn

Table 4. Binding of [^{3}H]CGS 19755 (30 nM) measured in autoradiographs of the mouse spinal cord

Region	Specific binding	% of total binding
Cervical/Thoracic spinal cord (C8/T1)		
Substantia gelatinosa	52.9 ± 1.6	49
Dorsal horn (excl. subst. gel.)	46.7 ± 1.8	46
Ventral horn	27.4 ± 2.3	36
Intermediate area	44.1 ± 3.9	43
Dorsal funiculus	11.1 ± 1.9	21
Lateral funiculus	8.6 ± 1.7	20
Ventral funiculus	3.2 ± 1.7	8
Lumbar spinal cord (L5)		
Substantia gelatinosa	63.1 ± 2.4	53
Dorsal horn (excl. subst. gel.)	37.1 ± 1.5	44
Ventral horn	26.6 ± 1.0	39
Intermediate area	42.6 ± 2.4	45
Dorsal funiculus	13.7 ± 1.2	25
Lateral funiculus	7.6 ± 1.2	18
Ventral funiculus	6.0 ± 0.8	14

Data are expressed as mean ± SEM fmol/mg tissue (specific binding) or % of total binding, n = 7. Specific binding was defined as total binding subtracted by binding in the presence of 10 μM L-glutamate.

Table 5. Binding of [^{3}H]TCP (30 nM) measured in autoradiographs of the mouse spinal cord

Region	Specific binding	% of total binding
Cervical/Thoracic spinal cord (C8/T1)		
Substantia gelatinosa	35.1 ± 1.1	60
Dorsal horn (excl. subst. gel.)	16.3 ± 0.7	40
Ventral horn	13.3 ± 0.9	35
Intermediate area	19.0 ± 1.1	44
Dorsal funiculus	2.3 ± 0.6	11
Lateral funiculus	1.6 ± 0.6	8
Ventral funiculus	0.4 ± 0.6	2
Lumbar spinal cord (L5)		
Substantia gelatinosa	35.7 ± 1.8	57
Dorsal horn (excl. subst. gel.)	13.4 ± 1.5	65
Ventral horn	16.7 ± 1.5	51
Intermediate area	18.6 ± 2.0	57
Dorsal funiculus	1.8 ± 0.8	8
Lateral funiculus	3.4 ± 1.0	14
Ventral funiculus	0.5 ± 1.2	2

Data are expressed as mean ± SEM fmol/mg tissue (specific binding) or % of total binding, n = 7–9. Specific binding was defined as total binding subtracted by binding in the presence of 10 μM PCP.

between the gray and white matter could be readily discerned. After incubation in the presence of 10 μM PCP, the labeling decreased markedly in the gray matter, however, the border between the white and gray matter could still be delineated. The specific binding in the gray matter ranged from 35 to 60% of the total binding. In the white matter, the specific binding constituted a few percent. The highest specific binding was seen in the substantia gelatinosa (Table 5).

Discussion

The present results show that the NMDA recognition site of the NMDA receptor complex can be visualized in rat and mouse spinal cord by using *in vitro* [^{3}H]CGS 19755 autoradiography. In addition, [^{3}H]CGS 19755 labeling of the NMDA receptor was also demonstrated in the rat brain.

It was observed in the initial experiments that the rinse time after [^{3}H]CGS 19755 incubation was critical in order to retain a high specific labeling and an acceptable low level of background staining. Hence, in order to reduce the problem of a rapid dissociation of the ligand, a very short rinsing time (15 sec) was used. In the protocol used first, a preincubation at 4°C for 60 minutes was employed, followed by another preincubation at 37°C for 30 min. Incubation with [^{3}H]CGS 19755 was thereafter performed for 30 minutes at room temperature. This protocol was found to be adequate for visualizing NMDA receptors in rat brain and in mouse spinal cord. However, in the rat spinal cord unacceptable low levels of specific binding were obtained in the gray

matter. The highest total binding of [^{3}H]CGS 19755 found in the spinal cord was much lower than that found in certain regions of the brain. In the brain regions with high total binding, a pronounced specific binding was seen, as defined by 10 μM of L-glutamate. On the other hand, in regions of the brain with low total binding, e.g. cerebellum, comparable relationships between total and specific binding were observed as in the spinal cord. Thus, low total binding, either in the spinal cord or in the brain, augment the relative proportion of the non-specific binding. The protocol was therefore revised to enhance the specific binding in the rat spinal cord. A more intense preincubation, at 22°C for 3 × 20 minutes, was used instead to better remove endogenous EAAs. Moreover, the incubation with [^{3}H]CGS 19755 was done at 4°C, for 60 minutes, to slow down the binding kinetics. By using this protocol, improved binding was obtained in the rat spinal cord. The compound used to define non-specific binding was also replaced in order to allow the use of glutamate dehydrogenase to further remove EAAs. However, this attempt to further enhance the [^{3}H]CGS 19755 labeling did not lead to any better results, in contrast to a previous study using [^{3}H]D,L-(E)-2-amino-4-propyl-5-phosphonopentenoic acid ([^{3}H]CGP 39653) as ligand (Jaarsma et al., 1993).

In the present study, a dense [^{3}H]CGS 19755 labeling was observed in the hippocampal formation and the cerebral cortex. A moderate labeling was seen in the thalamic region and striatum, while low labeling was seen in other regions, like the cerebellum. This finding is generally consistent with previous autoradiographic studies of the rat brain using L-[^{3}H]glutamate (Monaghan and Cotman, 1985; Greenamyre et al., 1984), D-[^{3}H]AP5 (Monaghan et al., 1988), [^{3}H]CGP 39653 (Laurie and Seeburg, 1994) or non-competitive ligands like [^{3}H]TCP (Gundlach et al., 1986). Thus, the observed distribution of the [^{3}H]CGS 19755 binding generally correlates with previous studies on the rat brain using other ligands for the NMDA receptor complex.

The present work shows significant binding of [^{3}H]CGS 19755 in the rat dorsal horn, in particular in the superficial layers, but notable binding was also observed in the intermediate area as well as in the ventral horn. There have been few reports on the distribution of NMDA receptors in the spinal cord and none so far with [^{3}H]CGS 19755. [^{3}H]Glutamate or [^{3}H]TCP autoradiography has been used to study EAA-receptor binding in rat and mouse spinal cord (Greenamyre et al., 1984; Aanonsen and Seybold, 1989; Gonzalez et al., 1993). Dense binding in gray matter, and particularly in the superficial layers of the dorsal horn, i.e. substantia gelatinosa, was found in these studies. Comparable results has also been obtained in the cat spinal cord (Mitchell and Anderson, 1991). A similar distribution of NMDA receptors were also demonstrated in both rat and human spinal cord by autoradiographic studies with [^{3}H]TCP or (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ([^{3}H]MK-801; Gundlach et al., 1986; Jansen et al., 1990; Kalb et al., 1992). Autoradiography of the rat spinal cord with the competitive NMDA antagonist [^{3}H]CGP 39653 has been described and compared to [^{3}H]glutamate and [^{3}H]MK-801 binding (Kalb et al., 1992). An intense binding in the dorsal horn was observed by using the three differ-

ent ligands, however the binding of [^{3}H]CGP 39653 and [^{3}H]MK-801 was almost exclusively located in substantia gelatinosa in contrast to the L-[^{3}H]glutamate binding.

Recent molecular cloning and expression studies have shown a heterogeneity in NMDA receptors (Nakanishi et al., 1992) and the NMDA receptor complex appears to consist of various combinations of subunits that are allocated to two main classes; the NMDAR1 (NR1) and NMDAR2 (NR2) families (Monyer et al., 1992; Sugihara et al., 1992; see also Nakanishi, 1992). Four related subunits belong to the NR2 family (NR2A-D), while only one NR1 subunit gene has been identified which can give rise to several mRNA splice variants (Sugihara et al., 1992). The anatomical organization of the different NR2 subunit mRNAs is highly heterogenous throughout the rat central nervous system, while the NR1 mRNA appears to be expressed ubiquitously (see Nakanishi, 1992). Also an ubiquitous distribution of NR1 mRNA as well as NR1 protein has been demonstrated in the rat spinal cord by in situ hybridization and immunocytochemistry (Tölle et al., 1993; Petralia et al., 1994). In contrast, Furuyama and co-workers (1993) were able to show, by using a gene expression technique, that the expression of the NR1 subunit, was actually much higher in the ventral horn than in the dorsal horn of the rat. In the study of Tölle and collaborators, no NR2A or NR2B mRNAs were found and the NR2C mRNA occured only in the substantia gelatinosa and at low levels, while a rather homogenous distribution of NR2D was found in the gray matter. Recently, Laurie and Seeburg (1994) were able to show that various NMDA ligands possess different affinities to recombinant NR1 homomeric and NR1-NR2 heteromeric receptors. This indicates that the slight dissimilarities observed in the distribution of the binding of various NMDA ligands in the rat spinal cord could reflect differences in their affinity to different compositions of NMDA subunits.

A similar distribution of the [^{3}H]CGS 19755 binding was found in the mouse spinal cord as compared to the rat spinal cord. However, the binding in the mouse spinal cord did not require modification of the original incubation protocol used. Previously, Gonzalez and colleagues (1993) showed significant binding of L-[^{3}H]glutamate to NMDA receptors in the superficial layers of the dorsal horn, particularly in the substantia gelatinosa, in the mouse; prominent binding was also found along the medial border of the dorsal horn, close to the central canal as well as in ventral horn regions. Taken together, these results suggest that in the mouse as well as in the rat [^{3}H]CGS 19755 labels sites that appears to overlap, at least partially, those labeled by L-[^{3}H]glutamate. Interestingly, the occurence and distribution of various NMDA mRNAs in the spinal cord appear to differ between adult mouse and rat. Similar to the rat an ubiquitous distribution of NR1 (ζ1) mRNA has been reported in the gray matter of the mouse spinal cord, while no NR2C (ε3) or NR2D (ε4) could be detected (Watanabe et al., 1994). Furthermore, a widespread distribution of the NR2A (ε1) mRNA was described, except for lamina 2, where NR2B (ε2) mRNA was detected instead. In spite of these described differences in subunit mRNA distribution, the [^{3}H]CGS 19755 binding appeared to be similar in both species.

A dense [^{3}H]TCP binding was found in the superfical layers of the dorsal horn, i.e. substantia gelatinosa, in the mouse spinal cord with moderate binding in the deep layers of the dorsal horn, intermediate area and ventral horn. Studies performed on human spinal cord have demonstrated a high density of NMDA receptors labeled with [^{3}H]TCP or [^{3}H]MK-801 in the dorsal horn and in particular lamina II, but considerable binding was also found in the ventral horn (Jansen et al., 1990; Krieger et al., 1993). In the rat spinal cord, Aanonsen and Seybold (1989) found decreasing [^{3}H]TCP binding density from rostral to more caudal levels. At variance, similar binding of [^{3}H]TCP was found in the gray matter of the cervical, thoracic and lumbal spinal cord of the mouse in the present study. It is therefore possible that certain species differences exist between rat and mouse in the distribution of TCP binding sites.

The specific binding of [^{3}H]CGS 19755 was higher than the specific [^{3}H]TCP binding in the mouse spinal cords, when the same concentration of the ligands was used. This may indicate that there is not a one-to-one relationship in the binding of the two ligands. However, differences in the characteristics of the ligands or experimental protocols is a more likely cause of this discrepancy. On the other hand, similar observations have been made in rat brain autoradiographs, when comparing cerebellar [^{3}H]CPP with [^{3}H]TCP binding (Jarvis et al., 1987). Furthermore, several studies have shown inconsistencies in the relationship between sites labeled with competitive antagonists (moderate densities) and sites labeled with ion-channel preferring antagonists (low densities) in the cerebellum (Maragos et al., 1988; Jacobson and Cotrell, 1993; Tacconi et al., 1993). It is therefore possible that the degree of specific [^{3}H]CGS 19755 binding compared with [^{3}H]TCP binding in the mouse spinal cord reflects true differences in the density of the various binding sites labeled by these ligands.

It is interesting that the highest levels of [^{3}H]CGS 19755 binding was found in the substantia gelatinosa of both rats and mice. The substantia gelatinosa of the dorsal horn is the major termination area for C-fiber afferents (Light and Perl, 1979). Even though not all C-fiber afferents are nociceptive (Kumazawa and Perl, 1978), the majority of them are classified as nociceptive (Lynn and Carpenter, 1982). C-fiber afferents and NMDA receptors are closely related in both wind-up (Davies and Lodge, 1987; Dickenson and Sullivan, 1987) and in activation of dorsal horn neurons (Nagy et al., 1993). The NMDA receptor has also been suggested to be involved in the process of central sensitization in the pathogenesis of pain (Woolf, 1992). Further anatomical characterization of the binding of different NMDA receptor ligands in the spinal cord are therefore warranted for our understanding of the involvement of EAAs in nociception.

In conclusion, the present study demonstrates that it is possible to use [^{3}H]CGS 19755 in autoradiographic studies to visualize NMDA recognition sites in rat and mouse spinal cord. Recently, the NMDA recognition site antagonist [^{3}H]CGP 39653 has been successfully utilized for autoradiography, by using a similar rinsing protocol as in the present study (Jaarsma et al., 1993; Kalb et al., 1992). This ligand appears to have a slightly slower dissociation constant, and may be more suitable for receptor-autoradiography. Future

autoradiographic studies will therefore be performed to compare [^{3}H]CGS 19755 binding with [^{3}H]CGP 39653.

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References

Aanonsen LM, Seybold VS (1989) Phencyclidine and sigma receptors in rat spinal cord: Binding characterization and quantitative autoradiography. Synapse 4: 1–10

Aanonsen LM, Lei S, Wilcox GL (1990) Excitatory amino acid receptors and nociceptive neurotransmission in rat spinal cord. Pain 41: 309–321

Davies SN, Lodge D (1987) Evidence for involvement of N-methylaspartate receptors in "wind-up" of class 2 neurones in the dorsal horn of the rat. Brain Res 424: 402–406

De Biasi S, Rustioni A (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. Proc Natl Acad Sci USA 85: 7820–7824

Dickenson AH, Sullivan AF (1987) Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. Neuropharmacology 26: 1235–1238

Furuyama T, Kiyama H, Sato K, Park HT, Maeno H, Takagi H, Tohyama M (1993) Region-specific expression of subunits of ionotropic glutamate receptors (AMPA-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociception. Mol Brain Res 18: 141–151

Gonzalez DL, Fuchs JL, Droge MH (1993) Distribution of NMDA receptor binding in developing mouse spinal cord. Neurosci Lett 151: 134–137

Greenamyre JT, Young AB, Penney JB (1984) Quantitative autoradiographic distribution of L-[^{3}H]glutamate-binding sites in rat central nervous system. J Neurosci 4: 2133–2144

Gundlach AL, Largent BL, Snyder SH (1986) Phencyclidine (PCP) receptors: autoradiographic localization in brain with the selective ligand [^{3}H]TCP. Brain Res 386:266–279

Jaarsma D, Sebens JB, Korf J (1993) Glutamate dehydrogenase improves binding of [^{3}H]CGS 39653 to NMDA receptors in the autoradiographic assay. J Neurosci Meth 46: 133–138

Jacobson W, Cotrell GA (1993) Rapid visualization of NMDA receptors in the brain: Characterization of (+)-3-[^{125}I]-iodo-MK-801 binding to thin sections of rat brain. J Neurosci Meth 46: 17–27

Jansen KL, Faull RL, Dragunow M, Waldvogel H (1990) Autoradiographic localisation of NMDA, quisqualate and kainic acid receptors in human spinal cord. Neurosci Lett 108: 53–57

Jarvis MF, Murphy DE, Williams M (1987) Quantitative autoradiographic localization of NMDA receptors in rat brain using [^{3}H]CPP: Comparison with [^{3}H]TCP binding sites. Eur J Pharmacol 141: 149–152

Kalb RG, Lidow MS, Halsted MJ, Hockfield S (1992) N-methyl-D-aspartate receptors are transiently expressed in the developing spinal cord ventral horn. Proc Natl Acad Sci USA 89: 8502–8506.

Kaplita PV, Ferkany JW (1990) Evidence for direct intractions between the NMDA and glycine recognition sites in brain. Eur J Pharmacol 188: 175–179

Krieger C, Wagey R, Shaw C (1993) Amyotrophic lateral sclerosis: Quantitative

autoradiography of [^{3}H]MK-801/NMDA binding sites in spinal cord. Neurosci Lett 159: 191–194
Kumazawa T, Perl ER (1978) Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: indications of their place in dorsal horn functional organization. J Comp Neurol 177: 417–434
Laurie DJ, Seeburg PH (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on the subunit composition. Eur J Pharmacol, Mol Pharm Sect 268: 335–345
Lehmann J, Hutchison AJ, McPherson SE, Mondadori C, Schmutz M, Sinton CM, Tsai C, Murphy DE, Steel DJ, Williams M, Cheney DL, Wood PL (1988) CGS 19755, a selective and competitive N-methyl-D-aspartate-type excitatory amino acid receptor antagonist. J Pharmacol Exp Ther 246: 65–75
Light AR, Perl ER (1979) Reexamination of dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. J Comp Neurol 186: 117–132
Lynn B, Carpenter SE (1982) Primary afferent units from the hairy skin of the rat hind limb. Brain Res 238: 29–43
Maragos WF, Chu DCM, Greenamyre T, Penney JB, Young AB (1986) High correlation between the localization of [^{3}H]TCP binding and NMDA receptors. Eur J Pharmacol 123: 173–174
Maragos WF, Penney JB, Young AB (1988) Anatomic correlation of NMDA and ^{3}H-TCP-labeled receptors in rat brain. J Neurosci 8: 493–501
Mitchell JJ, Anderson KJ (1991) Quantitative autoradiographic analysis of excitatory amino acid receptors in the cat spinal cord. Neurosci Lett 124: 269–272
Monaghan DT, Cotman CW (1985) Distribution of N-methyl-D-aspartate-sensitive L-[^{3}H]glutamate-binding sites in rat brain. J Neurosci 5: 2909–2919
Monaghan DT, Olverman HJ, Ngyen L, Watkins JC, Cotman CW (1988) Two classes of N-methyl-D-aspartate recognition sites: Differential distribution and differrential regulation by glycine. Proc Natl Acad Sci USA 85: 9836–9840
Monaghan DT, Bridges RJ, Cotman CW (1989) The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu Rev Pharmacol Toxicol 29: 365–402
Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH (1992) Heteromeric NMDA receptors: Molecular and functional distinction of subtypes. Science 256: 1217–1221
Murphy DE, Hutchison AJ, Hurt SD, Williams M, Sills MA (1988) Characterization of the binding of [^{3}H]CGS 19755: A novel N-methyl-D-aspartate antagonist with nanomolar affinity in rat brain. Br J Pharmacol 95: 932–938
Nagy I, Maggi CA, Dray A, Woolf CJ, Urban L (1993) The role of neurokinin and N-methyl-D-aspartate receptors in synaptic transmission from capsaicin-sensitive primary afferents in the rat spinal cord in vitro. Neurosci 52: 1029–1037
Nakanishi N (1992) Molecular diversity of glutamate receptors and implications for brain function. Science 258: 597–603
Nakanishi N, Axel R, Schneider NA (1992) Alternative splicing generates functionally distinct N-methyl-D-aspartate receptor. Proc Natl Acad Sci USA 89: 8552–8556
Näsström J, Karlsson U, Post C (1992) Antinociceptive actions of different classes of excitatory amino acid receptor antagonists in mice. Eur J Pharmacol 212: 21–29
Näsström J, Böö É, Ståhlberg M, Berge O-G (1993) Tissue distribution of two NMDA receptor antagonists, [^{3}H]CGS 19755 and [^{3}H]MK-801, after intrathecal injection in mice. Pharmacol Biochem Behav 44: 9–15
Petralia RS, Yokotani N, Wenthold RJ (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. J Neurosci 14: 667–696
Sandberg MP, Ask AL, Fowler CJ (1994) Characterisation of [^{3}H]CGS 19755 binding sites in the rat spinal cord. Neurochem Int 24: 329–337

Sugihara H, Moriyoshi K, Ishii T, Masu M, Nakanishi S (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. Biochem Biophys Res Comm 185: 826–832

Tacconi S, Ratti E, Marien MR, Gaviraghi G, Bowery NG (1993) Inhibition of [^{3}H]-(+)-MK 801 binding to rat brain sections by CPP and 7-chlorokynurenic acid: an autoradiographic analysis. Br J Pharmacol 108: 577–582

Tölle TR, Berthele A, Zieglgänsberger W, Seeburg PH, Wisden W (1993) The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. J Neurosci 13: 5009–5028

Watanabe M, Mishina M, Inoue Y (1994) Distinct spatiotemporal distributions of the N-methyl-D-aspartate receptor channel subunit mRNA in the mouse cervical cord. J Comp Neurol 345: 314–319

Watkins JC, Evans RH (1981) Excitatory amino acids. Annu Rev Pharmacol Toxicol 21: 165–204

Woolf CJ (1992) Excitability changes in central neurons following peripheral damage: Role of central sensitization in the pathogenesis of pain. Hyperalgesia and allodynia. Raven Press, Ltd. New York, pp 221–243

Young AB, Fagg G (1990) Excitatory amino acid receptors in the brain: membrane binding and receptor autoradiographic approaches. Trends Pharmacol Sci 11: 126–133

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