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# Evidence for presynaptic *N*-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn

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ABSTRACT **The *N*-methyl-D-aspartate (NMDA) receptor has been implicated in a variety of systems that undergo plastic changes in the central nervous system. We used electron microscopic immunocytochemistry with an antibody directed against an alternatively spliced exon near the C terminus of NMDAR1, the essential functional subunit of the NMDA receptor, to study the distribution of the NMDA receptor in the spinal cord and CA1 region of the hippocampus, two regions where NMDA-mediated long-term plasticity has been demonstrated. In CA1, we found that the NMDA receptor is exclusively expressed on postsynaptic structures. By contrast, in the spinal cord we found that in about one-third of labeled synapses, the receptor is located in the presynaptic terminal, immediately adjacent to the vesicle release site at the active zone. Using combined postembedding immunocytochemistry, we also showed that >70% of the NMDA receptor immunoreactive terminals are glutamate positive, which suggests that the presynaptic NMDA receptor is an autoreceptor. Nerve ligation studies demonstrated that the receptor is transported in dorsal roots and sciatic nerve to the spinal cord and periphery, respectively. These data indicate that an NMDA autoreceptor is located in terminals of primary afferent fibers, where it could facilitate the transmission of inputs to the spinal cord by increasing the release of neurotransmitter from the primary afferent terminal.**

The *N*-methyl-D-aspartate (NMDA) type of glutamate receptor has been implicated in long-term potentiation and depression in the hippocampus (1, 2), activity-dependent patterning of connections in development (3), neuronal excitotoxicity (4), degenerative disorders (5), and several neuropathic pain conditions that result from nerve injury (6). Despite evidence that NMDA-regulated synaptic plasticity involves changes in neurotransmitter release (7), it is generally assumed that presynaptic mechanisms of potentiation are initiated by NMDA receptor-mediated increases in intracellular $Ca^{2+}$ in the postsynaptic neuron (8, 9). With the exception of suggestive NMDA receptor labeling in mossy fiber axons of the primate hippocampus (10), studies to date have revealed only postsynaptic NMDA receptor labeling by immunocytochemistry (11). In the present study, we used an antibody (termed NR1-C1) directed against an alternatively spliced exon near the C terminus of NMDAR1 (12), the essential functional subunit of the NMDA receptor (13), and demonstrate that many glutamatergic terminals in the spinal cord dorsal horn express presynaptic NMDA receptors. These presynaptic receptors are critically located to contribute to long-term plastic changes by increasing neurotransmitter release from primary afferent fibers.

## MATERIALS AND METHODS

The affinity-purified rabbit anti-NMDAR1 antiserum (NR1-C1) has been described (12). It was raised against a 37-amino acid peptide (DRKSGRAEPDPKKKATFRAITSTLASSFKRRRTSKDT), corresponding to an alternatively spliced exon (residues 864–900) in the C terminus of the NMDAR1 subunit (13, 14). This exon segment is present in the majority of NMDAR1 splice variants (15) and, like a recently described N-terminal insert (16), is a predominant site for phosphorylation by protein kinase C (17). For Western blot analysis, we prepared crude lysed membrane fractions from brain, spinal cord, and trigeminal and dorsal root ganglia (DRG) of Sprague–Dawley rats as described (18). For the ganglia, we combined tissue from three rats. Approximately 40 μg of protein (predominantly membrane) was solubilized in SDS sample buffer, separated by SDS/7.0% PAGE, and electrophoretically transferred to nitrocellulose. Filters were blocked with 0.1% Tween 20/2% bovine serum albumin/2% normal goat serum/10% nonfat dry milk in Tris-buffered saline (10 mM Tris·HCl, pH 7.4/150 mM NaCl) for 1 hr at room temperature, and then incubated overnight at 4°C with primary antiserum at 1.5 μg/ml in the same buffer. After washing with Tris-buffered saline containing 0.1% Tween 20, we visualized the immunoreactive bands with a horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG using the enhanced chemiluminescence system (ECL, Amersham).

For immunocytochemistry, male Sprague–Dawley rats (240–260 g) were deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused through the ascending aorta with 100 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by a 0.1 M phosphate-buffered fixative solution containing 1.0% formaldehyde, 2.5% glutaraldehyde, and 0.2% picric acid (19). After perfusion, the brain, lumbar spinal cord, and DRG were removed and postfixed in the same solution for 2–4 hr. For light microscopy, the DRG were cryoprotected in 30% sucrose/Tris PBS overnight and then cut at 25 μm on a cryostat. The brain and spinal cord were cut transversely at 50 μm on a Vibratome. The sections were incubated in 50% ethanol in distilled water for 40 min to improve antibody penetration, washed in Tris PBS, and then blocked in 10% normal goat serum for 1 hr. With intervening washes, the sections were incubated with affinity-purified NR1-C1 antibodies (0.2 μg/ml; 1:1500) for 24–48 hr at room temperature, biotinylated goat anti-rabbit antiserum for 2–4 hr, and an avidin/biotin/peroxidase complex (20) (Vectastain; Vector Laboratories) for 1 hr. To identify the immunoreaction product, the HRP was visualized with diaminobenzidine and glucose oxidase, with nickel intensification.

For electron microscopic analysis, immunoreacted Vibratome sections through dorsal and ventral horns, or through the CA1 region of the hippocampus, were stained for 1 hr in 1% $OsO_4$, *en bloc* stained in 2% aqueous uranyl acetate for 30 min, dehydrated, and flat embedded in Durcupan. For some spinal cord ultrathin sections, we followed this with a

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Abbreviations: NMDA, *N*-methyl-D-aspartate; DRG, dorsal root ganglia; SP, substance P.

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postembedding immunogold procedure to simultaneously localize the distribution of glutamate immunoreactivity. After washing in Tris-buffered saline, containing 0.1% Triton X-100, the sections were incubated overnight in a rabbit anti-glutamate antiserum (1:30K, Arnell Products, New York). The sections were washed and then incubated for 1 hr in a 15-nm colloidal gold-labeled goat anti-rabbit IgG (Amersham). Finally, the sections were stained with uranyl acetate and lead citrate and examined in the electron microscope. A terminal was considered positively stained for glutamate when the number of gold particles in the terminal exceeded that in surrounding neuropil by at least 4-fold. Counts were made on photographic prints at ×100,000 magnification.

To determine whether the receptor that is synthesized in DRG neurons is transported to the central and/or peripheral terminals of primary afferent axons, in separate rats ($n = 6$) we ligated the dorsal roots or the sciatic nerve and looked for damming of the immunoreactivity at the ligature. After lumbosacral laminectomy and resection of the dura, dorsal roots $L_5$ and $L_6$ were identified and ligated with 6-0 silk nonabsorbable sutures, ≈1.0 cm proximal to the $L_5$ DRG. The sciatic nerve was exposed in the thigh and then two ligatures (3-0 silk), separated by ≈1.0 cm, were tied around the nerve. For both dorsal roots and sciatic nerves, the rats survived 48 hr, at which time they were perfused for light microscopy. The dorsal roots and sciatic nerves, with attached ligatures, were removed, embedded in OCT, and then cut longitudinally at 20 μm on a cryostat. The sections were immunoreacted with NR1-C1 antibody as described above.

## RESULTS

Previous studies reported that the NR1-C1 antibody recognizes a 120-kDa band in brain, which corresponds to the molecular mass of the NMDAR1 subunit (12, 17). In the present report, we found by Western blotting that the spinal cord and the trigeminal ganglia and DRG contain a single immunoreactive protein that exactly comigrates with the NR1 band detected in brain (Fig. 1). This strongly suggests that the NR1 is expressed in spinal cord and DRG. In agreement with ligand binding (21, 22), *in situ* hybridization (23), and light microscopic immunocytochemical analysis (11), we found dense NMDAR1 immunoreactivity in the superficial dorsal horn at all levels of the spinal cord (see Fig. 3*A*). The staining is of neuronal cell bodies, particularly in the inner part of the substantia gelatinosa, lamina II, and of many small, dark dendritic and axonal profiles (see below). There is also intense staining of ventral horn motoneuron somata and dendrites. Importantly, preabsorption of the primary antiserum for 24 hr with the peptide against which the antiserum was raised (at 3.0 μg per ml of diluted antiserum) completely eliminated staining.

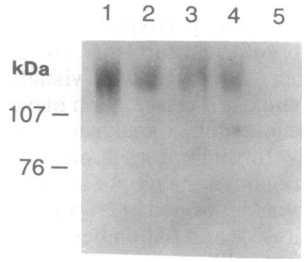


FIG. 1. Western blot analysis of membrane fractions from brain (lane 1), spinal cord (lane 2), trigeminal ganglia (lane 3), and DRG (lane 4) reveals a major band that migrates at ≈120 kDa, which corresponds to the size of the brain NMDAR1 protein. Control (lane 5) was loaded with DRG membrane proteins, but the primary antiserum was omitted.

At the electron microscopic level we found that most of the staining in the spinal cord was concentrated in small patches (average length, 0.12 μm) on the plasma membrane of cell bodies and dendrites; cytoplasmic labeling was also observed. Most commonly the membrane immunoreactivity was located at postsynaptic densities that apposed unlabeled synaptic terminals. In the CA1 region of the hippocampus, we found NMDAR1 immunoreactivity only in postsynaptic structures. Surprisingly, however, in the spinal cord we detected many presynaptic terminals that were NMDAR1 immunoreactive. In these terminals, the immunoreaction product was typically concentrated immediately adjacent to the presynaptic side of the synaptic specialization (Fig. 2). The synaptic cleft was always visible, even when there was both pre- and postsynaptic labeling at the same synaptic junction (Fig. 2*A*).

Presynaptic NMDAR1-immunoreactive labeling was common. In ≈35% of the NMDAR1 immunoreactive synapses in the superficial dorsal horn (295/855), the staining was located at the presynaptic terminal; in ≈62% (532/855), the immunoreaction product was associated with the postsynaptic density. In the remainder (3.3%), both pre- and postsynaptic labeling was present. In the ventral horn of the spinal cord, 37% (207/560) of labeled synapses contained immunoreaction product presynaptically; many of these synapses con-

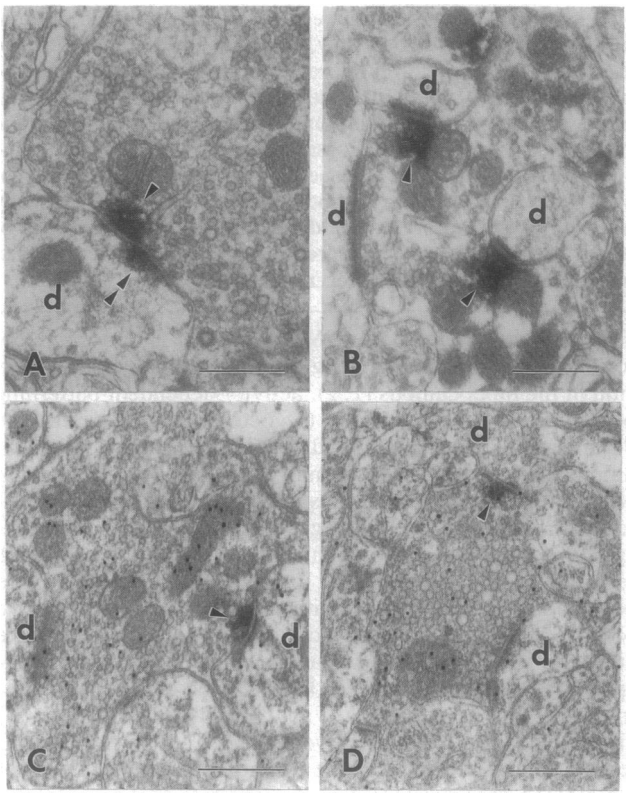


FIG. 2. NMDA receptor immunoreactivity is located in presynaptic terminals in the dorsal horn of the spinal cord and many of these terminals are glutamate positive. (*A*) This central terminal in lamina II of the dorsal horn contains NMDAR1 immunolabeling on both the presynaptic (arrowhead) and postsynaptic (double arrowhead) side of the synaptic specialization; the synaptic cleft separating the central terminal from the postsynaptic dendrite (d) is devoid of reaction product. (*B*) This central terminal in lamina II contains NMDAR1 immunoreaction product (arrowheads) at two of its three synaptic contacts with unlabeled dendrites (d). The immunolabel is readily distinguished from the postsynaptic densities, which denote the contacts. (*C* and *D*) Photomicrographs illustrate double labeling of the presynaptic NMDAR1 receptor (arrowheads) in terminals that contain glutamate immunoreactivity identified by colloidal gold labeling. (Bars = 0.2 μm.)

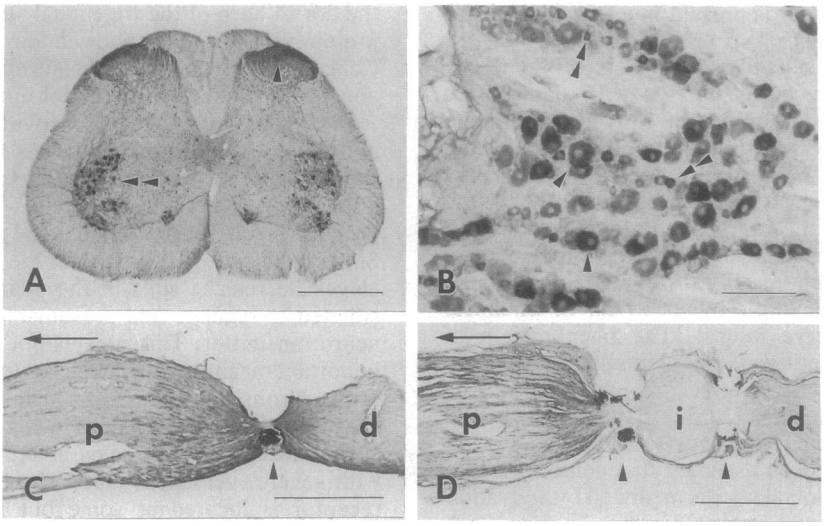


FIG. 3. (*A*) Transverse section through lumbar enlargement showing dense NMDAR1 immunoreactivity in the superficial dorsal horn and in ventral horn motoneurons (arrowheads). (*B*) Dense NMDAR1 receptor immunoreactivity in large-diameter (single arrowhead) and small-diameter (double arrowhead) neurons of the DRG. (*C*) After ligation of dorsal roots $L_{5-6}$, we found a buildup of NMDAR1 receptor immunoreactivity in the afferents proximal (p) to the ligature, which indicates that the receptor is transported in primary afferents to the spinal cord. (*D*) In the sciatic nerve, there is intense immunostaining of the NMDAR1 receptor proximal (p) to the first of two ligatures. There is absent or much less staining in the interligature segment of the nerve (i), which indicates that the receptor was transported beyond the second ligature during the postoperative survival period. There is also moderate NMDAR1 immunoreactivity at the distal (d) side of the second ligature, which suggests that the receptor is also retrogradely transported from the periphery. Staining of the distal side of the dorsal root was less apparent. Arrows in *C* and *D* point to location of the DRG. (*A* and *D*, bars = 200 μm; *B*, bar = 50 μm; *C*, bar = 150 μm.)

tacted heavily labeled cell bodies and proximal dendrites of motoneurons. Consistent with the labeling of presynaptic terminals, we also recorded patchy NMDAR1 immunoreactivity in unmyelinated and some thinly myelinated axonal profiles in laminae I–II; in the Lissauer's tract, which contains many primary afferent axons; and in the ventral horn (data not shown).

Interestingly, the presynaptic labeling was often recorded at more than one, but not at all, of the synapses made by the same terminal (Fig. 2 *B* and *C*). To determine how common this phenomenon was, we counted 54 immunoreactive terminals that made at least three synaptic contacts with different postsynaptic targets in a single plane of section. The total number of contacts made was 191; NMDAR1 labeling was found at 77 of them (≈40%). In none of the terminals was there label at all of the synaptic contacts.

What might be the source of the glutamate that could activate these presynaptic NMDA receptors? Using a postembedding immunogold approach, we found that the majority (71%; 124/175) of the presynaptic NMDAR1-labeled terminals in the superficial dorsal horn stained positively for glutamate (Fig. 2 *C* and *D*). In the ventral horn, we colocalized glutamate in 74% of the terminals (126/170) that contained presynaptic NMDAR1 immunoreactivity. These results suggest that the presynaptic receptors could act as autoreceptors—i.e., they are activated by glutamate that is released from the same terminal.

Since the presynaptic labeling of the superficial dorsal horn was commonly found in large, scallop-shaped terminals, which predominantly arise from nociceptive primary afferent fibers (24), we next examined the DRG. In agreement with *in situ* hybridization studies that reported NMDA receptor mRNA in DRG neurons (25), we found that the DRG contains many NR1-C1 immunoreactive neurons of both small and large diameter (Fig. 3*B*). After ligation of either the dorsal roots (Fig. 3*C*) or the sciatic nerve (Fig. 3*D*), we found significant buildup of NMDAR1 immunoreactivity on the side of the ligature proximal to the DRG neuron, which indicates that there was damming of immunoreactivity following axoplasmic transport of the NMDA receptor from the DRG. In the six rats studied, there was also buildup of receptor immunoreactivity, to a lesser degree, on the distal side of the second, more peripheral sciatic nerve ligature, which suggests that the NMDAR1 protein is also transported in a retrograde direction from the peripheral terminal to the cell body, where it is probably degraded.

## DISCUSSION

The present study demonstrates that the NMDA receptor, revealed with an antiserum directed against a C terminus domain of NMDAR1, its essential subunit, is located in both pre- and postsynaptic components of synapses in the dorsal and ventral horns of the rat spinal cord. The accumulation of immunoreaction product on the intracellular side of the synaptic membrane is consistent with the C terminus of the NMDAR1 subunit being on the cytoplasmic side of the membrane, as predicted from phosphorylation studies (17). The fact that the presynaptic labeling is concentrated in terminals that could be stained with antisera directed against glutamate, taken together with the preferential location of the receptor labeling near the active zone—i.e., at vesicle release sites—suggests that the presynaptic NMDA receptor functions as an autoreceptor that can regulate neurotransmitter release.

The fact that the NMDAR1 immunoreactivity is transported both centrally and peripherally from the DRG is consistent with a previous study that demonstrated bidirectional transport of NMDA binding sites described in the vagus nerve (26). Taken together with the typical scalloped morphology of the labeled terminals in the superficial dorsal horn, these data indicate that the receptor is located on small-diameter primary afferent fibers. However, since both small and large diameter neurons were labeled in the DRG, it is likely that the receptor is expressed on terminals of both small-diameter nociceptive and large-diameter nonnociceptive primary afferents. It is, in fact, possible that some of the presynaptic labeling in the ventral horn is located on Ia spindle afferents. Furthermore, since there is dense motoneuron labeling it is likely that some of the sciatic nerve

NMDAR1 immunoreactivity is present in efferent as well as in afferent (sensory) axons.

Based on studies using selective antagonists, it is generally agreed that non-NMDA receptor spinal cord mechanisms are involved in the transmission of acute (reflex evoking) nociceptive messages from normal tissue—e.g., heat-evoked tail flick (27)—and that the NMDA receptor comes into play when there is a persistent noxious stimulus (6, 27–29), the result of which is facilitation of second-order neurons and hyperalgesia—i.e., increased pain. The latter can be evoked by a variety of stimuli, that usually involve intense stimulation at C-fiber strengths (e.g., by electrical or chemical stimulation, burn or nerve injury). The phenomenon of "wind-up," in which repetitive C-fiber stimulation results in progressively greater discharge of second-order neurons, is also NMDA receptor dependent (30, 31). By analogy with the proposed mechanism underlying long-term potentiation in the hippocampus, it has been assumed that central facilitation/sensitization in the dorsal horn is mediated by activation of postsynaptic NMDA receptors (32). This permits $Ca^{2+}$ influx through the NMDA receptor, which is followed by a cascade of second messenger-linked events that contribute to the long-term plastic changes that underlie the central facilitation. Long-term potentiation has, in fact, recently been described in the dorsal horn (33). Consistent with the hypothesized postsynaptic NMDA receptor-mediated mechanisms in the hippocampus, we found postsynaptic NMDAR1 labeling only in the CA1 region of the hippocampus. By contrast, we found both pre- and postsynaptic labeling at synapses in the spinal cord.

The release of glutamate from the primary afferent terminal would not only depolarize the postsynaptic neuron but could contribute to enhanced glutamate release in response to subsequent stimuli, because of the relatively prolonged increase in the primary afferent terminal concentration of $Ca^{2+}$ that would result from positive feedback at the presynaptic NMDA autoreceptor. If the stimulus persists, as under inflammatory conditions, the $Ca^{2+}$ levels would continue to increase considerably beyond that which might accumulate via voltage-dependent $Ca^{2+}$ channels. Furthermore, since substance P (SP), calcitonin gene-related peptide (CGRP), and glutamate co-occur in many primary afferent terminals (34), it is possible that the presynaptic NMDA autoreceptor also regulates the release of primary afferent neuropeptides. Interestingly, Garrison *et al.* (35) reported that rats with peripheral nerve injury and neuropathic pain had decreased SP levels in the dorsal horn and that the noncompetitive NMDA antagonist MK-801 increased the levels of SP. They concluded that activation of postsynaptic NMDA receptors on SP-containing interneurons mediated the depletion of SP. Our results suggest that the nerve injury-evoked decrease of SP could reflect an action on the SP-containing primary afferent terminal. In fact, the buildup of NMDAR1 immunoreactivity on the sciatic nerve suggests that a peripheral NMDA receptor may also regulate the release of primary afferent neurotransmitters (namely, SP and CGRP) that contribute to neurogenic inflammation (36).

Some studies failed to find NMDA receptor-mediated currents in cell bodies of DRG neurons (37, 38). On the other hand, NMDAR1 subunits can form a functional receptor by homooligomerization (13) and Lovinger and Weight (39) demonstrated a $Mg^{2+}$ and 2-amino-5-phosphonovaleric acid-sensitive NMDA-induced depolarization of adult DRG neurons. The fact that we revealed a presynaptic NMDA receptor with antibodies directed against a region that contains protein kinase C phosphorylation sites suggests that the presynaptic receptor may be regulated by phosphorylation. It is of interest in this regard that, although opioids generally inhibit the release of peptides from primary afferent fibers, Chen and Huang (40) described an excitatory opioid effect in the dorsal horn of the trigeminal system that is mediated by protein kinase C phosphorylation of the NMDA receptor. Conceivably similar mechanisms may underlie the somewhat anomalous excitatory effects of opioids on DRG cells (41).

In conclusion, our results provide evidence for a presynaptic NMDA autoreceptor that is located on glutamatergic primary afferent terminals in the spinal cord. This receptor differs from syntactin, a protein with NMDA receptor-like properties that is also located in the presynaptic terminal (42). Since the NMDAR1 subunit of the receptor is concentrated at vesicle release sites in the synaptic terminal, it is well situated to regulate and presumably increase the release of neurotransmitter. This presynaptic mechanism provides an important means through which the responsiveness of dorsal horn neurons could be enhanced and may underlie the central sensitization of dorsal horn neurons, a phenomenon that contributes to a variety of pathological conditions associated with severe, persistent pain. The fact that the presynaptic receptor is located at some but not all of the dendritic contacts made by a single synaptic terminal suggests a potentially novel mechanism for the selective strengthening of a subset of synaptic connections that arises from the same terminal.

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