

## N-Methyl-D-aspartate receptors are transiently expressed in the developing spinal cord ventral horn

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**ABSTRACT** Quantitative receptor autoradiography was used to map the distribution of *N*-methyl-D-aspartate (NMDA) receptors in the developing rat spinal cord. Three different specific ligands, which label partially overlapping subpopulations of NMDA receptors, were used: an agonist (L-[<sup>3</sup>H]glutamate), a noncompetitive antagonist ([<sup>3</sup>H]MK-801), and a competitive antagonist ([<sup>3</sup>H]CGP-39653). In the adult, NMDA receptors labeled with all three ligands are restricted to the substantia gelatinosa in the spinal dorsal horn. In marked distinction, at postnatal day 7 NMDA receptors labeled with L-[<sup>3</sup>H]glutamate and [<sup>3</sup>H]MK-801 are present throughout the spinal gray matter. NMDA receptors in the neonatal spinal ventral horn have a higher affinity for L-[<sup>3</sup>H]glutamate than those in the adult substantia gelatinosa. Over the second and third postnatal weeks, NMDA receptors are lost from all areas of the spinal gray matter except for the substantia gelatinosa. Neonatal NMDA receptors identified with [<sup>3</sup>H]CGP-39653 are restricted to the substantia gelatinosa. These results show that the immature ventral horn contains a subpopulation of NMDA receptors and raise the possibility that motor neurons transiently express NMDA receptors in early postnatal life. Ventral horn NMDA receptors may be a component of the mechanisms by which the mature phenotype of motor neurons is acquired through activity-dependent processes. The loss of NMDA receptors over the course of development may play a role in limiting the period of motor neuron plasticity.

Many of the mature properties of motor neurons are acquired during the early postnatal period. For example, the large number of synapses made onto motor neurons early in development is reduced by approximately half over the first several weeks of postnatal life in the cat (1, 2). The reduction in synaptic inputs is accompanied by regressive changes in motor neuron dendritic structure (3). Further major synaptic reorganization does not occur in the adult animal. Studies from the visual system have provided compelling evidence that synaptically driven neuronal activity can control the synaptic rearrangements and axonal remodeling that occur during circumscribed periods in early development (4, 5).

Although the role of activity in the development of the vertebrate visual system is well-accepted, how general such processes are in neuronal development is not known. We have provided evidence that the acquisition of normal molecular properties of motor neurons and of neurons in central visual areas requires normal patterns of activity during critical periods in postnatal development (6–10). Our studies of activity-dependent development in the cat visual system have shown that expression of the surface-associated proteoglycan recognized by monoclonal antibody Cat-301 requires normal visual input during the first 3 postnatal months. Similarly, the expression of the Cat-301 proteoglycan on spinal motor neurons in the hamster requires normal patterns of neuromuscular activity during the first 2 weeks of life (11).

Glutamate, the major excitatory neurotransmitter in the vertebrate central nervous system, has been suggested to play an important role in developmental plasticity (12, 13). Recent studies indicate that activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor is required for activity-dependent development in both the frog retinotectal projection (13–15) and in the cat visual cortex (12, 16). Our previous experiments demonstrated that NMDA receptor activation is required for activity-dependent development of motor neurons (7, 8). Although we were able to show that the site of action of the NMDA receptor in motor neuron development is probably at the spinal segmental level (10), the resolution of the techniques used was not sufficient to determine the subregion of a spinal segment in which this receptor might be active. In the adult spinal cord NMDA receptors are most densely distributed in the dorsal layers of the dorsal horn (17).

In the present study we have examined the distribution of NMDA receptors in the spinal cord during the early postnatal period. We show that NMDA receptors are expressed in the ventral horn in young animals and that the distribution changes dramatically over the first 2 weeks of life. These results are consistent with a role for the NMDA receptor in motor neuron development during the critical period that we previously described.

### METHODS AND MATERIALS

Rats were anesthetized with Nembutal (2 mg/g) and perfused with phosphate-buffered saline, pH 7.4, followed by 0.1% paraformaldehyde containing increased concentrations of sucrose (up to 20%). Twenty-micron-thick cryostat sections from the lumbar and cervical enlargements were mounted on polylysine-coated slides and stored at  $-70^{\circ}\text{C}$  for no more than 2 weeks before use. The fixation procedure improved tissue adherence to slides and had minimal effect on ligand binding.

In binding assays performed with L-[<sup>3</sup>H]glutamate, all incubations were in 50 mM Tris acetate buffer, pH 7.2. Before labeling, sections were preincubated for 10 min in 0–4°C buffer and then transferred for 10 min into 37°C buffer containing 0.04% Triton X-100 (to remove endogenous glutamate). Tissue sections were subsequently washed three times for 10 min in ice-cold buffer. Incubation with various concentrations of L-[<sup>3</sup>H]glutamate (10–200 nM for neonates and 10–1200 nM for adults) was conducted for 10 min at 0–4°C in the same buffer to which 700 nM kainate and 400 nM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) were added. These agents were added in all incubation solutions to block non-NMDA receptors (18–20). To determine nonspecific binding, parallel sections were incubated in the presence of 100  $\mu\text{M}$  NMDA. After incubation, tissue sections were

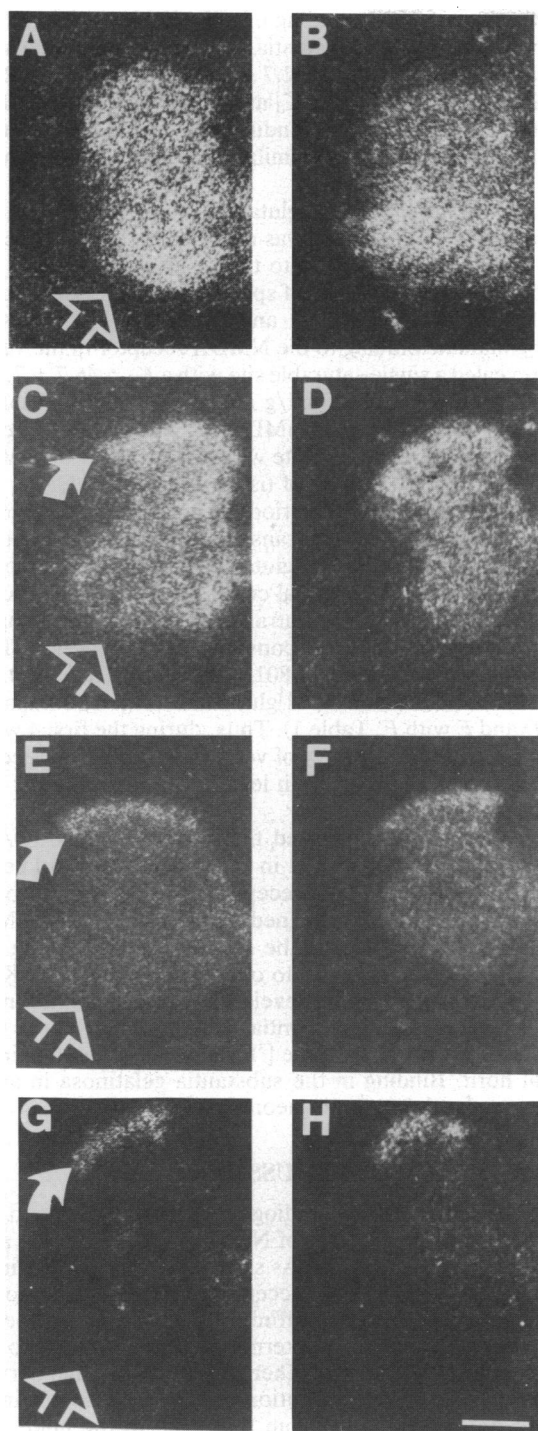


FIG. 1. Binding of L-[<sup>3</sup>H]glutamate and [<sup>3</sup>H]MK-801 to the NMDA receptor in the spinal cord is developmentally regulated. Spinal cords from animals of various postnatal ages were labeled with L-[<sup>3</sup>H]glutamate in the presence of kainate and CNQX (A, C, E, and G) or [<sup>3</sup>H]MK-801 (B, D, F, and H), according to the described protocol. (A) At postnatal day 7 (P7), intense labeling with L-[<sup>3</sup>H]glutamate is seen throughout the spinal cord gray matter, including the ventral horn (open arrow). (B) At P7, [<sup>3</sup>H]MK-801 binding reveals the same pattern as L-[<sup>3</sup>H]glutamate. (C) By P14, the L-[<sup>3</sup>H]glutamate label has diminished in all regions of the spinal gray matter, including the ventral horn (open arrow), except for the substantia gelatinosa in the dorsal horn (curved solid arrow). (D) At P14, [<sup>3</sup>H]MK-801 reveals a very similar staining pattern. There is less binding than observed at P7 in all regions of the spinal gray except the substantia gelatinosa. (E) At P21, there is a further decrease in L-[<sup>3</sup>H]glutamate in the ventral horn (open arrow), whereas the level of binding in the substantia gelatinosa of the dorsal horn remains high (curved solid

rinsed in 6 ml of iced buffer followed by 2 ml of iced acetone and dried under a stream of cold air. Dried slides with tissue sections were apposed to <sup>3</sup>H-sensitive Ultrafilm for 3 weeks. Under these conditions, the specific binding (total binding minus binding in the presence of 100  $\mu$ M NMDA) at a concentration of the radioligand near the  $K_d$  was >74% of total binding.

In binding assays using [<sup>3</sup>H]CGP-39653 (which is CIBA-Geigy pharmaceutical DL-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid), slides were preincubated as described for L-[<sup>3</sup>H]glutamate, incubated with ligand (0.5–13 nM) in 30 mM Tris-HCl, pH 7.1, for 60 min at 0–4°C, and rinsed with the same buffer (30 sec twice) before placement against film for 2 months. Nonspecific binding was determined in the presence of 100  $\mu$ M NMDA.

In binding assays using [<sup>3</sup>H]MK-801, all incubations were done as described (21), except that ligand binding was done in the presence of 10  $\mu$ M glutamate and 10  $\mu$ M glycine. Dried slides with tissue sections were apposed to <sup>3</sup>H-sensitive Ultrafilm for 4 weeks (21). After films were developed, tissue sections were stained with cresyl violet and used for regional identification within the spinal cord. Under these conditions, the specific binding (total binding minus binding in the presence of 5  $\mu$ M MK-801) at a concentration of the radioligand near the  $K_d$  was >81% of total binding.

Densitometry was performed on a computer imaging system (Imaging Research, St. Catherine, ON, Canada) (22), and saturation was analyzed with the nonlinear curve-fitting computer programs KINETIC/EDBA/LIGAND/LOWRY from Elsevier-Biosoft (Cambridge, U.K.). A minimum of 10 separate measurements were averaged per single concentration from each of at least three animals in the determination of the optical density at a given ligand concentration. Both  $B_{max}$  and  $K_d$  values represent the results averaged from at least three separate experiments. We quantitatively estimated the error in these calculations by determining a coefficient of variation, the  $SD(E_{rad})$  (23). The average  $SD(E_{rad})$  values for L-[<sup>3</sup>H]glutamate and [<sup>3</sup>H]MK-801 autoradiography were 11.9% and 6.8%, respectively. The relatively high  $SD(E_{rad})$  for L-[<sup>3</sup>H]glutamate is unavoidable due to the low intrinsic affinity of the ligand necessitating short wash times and to the inherent difficulty in determining optical density of a small region of interest.

In most previous studies, the  $K_d$  and  $B_{max}$  of ligand binding to the NMDA receptor were determined for a single region of the central nervous system by autoradiography (24) or by the analysis of tissue homogenates (25, 26). These studies assume that the  $K_d$  is uniform in all regions. Based on this analysis, ligand binding of a single ligand concentration was used to quantify regional differences in receptor density (17). This approach, however, does not permit analysis of any changes in ligand affinity between areas or over time. To overcome this potential problem, we determined the  $K_d$  and  $B_{max}$  (by saturation analysis) for each subregion of interest within the spinal cord and at each age studied.

## RESULTS

The distribution of NMDA-specific L-[<sup>3</sup>H]glutamate binding in the adult rat spinal cord was not uniform: a high level of specific binding was seen in the substantia gelatinosa, and a much lower level was seen in the ventral horn (Fig. 1G). This

arrow). (F) At P21, [<sup>3</sup>H]MK-801 reveals a very similar staining pattern with a decrease of label in all regions of the spinal gray except the substantia gelatinosa. (G) In the adult spinal cord, L-[<sup>3</sup>H]glutamate binding reveals no label in the ventral horn (open arrow) and persistent labeling in the substantia gelatinosa (curved solid arrow). (H) In the adult spinal cord, [<sup>3</sup>H]MK-801 binding has a very similar pattern with label restricted to the substantia gelatinosa. (Bar = 50  $\mu$ m.)

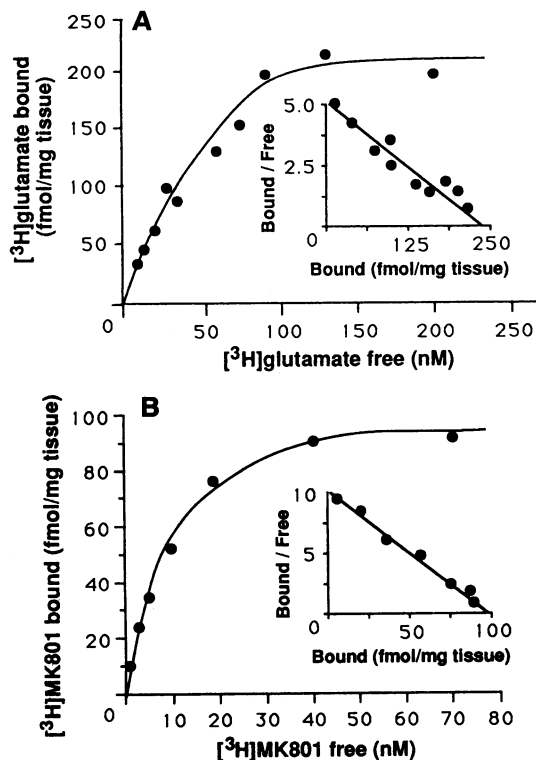


FIG. 2. (A) Representative saturation and Scatchard plot (*Inset*) of NMDA-specific L-[<sup>3</sup>H]glutamate binding to the neonatal ventral horn. Slides with P7 spinal cords were incubated with various concentrations of L-[<sup>3</sup>H]glutamate (10–200 nM) with kainate and CNQX, then washed, and apposed to Ultrofilm with tritium standards. Each point represents specific binding (average of at least 10 replicates minus readings from adjacent sections incubated with 100  $\mu$ M NMDA).  $K_d = 45.5$  nM;  $B_{max} = 231.5$  fmol/mg of tissue. (B) Representative saturation and Scatchard plot (*Inset*) of [<sup>3</sup>H]MK-801 binding to the neonatal ventral horn. Slides with P7 spinal cords were incubated with various concentrations of [<sup>3</sup>H]MK-801 (1–70 nM), then washed, and apposed to <sup>3</sup>H-sensitive Ultrofilm with tritium standards. Each point represents specific binding (average of at least 10 replicates minus readings from adjacent sections incubated with 5  $\mu$ M unlabeled MK-801).  $K_d = 9.4$  nM;  $B_{max} = 99.5$  fmol/mg of tissue. Computerized image analysis and quantitative analysis with nonlinear curve-fitting computer program are described in text.

pattern of L-[<sup>3</sup>H]glutamate binding was the same as described previously (17). Saturation analysis of L-[<sup>3</sup>H]glutamate binding to the NMDA receptor in the adult substantia gelatinosa revealed a single saturable site with a  $K_d = 315.6 \pm 44.6$  nM and  $B_{max} = 376.3 \pm 37.8$  fmol/g of tissue. There was no specific binding of NMDA-sensitive L-[<sup>3</sup>H]glutamate to the adult ventral horn.

A very similar pattern of binding to the adult spinal cord was obtained by using [<sup>3</sup>H]MK-801; specific binding to the substantia gelatinosa was high, and specific binding elsewhere in the spinal gray matter was minimal (Fig. 1*H*).

Saturation analysis of binding of [<sup>3</sup>H]MK-801 to the NMDA receptor in the adult substantia gelatinosa revealed a single saturable site with a  $K_d = 12.7 \pm 1.1$  nM and  $B_{max} = 207 \pm 11.9$  fmol/g of tissue. The  $K_d$  and  $B_{max}$  values of L-[<sup>3</sup>H]glutamate and [<sup>3</sup>H]MK-801 binding to the NMDA receptor determined here are quite similar to values reported for the adult hippocampus (21).

The distribution of L-[<sup>3</sup>H]glutamate and [<sup>3</sup>H]MK-801 binding to the NMDA receptor was next assayed in the P7 spinal cord. In marked distinction to the results in the adult, both ligands showed a high level of specific binding throughout the spinal gray matter (Fig. 1*A* and *B*). Saturation analysis of L-[<sup>3</sup>H]glutamate binding to the NMDA receptor in the ventral horn revealed a single saturable site with a  $K_d = 46.7 \pm 7.3$  nM and  $B_{max} = 240 \pm 24.6$  fmol/g of tissue (Fig. 2*A*, Table 1). [<sup>3</sup>H]MK-801 binding to the NMDA receptor in the P7 ventral horn had a single saturable site with a  $K_d = 8.9 \pm 0.9$  nM and  $B_{max} = 100.7 \pm 5.1$  fmol/g of tissue (Fig. 2*B*, Table 1).

To determine the time period during which the neonatal pattern of NMDA receptor transforms into the adult pattern, saturation analysis of L-[<sup>3</sup>H]glutamate binding in the ventral horn was performed on spinal cords from P14, P21, and P28 animals. The density of ventral horn NMDA receptor declines while the  $K_d$  remains constant over this period (Table 1). The decline in [<sup>3</sup>H]MK-801 binding was quantitatively very similar to that of L-[<sup>3</sup>H]glutamate (Fig. 1, compare *C* with *D* and *E* with *F*; Table 1). Thus, during the first 4 weeks of postnatal life, the density of ventral-horn NMDA receptor declines from an initially high level to the much lower level seen in the adult.

Recent work has suggested that subtypes of NMDA receptors can be distinguished in receptor-binding studies by using radiolabeled NMDA receptor agonists or antagonists (27). We quantitatively examined the distribution of NMDA receptor antagonist sites in the spinal cord by labeling with [<sup>3</sup>H]CGP-39653. In contrast to our results with [<sup>3</sup>H]MK-801 and L-[<sup>3</sup>H]glutamate, high levels of specific ligand binding were only found in the substantia gelatinosa of neonates (Fig. 3*A*). There was no detectable [<sup>3</sup>H]CGP-39653 binding in the ventral horn. Binding in the substantia gelatinosa in adults was somewhat less than in neonates (Fig. 3*B*).

## DISCUSSION

Quantitative receptor autoradiography was used here to map the distribution and density of NMDA receptors in the adult and developing spinal cord. As shown previously (17), in the adult spinal cord NMDA receptors are restricted to the substantia gelatinosa. The affinity of receptors for specific ligands in the spinal cord determined here is similar to that found in previous studies in other regions of the adult nervous system (17, 21). The distribution of NMDA receptor in the neonate differs markedly from that seen in the adult. The spinal gray matter, in general, and the ventral horn, in particular, show a high density of NMDA receptors identified with [<sup>3</sup>H]MK-801 and L-[<sup>3</sup>H]glutamate. The density of ventral

Table 1. Density and affinity of L-[<sup>3</sup>H]glutamate and [<sup>3</sup>H]MK-801 binding to the NMDA receptor in adult and neonatal spinal cords

Age	Region	Ligand	$B_{max}$ , fmol/mg of tissue	$K_d$
P7	Ventral horn	L-[ <sup>3</sup> H]Glutamate	240.4 $\pm$ 24.6	46.7 $\pm$ 7.3
P14	Ventral horn	L-[ <sup>3</sup> H]Glutamate	118.8 $\pm$ 9.7	39.8 $\pm$ 4.0
P21	Ventral horn	L-[ <sup>3</sup> H]Glutamate	65.9 $\pm$ 3.90	42.6 $\pm$ 4.8
Adult	Substantia gelatinosa	L-[ <sup>3</sup> H]Glutamate	376.3 $\pm$ 37.8	315.6 $\pm$ 44.6
P7	Ventral horn	[ <sup>3</sup> H]MK-801	100.7 $\pm$ 5.1	8.9 $\pm$ 0.9
P14	Ventral horn	[ <sup>3</sup> H]MK-801	63.9 $\pm$ 9.0	11.1 $\pm$ 2.7
P21	Ventral horn	[ <sup>3</sup> H]MK-801	36.9 $\pm$ 2.1	9.5 $\pm$ 1.6
Adult	Substantia gelatinosa	[ <sup>3</sup> H]MK-801	207.4 $\pm$ 11.9	12.7 $\pm$ 1.1

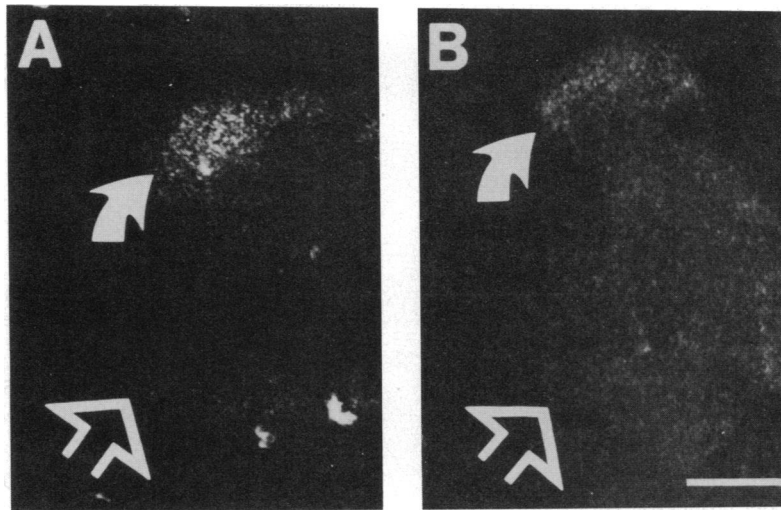


FIG. 3. Distribution of [ $^3\text{H}$ ]CGP-39653 sites is not developmentally regulated between P7 and adult in the rat spinal cord. Spinal cords from P7 (A) and adult (B) rats were labeled with [ $^3\text{H}$ ]CGP-39653 as described. (A) Intense labeling with [ $^3\text{H}$ ]CGP-39653 is present in the neonatal substantia gelatinosa (curved solid arrow), while the ventral horn is devoid of labeling (open arrow). (B) Similarly in adults, [ $^3\text{H}$ ]CGP-39653-binding sites are found in the substantia gelatinosa (curved solid arrow) and are not present in the ventral horn (open arrow). (Bar = 50  $\mu\text{m}$ .)

horn NMDA receptors is only 2-fold less than the density found in the adult substantia gelatinosa. Over the first month of life the level of NMDA receptor falls in all regions of the spinal gray matter, with the exception of the substantia gelatinosa, such that by P28, the adult pattern of NMDA-receptor distribution within the spinal cord is achieved.

It is likely that the binding demonstrated here with [ $^3\text{H}$ ]MK-801 and L-[ $^3\text{H}$ ]glutamate represents *bona fide* NMDA receptor for several reasons: (i) Two different radioactive ligands previously shown to bind to different domains of the NMDA receptor were used: L-[ $^3\text{H}$ ]glutamate, which binds to the putative endogenous neurotransmitter site, and [ $^3\text{H}$ ]MK-801, which binds to an open-channel site within the receptor-ionophore complex (28). In all instances, the tissue distribution was the same for both ligands. (ii) The affinities of radioligand binding determined here are quite close to values previously reported in other brain regions. (iii) All incubations with L-[ $^3\text{H}$ ]glutamate were done in the presence of CNQX and kainic acid to block non-NMDA receptors, and saturation analysis from all regions demonstrated binding to a single site. (iv) The ratio of L-[ $^3\text{H}$ ]glutamate sites to [ $^3\text{H}$ ]MK-801 sites was  $\approx 2:1$  in all instances, matching that previously reported (29). In sum, these results indicate that the ligand-binding patterns we observe represent the distribution of functional NMDA receptors.

The affinity of L-[ $^3\text{H}$ ]glutamate binding in the adult substantia gelatinosa ( $K_d = 315.6 \pm 44.6$  nM) differs from the affinity of binding in the neonatal ventral horn ( $K_d = 46.7 \pm 7.3$  nM), suggesting the presence of two distinct subtypes of receptor. This hypothesis is further supported by our observation that two distinct populations of NMDA receptors can be identified in the neonatal spinal cord: one population, labeled with L-[ $^3\text{H}$ ]glutamate and [ $^3\text{H}$ ]MK-801, is present throughout the spinal gray matter, and another population, labeled with [ $^3\text{H}$ ]CGP-39653, is present only in the substantia gelatinosa. Further characterization is needed to determine the relationship between the developmentally regulated spinal cord NMDA receptors we report and the previously described subtypes of NMDA receptors (27). In spite of the fact that we were able to distinguish autoradiographically the distribution of agonist-preferring and antagonist-preferring NMDA receptor sites, the affinity of competitive antagonists, such as DL-amino-5-phosphonovalerate, for these binding sites differs by less than an order of magnitude (27). There-

fore, it is likely that DL-amino-5-phosphonovalerate can act as an antagonist at both subtypes of NMDA receptors.

It is interesting that, although two receptor populations are suggested by L-[ $^3\text{H}$ ]glutamate and [ $^3\text{H}$ ]CGP-39653 binding, the affinity of [ $^3\text{H}$ ]MK-801 to the NMDA receptor is essentially constant in all tissues, regions, and ages examined. Therefore, under the binding conditions used here, there is evidence for only one type of ion channel associated with the NMDA receptor. Variations in NMDA-receptor subunit composition across different areas and over the course of development may account for the differences in ligand affinity we find with L-[ $^3\text{H}$ ]glutamate. The constancy of [ $^3\text{H}$ ]MK-801 binding suggests that the structure of the ionophore domain is conserved, regardless of region or developmental stage.

Recently, several cDNAs have been cloned that encode NMDA-receptor subunits (30, 31, 39, 40). Different heteromeric combinations of these subunits produce characteristic electrophysiological properties (31, 39, 40). The receptor autoradiographic results presented here predict that particular combinations of NMDA-receptor subunits are spatially and temporally regulated in the spinal cord.

The demonstration of developmental regulation of NMDA receptor distribution, while unusual for spinal cord, has been suggested to be important for synaptic plasticity in the developing mammalian visual cortex. Localization of the NMDA receptor by electrophysiology (32) and by receptor autoradiography (33) in the developing and adult cat indicates that the level of NMDA receptor is highest in cortical layers that undergo synaptic rearrangement during the period of maximal synaptic plasticity.

In previous work we have reported the activity-dependent regulation of expression of the motor neuron cell-surface proteoglycan recognized by monoclonal antibody Cat-301. Our studies indicate that a critical period for motor neuron development exists between P7 and P14 (7, 8). Both descending and spinal segmental afferents must be intact during this period for the development of normal levels of Cat-301 expression on motor neurons. We recently demonstrated that NMDA-receptor activation at the spinal segmental level during this critical period is also required for the induction of Cat-301 expression on motor neurons (10). The present results extend our previous observations by showing a transient population of NMDA receptors in the ventral horn that

could mediate the NMDA-dependent aspects of motor neuron development.

During development, several cortical and brainstem regions transiently over-express neurotransmitter receptors. The early postnatal rodent cortex, for example, has a high density of serotonin, kainate, quisqualate, and NMDA subtypes of glutamate receptors (34, 35). Similarly, in the primate cortex, a variety of neurotransmitter receptors are transiently expressed during development (36). Each receptor type has a distinct temporal and spatial pattern of expression. These receptors may have a trophic influence on neuronal growth or play a role in other maturational events, such as synapse formation or stabilization. Our present experiments suggest that motor neurons transiently express NMDA receptors during a developmental critical period. Previous work has shown that embryonic and early postnatal motor neurons do express functional NMDA receptors (37, 38). Once motor neurons have acquired their mature features, NMDA-receptor expression may be reduced. The reduction of NMDA receptors could reflect the close of a plastic period in motor neuron development. Parallels in the activity-dependent development of vertebrate visual and neuromuscular systems suggest that general cellular and molecular events underlying activity-dependent neuronal development may be accessible to study in spinal motor neurons.

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