Functional and Immunocytochemical Identification of Glutamate Autoreceptors of an NMDA Type in Crayfish Neuromuscular Junction

N. FEINSTEIN, D. PARNAS, H. PARNAS, J. DUDEL, AND I. PARNAS

1The Otto Loewi Center for Cellular and Molecular Neurobiology and Department of Neurobiology; and 2Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel 91904; and 3Department of Physiology, The Technical University, Munich, Germany

Feinstein, N., D. Parnas, H. Parnas, J. Dudel, and I. Parnas. Functional and Immunocytochemical Identification of Glutamate Autoreceptors of an NMDA Type in Crayfish Neuromuscular Junction. J. Neurophysiol. 80: 2893–2899, 1998. N-Methyl-D-aspartate (NMDA) receptors reduce release from crayfish excitatory nerve terminals. We show here that polyclonal and monoclonal antibodies raised against the mammalian postsynaptic NMDA receptor subunit 1 stain specifically the presynaptic membrane of release boutons of the crayfish neuromuscular junction. In crayfish ganglionic membranes, the polyclonal antibody recognizes a single protein band that is somewhat larger (by ~30 kD) than the molecular weight of the rat receptor. Moreover, the monoclonal (but not the polyclonal) antibody abolishes the physiological effect of NMDA on glutamate release. The monoclonal antibody did not prevent the presynaptic effects of glutamate, which also reduces release by activation of quisqualate presynaptic receptors. Only when 6-cyano-7-nitroquinocaxaline-2,3-dione (CNQX) was added together with the monoclonal antibody was the presynaptic effect of glutamate blocked. These results show that presynaptic glutamate receptors of the crayfish NMDA type are involved in the regulation of neurotransmitter release in crayfish axon terminals. Although the crayfish receptor differs in its properties from the mammalian NMDA receptor, the two receptors retained some structural similarity.

INTRODUCTION

Glutamate receptors of the N-methyl-D-aspartate (NMDA) type are involved in long-term synaptic modulation in the mammalian brain (Bliss and Collingridge 1993; Collingridge and Singer 1990; Mulkey and Malenka 1992). Such NMDA receptors were thought to exist only in vertebrates (Mayer and Westbrook 1987), but evidence suggests that NMDA acts also in invertebrates (Dale and Kandel 1993; Evans et al. 1992; Parnas et al. 1994, 1996; Pfeiffer-Linn and Glantz 1991; Ultsch et al. 1993). Recently, it was shown that NMDA reduces release of transmitter from crayfish terminals at concentrations as low as 10^{-7} M (Parnas et al. 1996). These crayfish NMDA receptors whose activation affects—directly or indirectly—the release of transmitter differ in their physiological properties from the mammalian NMDA receptor. Their activation is not associated with an increase in membrane conductance, there is no increase in intracellular Ca^{2+} concentration, and a change in extracellular Mg^{2+} concentration does not affect the magnitude of their response (Parnas et al. 1994).

The finding that NMDA reduces release from crayfish axon terminals (Parnas et al. 1994, 1996) does not necessarily mean that receptors are located directly on the presynaptic terminal. It is possible that NMDA acts on receptors located on other cells, such as the postsynaptic membrane or glial cells (Conti et al. 1997), and that the latter affect release indirectly.

This study uses immunostaining techniques to obtain evidence for the presence of presynaptic NMDA receptors in crayfish axon terminals. Because specific antibodies for crayfish NMDA receptors are not available, we tried two antibodies (available commercially) raised against the mammalian NMDA receptor. Although the two receptors have different physiological properties, they may have retained some structural similarity because in many cases channels and receptors evolved each from a common ancestor (Hille 1992). Such an assumption gains support from the finding of some structural similarity between the NMDA receptor of arthropods (Drosophila) and the mammalian NMDA receptors (Ultsch et al. 1993).

One of the antibodies is an anti-NMDA receptor subunit 1 (NMDAR1), monoclonal antibody (mAb) (Siegel et al. 1994). The other is a polyclonal antibody (pAb) raised against splice variants NR1-1a, NR1-1b, NR1-2a, and NR1-2b of NMDAR1 (Petralia et al. 1994).

By electron microscopy, we identified positive immunostaining for NMDA receptors on the presynaptic membrane of crayfish release boutons. Staining was not found on muscle or nonneuronal elements. We further show by Western analysis that the pAb recognizes a single protein band that is somewhat larger (by ~30 kD) than the mammalian NMDAR1. Moreover, the mAb (but not the polyclonal antibody) completely abolished the physiological effect of NMDA. The mAb did not block the physiological effects of glutamate that also acts via quisqualate presynaptic receptors (Parnas et al. 1996). To abolish the presynaptic effect of glutamate the combined action of the mAb together with 6-cyano-7-nitroquinocaxaline-2,3-dione (CNQX) was required.

Our results indicate that, in the crayfish, feedback inhibition of glutamate release is mediated, in part, by crayfish NMDA receptors located on presynaptic axon terminals. Although these receptors differ in their properties from the mammalian receptor, the two receptors (crayfish and mammalian) probably share some structural similarity.
METHODS

Preparation

The deep extensor abdominal muscles (DEAM) (Parnas and Atwood 1966) of the crayfish Procambarus clarkii were used for immunostaining. For electrophysiological experiments, the DEAM system of both P. clarkii and Astacus astacus was used. (For details see Parnas et al. 1994, 1996). Abdominal ganglia of P. clarkii were used for Western blots. To obtain the abdominal ganglia, we separated the abdomen of the crayfish from the cephalothorax. The cephalothorax was then rapidly crushed. The abdominal ganglia were then isolated for the Western blots. As a control for Ab binding, we used rat brains. Male Sprague-Dawley rats, 180–200 g, were killed by CO₂ inhalation in accordance with guidelines set forth by the National Institutes of Health.

Western blotting

Crayfish abdominal ganglia, crayfish DEAM muscles, and rat brain tissue were each homogenized in a glass homogenizer in Krebs buffer supplemented with protease inhibitors (4-2-amino ethylbenzenesulfonyl fluoride, 1 mM; aprotinin, 1 μg/ml; leupeptin, 5 μg/ml). Nuclei were precipitated (350g, 15 min, 4°C), and the resulting supernatant was centrifuged at 300,000g for 20 min at 4°C. The membranous fraction (pellet) was resuspended in solubilization buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris, pH 8), and the supernatant was precipitated with 80% acetone.

Protein concentration was determined by the microBCA method (Pierce, Rockford, IL). Thirty milligrams of protein from each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel. The pAb antibody (Chemicon International, Temecula, CA) was diluted 1:50. Detection was by enhanced chemiluminescence.

Electrophysiological measurements

Electrophysiological measurements were carried out as described by Parnas et al. (1994, 1996). Briefly, single quanta events were recorded extracellularly by means of the macropatch focal recording technique (Dudel 1981). The same macropatch electrode (12-μm openings) was used to depolarize a single, or at most two, release boutons (Fischer and Parnas 1996) as well as to record quantal responses. Samples of such recordings are shown in Fig. 5. The preparation was treated with 5 × 10⁻⁷ M tetrodotoxin. The depolarizing pulse was 1 ms, −0.4 μA. The temperature was 11–12°C.

Testing for the effects of antibodies

To give the antibodies ample time to reach the presynaptic release boutons, the preparation was incubated for ≥2 h with the antibody. Because of the long incubation period and the relatively larger volumes required to perfuse a bouton through the macropatch electrode (Dudel 1989), we could not perform the antibody-treated -untreated experiments while recording from the same release site as in previous experiments (Parnas et al. 1994, 1996). Instead, the left and right hemisegments containing only the L₁ and L₂ bodies before any fixation procedure and consequently without any pretreatment and -untreated experiments while recording from the same release antibody binds with the receptor under these conditions. We therefore used Sprague-Dawley rats, 180–200 g, were killed by CO₂ inhalation in accordance with guidelines set forth by the National Institutes of Health.

Calculation of quantal content

Single quanta events are clearly distinguishable at 12°C (see Fig. 5). Counting the number of quanta and dividing by the number of pulses provides a direct measure of quantal content. The quantum size measured was not influenced by postsynaptic effects, as there are no postsynaptic NMDA receptors in the DEAM, and the concentration of glutamate used here (1 × 10⁻⁶ M) does not cause appreciable desensitization (Parnas et al. 1994, 1996). Recordings were digitized at 50 kHz (LabVIEW interface, National Instruments, Austin, TX) and analyzed with a Pentium computer. In the crayfish, synapses on the same muscle show large differences in quantal content (Cooper et al. 1995; Parnas et al. 1996). For this reason, and to present observations from different synapses together, we normalized our results, presenting quantal content as a percentage of the control at each site.

Prefixation immunogold staining

Because we found that incubation of the live preparation with the mAb blocks the physiological effect of NMDA, we assumed that the antibody binds with the receptor under these conditions. We therefore exposed the muscle hemisegments to the primary and secondary antibodies before any fixation procedure and consequently without any permeabilization of the membranes. As shown, this approach was found successful for both antibodies. The live preparation was incubated at room temperature for 1 h in VH solution containing 2% BSA for the mAb or in 5% NGS for the pAb. The muscle was then transferred to VH solution containing 15 μg/ml mAb anti-NMDAR1 or 5 μg/ml pAb for ≥2 h. Then the preparation was washed several times in VH solution and transferred to the proper secondary antibody for 2 h. Subsequently, the hemisegments were incubated in IgG (Fab fragment) coupled to 1.4 nm gold (Nanoprobes, Stonybrook, NY). After this incubation, the muscle specimens were washed several times in VH solution and transferred to the proper secondary antibody for 2 h. Subsequently, the hemisegments were incubated in IgG (Fab fragment) coupled to 1.4 nm gold (Nanoprobes, Stonybrook, NY). After this incubation, the muscle specimens were washed several times in VH solution and transferred to the proper secondary antibody for 2 h. Subsequently, the hemisegments were incubated in IgG (Fab fragment) coupled to 1.4 nm gold (Nanoprobes, Stonybrook, NY). After this incubation, the muscle specimens were washed several
boutons (multiterminal innervation) (Atwood 1977). Many of these release boutons are embedded in dimples in the muscle fiber (Fig. 1). Each release bouton or varicosity contains several release sites (Wojtowicz et al. 1994). Figure 1 shows that the postsynaptic membrane is at some distance from the myofibrils, and the peripheral extracellular sarcoplasm is occupied by large mitochondria and other sarcoplasmic elements. In Fig. 1, the presynaptic and postsynaptic membranes are clearly visible. The longitudinal section through this axon shows many vesicles. A short stalk connects the axon terminal to a bouton filled with synaptic vesicles. It should be noted that none of the sections presented in this and the following figures were stained with uranyl acetate and lead citrate; thus membranes are not as clear as in stained preparations.

**Staining with the pAb**

Intense staining with the pAb was obtained with the silver-enhanced method (Fig. 2). In many cases, there was a clear distinction between the pre- and the postsynaptic membranes, and clusters of particles were visible in the presynaptic membranes (Fig. 2).

Five animals were used for the staining with the pAb. Nine hemisegments were taken, and in these we found 51 stained terminals with 94 presynaptic clusters.

**Staining with the mAb**

Staining by the mAb was less prominent. Figure 3 shows two terminals, each with only one cluster. In Fig. 3B, the presynaptic localization of this cluster is particularly clear, as the cluster is located near Ω-shaped structures (arrowheads) that may indicate fusing vesicles or endocytotic structures. We used 6 animals with 11 hemisegments for the mAb. In 34 terminals, we found staining with mAb, containing 44 clusters.

**Western analysis**

Because the mass of muscle tissue and its membranes was much larger than the membranes of the embedded small release boutons, we performed Western analysis on crayfish ganglia, which are rich in synaptic connections and were also immunostained by the mAb and pAb (not shown). We
used crayfish muscles as controls and compared the results with those obtained with rat brain. In each preparation, we compared the staining in the membranal and soluble fractions.

By using the pAb, a single band of \(~150\,\text{kD}\) was detected in the membranal fraction of the crayfish ganglia (cfgp) (Fig. 4). The response was very specific, as staining was not observed in the soluble fraction of the ganglia (cfgs) or in the two fractions of the crayfish muscle (cfm). The membranal fraction of rat brain (rbp) showed intense staining, and as expected the soluble fractions showed no staining.

Similar experiments with the mAb were not successful. No detectable bands were observed in the crayfish membrane fractions when the mAb was used. This may have resulted from the more sporadic binding of the mAb as seen with the immunogold staining (Fig. 3).

The detection of a single band by the pAb together with the immunostaining with both antibodies demonstrate the presence of presynaptic receptors that interact with NMDA in the crayfish axon terminals. It seems that a part of the receptor molecule was conserved during evolution and that the two receptors (crayfish and mammalian) have some structural similarity. The two receptors are certainly not identical, as the crayfish NMDA receptor is somewhat larger (by \(~30\,\text{kD}\)) and has different physiological properties (Parnas et al. 1996).

**The mAb abolishes the physiological effects of NMDA**

Antiglutamate receptor antibodies were used previously for physiological studies. For example, Shigemoto et al. (1994) demonstrated that an ant metabotropic glutamate receptor, anti-mGluR1 polyclonal antibody, blocked the induction of long-term depression of glutamate-induced currents in cultured rat Purkinje cells. We tested whether the polyclonal and monoclonal antibodies block the physiological effects of NMDA.

Figure 5 shows the effects of NMDA on release in treated and untreated muscles. The traces in the top right column show sample recordings. One trace shows a failure in release, and the other two show one and two quanta. Figure 5A shows results obtained in eight synapses from a hemisegment incubated in BSA (3 preparations were also incubated with the preimmune mouse serum). Note that, in all eight synapses, after addition of \(10^{-6}\,\text{M}\) NMDA (down arrow), quantal content declined to values \(<50\%\) of the control (before addition of the NMDA). After the preparations were washed (up arrow), quantal content continued to decline for two to three points and then recovered. These results are similar to those obtained by Parnas et al. (1996) in muscles that underwent no treatment. This indicates that the BSA, like the NGS and the mouse preimmune serum (important as a control for the mAb effects), did not affect the ability of NMDA to reduce release. We recorded from a total of 26 synapses from 5 muscles without antibodies (pooled results of 3 muscles incubated with BSA and preimmune serum and 2 muscles incubated with NGS). In all synapses, \(10^{-6}\,\text{M}\) NMDA reduced release to \(<50\%\) of the control. Recovery, after washing of the NMDA, reached \(\geq 85\%\) of the control.

Similar results were obtained in muscles treated with the...
The hemisegments were treated with 5-, 15-, or 50-μg/ml solutions. The recordings from muscles treated with 50 μg/ml deteriorated rapidly. We therefore limited our measurements to hemisegments treated with 5 or 15 μg/ml and did not investigate further the problem of deterioration. Figure 5B shows the quantal content obtained in eight synapses from a hemisegment treated with 15 μg/ml pAb. As in muscles not treated with antibody, addition of 10⁻⁶ M NMDA reduced quantal content with good recovery after washing. We tested the effect of NMDA on 16 synapses from pAb-treated muscles. Quantal content was always reduced by >50%, with complete recovery after washing of the NMDA. Similar results were obtained in eight synapses in a hemisegment treated with 5 μg/ml pAb. Thus, at these concentrations, the polyclonal antibody did not block the presynaptic effects of 10⁻⁶ M NMDA.

A different result was obtained in synapses from the mAb-treated muscles (Fig. 5C). We tested hemisegments incubated with 5-, 15-, and 50-μg/ml mAb solutions. Synapses from hemisegments treated with 5 μg/ml mAb remained sensitive to 10⁻⁶ M NMDA. Quantal content declined in a manner similar to that shown in Fig. 5, A and B. In eight such synapses, release was reduced to levels 50% lower than the control, with recovery after washing (not shown). When other hemisegments were incubated with 15 μg/ml (same batch of mAb), application of 10⁻⁶ M NMDA was ineffective in reducing quantal content. Figure 5C shows results of eight such experiments. It is clear that quantal content fluctuated around the control level even after NMDA was added. In preparations washed for very long periods of ≥2 h after removal of the antibody, NMDA was still ineffective; the blocking effect of the mAb was irreversible. Similar results were obtained from muscles treated with 50 μg/ml mAb. We tested the effect of NMDA on a total of 23 synapses from mAb-treated hemisegments (8 from muscles treated with 15 μg/ml and 15 from muscles treated with 50 μg/ml). Only in 2 of 15 synapses from hemisegments treated with 50 μg/ml mAb did 10⁻⁶ M NMDA reduce release. In the other 21 synapses, 10⁻⁶ M NMDA was ineffective in reducing release.

These results show that synapses from hemisegments treated with BSA + preimmune serum, NGS, and pAb (the hemisegments were incubated for the same period of time as with the mAb) were sensitive to NMDA, whereas synapses from mAb-treated muscles were insensitive to NMDA.

mAb did not block the physiological effect of glutamate

The presynaptic excitatory terminals of the crayfish neuro-muscular system contain at least two types of glutamate receptors, those activated by NMDA and blocked by (±)-2-amino-5-phosphonovaleric acid (APV) and receptors activated by quisqualate and blocked by CNQX (Parnas et al. 1996).

The finding that the mAb stains presynaptic terminals and also blocks the physiological effect of NMDA suggests that the effect is specific. However, these results do not exclude the possibility that the mAb raised against the mammalian NMDA receptors may also bind to other glutamate receptor types in crayfish axon terminals. It was therefore necessary to test whether the mAb blocks the physiological effect of glutamate.

Figure 5D shows that, for hemisegments incubated with 15 μg/ml mAb, the quantal content declined after addition of 10⁻⁶ M glutamate, with recovery after washing. Similar results were obtained in hemisegments treated with 50 μg/ml mAb (12 synapses from 3 hemisegments; not shown). It should be emphasized that 10⁻⁶ M NMDA did not reduce...
release in synapses of hemisegments where glutamate did. This suggests that, whereas the NMDA type receptors were blocked by the mAb, the quisqualate type receptors were not. These results resemble those obtained in muscles incubated with APV. There too, the effect of NMDA, but not that of glutamate, was blocked by APV (Parnas et al. 1996).

We therefore expected that application of CNQX together with mAb would render the synapses insensitive to glutamate, and this indeed is what occurred (Fig. 5E). Here the hemisegments were incubated with 15 μg/ml mAb. The fluid circulating during the electrophysiological measurements included 10⁻⁶ M CNQX. Under these conditions, 10⁻⁶ M glutamate (down arrow) did not reduce quantal content (6 synapses).

The findings that 15 μg/ml mAb completely abolished the physiological effect of 10⁻⁶ M NMDA, but not that of 10⁻⁶ M glutamate, and that even 50 μg/ml mAb did not abolish the blocking effect of glutamate further reduces the likelihood that the effect of the mAb is nonspecific. The additional finding that the mAb applied together with CNQX did block the effect of glutamate reinforces earlier indications (Parnas et al. 1996) that these two types of crayfish presynaptic glutamate autoreceptors, an NMDA receptor and a non-NMDA receptor, are involved in the feedback inhibition of release.

**DISCUSSION**

We investigated whether the effect of NMDA on glutamate release in the crayfish is achieved by directly activating an NMDA-type receptor located on the presynaptic excitatory terminals.

The finding that the effect of NMDA and glutamate on release depends on the amplitude of the depolarizing pulse (Parnas et al. 1994, 1996) demands that the presynaptic glutamate receptors reside in the presynaptic membrane. Otherwise, how could they be affected by the invading action potential? Our results show that the NMDA-type receptor is indeed located on presynaptic nerve terminals.

Because we did not find any evidence that NMDA-type receptors are located on the muscle or glial cell membranes, the possibility that glutamate inhibits its own release by indirect action is less likely.

Our method of prefixation incubation did not include permeabilization treatments. This is the reason that only the presynaptic membrane was stained and no staining was observed within the terminal, as found, for example, in the cerebral cortex (Debiasi et al. 1996). Under conditions of prefixation incubation, an antibody can bind only to molecules on the external face of the membrane. This method worked for both the mAb and the pAb. The staining was found to be very specific and limited to presynaptic membranes.

The results presented here are surprising for several reasons. First, antibodies raised against mammalian NMDAR1 stained presynaptic terminals of crayfish, although the physiological properties of the crayfish receptor differ from the physiological properties of mammalian receptors. Crayfish receptors do not behave as ionotropic receptors, as their activation is not associated with a conductance increase. Intraterminal Ca²⁺ accumulation is not altered, and it takes several minutes to establish the effect and >20 min to recover after washing (Parnas et al. 1994, 1996). Second, the pAb was reported to be raised against an internal domain of the mammalian receptor (Petralia et al. 1994). Nevertheless, this pAb immunostained crayfish terminals while exposing them to the antibody without permeabilization of the membrane. This means that the pAb recognized an extracellular domain or domains. Binding to these domains did not, however, block the physiological activity of NMDA. It is possible that the crayfish receptor, which has different physiological properties, also differs somewhat in its structure from the mammalian receptor. This possibility is strengthened by the finding that the crayfish receptor is larger. With respect to the mAb, the physiological results agree with current views concerning the recognition site. The mAb was thought to recognize an intracellular loop between transmembrane regions III and IV (Hollman et al. 1989; Keinänen et al. 1990; see Hollmann and Heinemann 1994). However, it has been shown (Hollman et al. 1994) that this domain is external in the ionotropic non-NMDA receptor (GluR1). Because the NMDA receptor has a similar structure (Moriyoshi et al. 1991), it is possible that this domain is external in the crayfish receptor as well.

The physiological effect of the mAb was specific for NMDA. It did not block the effect of glutamate, which also activates quisqualate receptors. This indicates that in the crayfish there are fundamental differences in the structure of the receptor that binds NMDA and the one that binds quisqualate.

Presynaptic NMDA receptors were found in the presynaptic terminals of mammals. Such receptors were found in the spinal cord (Liu et al. 1994), and recently, Conti and colleagues (Conti et al. 1997; DeBiasi et al. 1996) identified presynaptic NMDA receptors in the cerebellar cortex, with the same polyclonal antibody. The physiological role of the presynaptic NMDA receptors in mammals is not known. It is possible that in mammals, as in crayfish, they are involved in inhibition of release of glutamate from nerve terminals.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, SFB 391, Germany. We thank the Anna Lea Foundation for its continuous support. I. Parnas is the Greenfield Professor of Neurobiology.

Received 13 April 1998; accepted in final form 14 September 1998.

**REFERENCES**


Dale, N. and Kandel, E. A. L-Glutamate may be the fast excitatory trans-
Hollmann, M., Maron, C., and Heinemann, S. 
N-glycosylation site tag.


