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Rat Brain *N*-Methyl-D-Aspartate Receptors Expressed in *Xenopus* Oocytes

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N-methyl-D-aspartate (NMDA) activates a class of excitatory amino acid receptor involved in a variety of plastic and pathological processes in the brain. Quantitative study of the NMDA receptor has been difficult in mammalian neurons, because it usually exists with other excitatory amino acid receptors of overlapping pharmacological specificities. *Xenopus* oocytes injected with messenger RNA isolated from primary cultures of rat brain have now been used to study NMDA receptors. The distinguishing properties of neuronal NMDA receptors have been reproduced in this amphibian cell, including voltage-dependent block by magnesium, block by the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid, and potentiation by glycine. This preparation should facilitate the quantitative study of the regulation of NMDA receptor activation and serve as a tool for purification of the encoding messenger RNA.

OF THE THREE EXCITATORY AMINO acid (EAA) receptors identified pharmacologically (*1*), the *N*-methyl-D-aspartate (NMDA) receptor has received the most attention. This is due to its potential roles in long-term potentiation (*2*), hypoxic damage (*3*), and epileptic discharges (*4*); to the availability of selective and potent antagonists such as D-2-amino-5-phosphonovaleric acid (D-APV) (*5*), MK-801 (*6*), and 3(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP+) (*7*); and to its large conductance (45 to 50 pS) (*8, 9*), which facilitates single-channel studies. Indeed, patch clamp experiments have revealed a rich complexity in the activation of NMDA receptors that render it well suited to regulation under different physiological conditions. The open NMDA channel appears to be blocked by Mg^{2+} (*8, 10*) and ketamine (*11*) in a voltage-dependent manner, and the frequency of opening of NMDA channels is greatly increased in the presence of low concentrations of glycine (*12*). Single-channel studies (*9*) raise the possibility that the three pharmacologically identified EAA receptors (for NMDA, kainate, and quisqualate) are not independent entities but may instead reflect the use of a single-channel type by the three receptors. The presence of four or more major conductance states for each agonist; preferential activation of different conductance states by the agonists NMDA, quisqualate, and kainate; frequent transitions among the different conductance states; and selective block of only the highest conductance by D-APV and Mg^{2+} favor this hypothesis of receptor sites that share channel properties. Clearly, to investigate the structural and functional relations among the three EAA receptors it would be advantageous to have a preparation that expresses all three receptors but in which receptor expression could be systematically manipulated.

The *Xenopus* oocyte translation system, originally developed by Gurdon and colleagues (*13*) to study messenger RNA (mRNA) that encodes soluble proteins, has been used to translate mRNA encoding neurotransmitter receptors. We (*14*) and others (*15*) have shown that mRNA from rat or chick brain, when injected into oocytes, readily induces responses to kainate and quisqualate, but heretofore NMDA responses have not been observed. We now report that mRNA extracted from adult rat brain or primary cultures of fetal rat brain (*16*) encodes NMDA receptors when injected into oocytes. Our results were mainly from 19 separate experiments in which six independent mRNA preparations were used. An abstract of some of this work has been published (*17*).

Oocytes injected with mRNA from rat brain were voltage-clamped with two microelectrodes, and drugs were applied by perfusion in medium similar to that used to culture the oocytes. At a holding potential of −60 mV, 100 μ*M* NMDA evoked a smooth, readily reversible, nondesensitizing inward current that ranged from 5 to 220 nA (mean ± SEM, 41 ± 5 nA, $n = 73$ cells). In six mRNA preparations that encoded NMDA receptors, 73 of 94 oocytes tested responded to NMDA, whereas uninjected or water-injected oocytes ($n = 12$), or oocytes injected with eight mRNA preparations that encoded only kainate receptors ($n = 36$), did not respond (<5 nA) to NMDA at up to 300 μ*M* concentration. In seven cells, increasing concentrations of NMDA were sequentially applied to construct a concentration response curve; the curve indicated a single component response with a half-maximally effective concentration (EC_{50}) of 38 ± 8 μ*M* (mean ± SEM). The selective NMDA receptor antagonist D-APV (10 μ*M*) reduced the amplitude of the ionic current evoked by 100 μ*M* NMDA by 83 ± 1% ($n = 6$ cells) (Fig. 1A).

Current-voltage curves of NMDA responses constructed in the presence of 1 m*M* Mg^{2+} showed a marked nonlinear region between −80 and −30 mV, similar to results obtained with neuronal NMDA receptors (*8, 10*) (Fig. 1B). As expected, the nonlinear behavior was nearly eliminated when Mg^{2+} was omitted from the perfusion

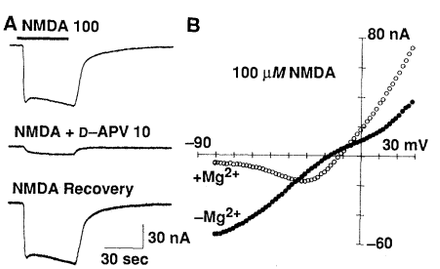

Fig. 1. Block of NMDA-evoked current by D-APV and Mg^{2+}. (**A**) An oocyte injected with approximately 50 ng of mRNA 48 hours before was voltage-clamped with two microelectrodes to a holding potential of −60 mV and superfused continuously with modified Barth's solution (*16*) ($MgSO_4$ replaced by Na_2SO_4 and supplemented with 0.5 m*M* $CaCl_2$ plus 3 μ*M* glycine). During the periods indicated by the solid bar in (A), the superfusion solution was changed to include 100 μ*M* NMDA with or without previous exposure to 10 μ*M* D-APV, as indicated. The input resistance of this cell, measured at its resting potential (−70 mV), was 1.2 Mohm. (**B**) In a different cell the ionic current produced by 100 μ*M* NMDA was plotted as a function of voltage in the absence (●) and presence (○) of 1 m*M* Mg^{2+}. The perfusion solution also contained 3 μ*M* glycine. Current-voltage curves were constructed by ramping the voltage slowly (55 mV per second) from −80 to +30 mV in the presence and absence of 100 μ*M* NMDA. Under these conditions the capacitive current associated with the ramp was small. Leakage and capacitive currents at each voltage were then subtracted from the membrane currents measured in the presence of NMDA. The resting potential and input resistance of this cell were −50 mV and 0.6 Mohm, respectively.

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fluid. The ratio of chord conductances at −60 and −30 mV was 0.13 ± 0.02 in Mg^{2+} but 0.91 ± 0.06 without Mg^{2+} (n = 5), which attests to the strong voltage-dependent block by Mg^{2+} of NMDA receptors expressed in oocytes. Another measure of the voltage dependence of NMDA currents is the rectification ratio (*10*) of chord conductances at +20 and −70 mV, which was 37 ± 18 (range, 16 to 98) in Mg^{2+} and 0.44 ± 0.14 (range, 0.15 to 0.78) in the absence of Mg^{2+}. The NMDA current-voltage relation was similar although not identical to that reported in neurons. A smaller NMDA chord conductance was noted consistently in the depolarizing limb of the current-voltage curve in the absence of Mg^{2+}, and the reversal potential was slightly more negative (−12 ± 4 mV, n = 7) than in neurons. It is not known if these discrepancies between oocytes and neurons reflect differences in post-translational processing of the NMDA receptor in the two cell types, or if they are merely consequences of differences in ionic gradients in the two cells or differences in experimental conditions (*18*).

Johnson and Ascher (*12*) discovered that the opening rate of NMDA channels studied by patch clamp in cultured neurons was increased by 1 μ*M* glycine. This action of glycine was not blocked by 10 μ*M* strychnine, was not mimicked by most other amino acids, and appears to reflect a novel allosteric facilitation of NMDA receptor activation. We found that in mRNA-injected oocytes, 3 μ*M* glycine potentiated the current produced at −60 mV by 30 to 100 μ*M* NMDA by 4- to 15-fold or more (n = 11) (Fig. 2, inset). Full concentration response curves for NMDA were constructed in the presence and absence of 3 μ*M* glycine in three cells. The maximum current evoked by NMDA was increased 9.0 (± 3.3)-fold by glycine, without a marked lateral shift of the NMDA concentration response curve (Fig. 2). This result indicates that the increase in opening rate of NMDA channels in the presence of glycine (*12*) was probably not due to an increase in the number of successful agonist-receptor interactions, which would have shifted the curve to the left, but instead was due to an increase in the probability of opening of the NMDA-receptor-glycine complex. Our results are consistent with the single-channel records of Johnson and Ascher (*12*), but are at apparent variance with those of Murphy *et al.* (*19*), who found that glycine increased the potency of NMDA for elevating cytoplasmic-free Ca^{2+} in cultured striatal neurons. Although the reason for this apparent discrepancy is not understood, fluctuations in the concentration of endogenous glycine or other factors in the medium bathing cultured neurons (*19*) may have influenced the action of exogenous glycine.

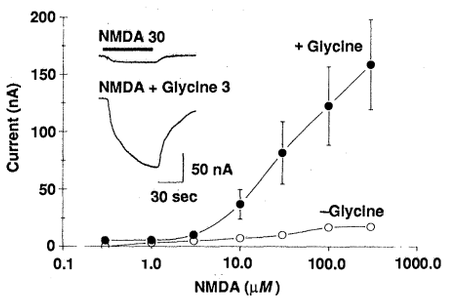

Fig. 2. Potentiation of NMDA-evoked current by glycine. An mRNA-injected oocyte was voltage-clamped to −60 mV and perfused with modified Barth's solution containing no added Mg^{2+}. During the periods indicated by the solid bar (inset) the perfusion solution was changed to include 30 μ*M* NMDA with or without previous exposure of the oocyte to 3 μ*M* glycine, as indicated. The input resistance of this oocyte was 0.8 Mohm at its resting potential (−53 mV). The mean (± SEM) concentration response curve for NMDA-evoked membrane current was measured at −60 mV in the presence and absence of 3 μ*M* glycine (n = 3 oocytes). The standard errors of the response in the absence of glycine were smaller than the symbol size. Although the magnitude of the NMDA response in the presence of glycine was variable from cell to cell, the potentiation of the maximum effect by glycine was clear.

Unexpectedly, washing out the Mg^{2+} in glycine-containing medium produced a steady inward current in 21 of 133 mRNA-injected cells. This current was partially to fully reversible by 10 μ*M* D-APV in four of five oocytes tested, and so was presumably mediated in part by NMDA receptors. Only 2 of 14 uninjected or water-injected oocytes responded similarly, and in neither of these cells was the current blocked by D-APV. The origin of this NMDA receptor response in oocytes is unclear, but it may be due to release of an NMDA receptor agonist such as glutamate from follicle cells or the oocytes themselves.

Our results demonstrate that neuronal NMDA receptors that possess both of the native regulatory sites (for Mg^{2+} and glycine) are expressed in *Xenopus* oocytes injected with the appropriate mRNA. Further, the potentiation by glycine is due to an increase in maximum NMDA response rather than to an increase in agonist affinity. The mRNA preparations that encoded NMDA receptors also encoded kainate receptors, but other mRNA preparations encoded only kainate receptors (*14*). The ability to make mRNA preparations that encode only one type of EAA receptor in oocytes indicates that the three receptor subtypes can operate independently, and we anticipate that each will eventually be studied in isolation. Although some aspects of the NMDA conductance mechanism in oocytes remain unresolved, this preparation lends itself to quantitative studies of the regulation of NMDA receptor activation. It should also prove useful for the development of novel agonists and antagonists of the allosteric and agonist binding sites and for the purification of mRNA encoding these receptors.

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16. Primary cultures were prepared from whole brain (minus cerebellum) of 18- to 19-day-old fetal rats as described for hippocampal cultures [D. B. Hoch and R. Dingledine, *Dev. Brain Res.* **25**, 53 (1986)]. Cells were plated into 75-cm^2 polylysine-coated flasks and fed with serum-containing medium for 7 to 10 days, then rinsed with minimum essential medium. RNA was extracted by a variation of the Chirgwin [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)] method. A solution of 4*M* guanidinium thiocyanate (Fluka), 25 m*M* sodium citrate, *p*H 7, 0.1% antifoam (Sigma), 0.5% sodium lauroyl sarcosine (Sigma), and 0.56% β-mercaptoethanol was flowed onto the cultures and allowed to lyse the cells. No scraping or homogenization was done. Alternatively, whole brain from adult rats was homogenized in the same solution. The resulting mixture was then layered over 5.7*M* CsCl plus 25 m*M* sodium citrate, *p*H 5, and centrifuged at 83,000*g* for 18 hours to pellet total RNA. The RNA pellet was extracted once with phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (24:1), then mRNA was selected by one round of oligo(dT) cellulose chromatography [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)]. Messenger RNA was dissolved in water and stored in aliquots at −70°C until used. Stage V or VI oocytes were manually dissected from *Xenopus*, microinjected with 40 to 80 ng of mRNA, and cultured in modified Barth's medium for 1 to 4 days before assay. Oocytes were studied in which the follicle cell layer was left intact or was removed by treatment for 1 to 2 hours with type 1A collagenase (2 mg/ml) (Sigma) in Ca^{2+}-free medium. Results were the same in either case. The composition of the

culture medium was 88 m*M* NaCl, 1.0 m*M* KCl, 2.4 m*M* $NaHCO_3$, 10 m*M* Hepes, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.91 m*M* $CaCl_2$, penicillin (0.01 mg/ml), and streptomycin (0.01 mg/ml); the *p*H was 7.3 to 7.4.

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18. The measured value of the reversal potential would be in error by the amount of the liquid junction potential arising at the tip of the voltage-sensing electrode as it penetrated an oocyte. The value of the liquid junction potential is likely to be at least several millivolts, because a potential of up to about −10 mV (typically −2 to −3 mV) could be observed on withdrawing the pipette, and this potential often decayed toward zero over several minutes as the electrode sat in the bathing fluid. It is unclear if the rectification in the positive limb of the current-voltage curve in Mg^{2+} (Fig. 1B) represents an actual difference from the properties of neuronal NMDA receptors, because a similar phenomenon has been observed in neurons (M. L. Mayer, personal communication).
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Autoreactive Epitope Defined as the Anticodon Region of Alanine Transfer RNA

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Autoantibodies to aminoacyl–transfer RNA (tRNA) synthetases are common in the human autoimmune diseases polymyositis and dermatomyositis. Sera of the PL-12 specificity contain separate antibodies reacting with alanyl-tRNA synthetase and alanine tRNA ($tRNA^{Ala}$). The antibodies to tRNA recognize at least six distinguishable human $tRNA^{Ala}$ species grouped into two sequence families. The antibody-reactive determinants on the tRNA were identified through ribonuclease protection and oligonucleotide binding experiments. The antibody binding site is a seven- to nine-nucleotide sequence containing the anticodon loop and requires an intact anticodon. No requirement for anticodon stem structure or sequence is observed, although the 5′ portion of the stem is protected from nuclease attack. Antibodies from several patients appear to share the same specificitym, indicating that the antibodies are induced by a unique sequence feature in the immunogen.

NATIVE NUCLEIC ACIDS DO NOT stimulate significant antibody production when injected into experimental animals, but such antibodies can be generated against nucleic acid in association with protein (*1*) and are common in human systemic lupus erythematosus (SLE) and its murine equivalents. In these diseases, where the nature of the immunogen is unknown, characterization of the reactive epitopes may provide clues to the identity of potential etiologic agents. Antibodies to DNA are particularly prevalent in SLE and have been extensively investigated. They are heterogeneous and can recognize numerous features, such as heterocyclic bases, nucleosides, nucleotides, simple repeating sequences, and the configuration of the sugar-phosphate backbone (*2, 3*). Similarly, RNA molecules, including ribosomal, transfer, and U1 species, are the target of the autoimmune response in up to 30% of patients with autoimmune disorders (*4–9*). Here both sequential and conformational antigenic determinants are thought to occur, but they are less well defined than those in DNA. Three antibody systems that involve transfer RNA (tRNA) occur in patients with myositis, an inflammatory muscle disease. In two such systems, known as Jo-1 and PL-7, the antigenic components are associated protein molecules, the aminoacyl-tRNA synthetases specific for histidine (Jo-1) and threonine (PL-7) (*7*). In the third system, PL-12, antibody recognizing alanyl-tRNA synthetase coexists with another antibody that directly recognizes alanine tRNA ($tRNA^{Ala}$) (*8*). This unusual interaction of an autoantibody with a specific set of tRNA species has allowed us to identify the antibody binding site (epitope) of an autoantigen.

The RNA antigen precipitated by the PL-12 antibody is predominantly, if not exclusively, $tRNA^{Ala}$, and other sera containing antibodies to DNA, RNA, or ribonucleo proteins (RNPs) do not precipitate these molecules (*6, 8, 9*). We fractionated the tRNA in the precipitate and determined the primary nucleotide sequences of two related species and the anticodon stem and loop sequence of a third species (*9*). The antibody recognizes two families of human $tRNA^{Ala}$, named "slow" and "fast" for their relative electrophoretic mobility in denaturing polyacrylamide gels. They contain the same anticodon loop sequence but differ in the anticodon stem region and in other parts of the molecule. To determine whether the binding of antibody could confer resistance to ribonuclease digestion, we formed immune complexes between uniformly radiolabeled tRNA and the PL-12 antibody from several patients. The complexes were isolated on protein A–Sepharose and digested with ribonuclease T_1, ribonuclease A, or a mixture of both. After the matrix had been washed to remove released digestion products, the residual bound RNA was eluted. In all cases, gel electrophoresis revealed the presence of protected fragments ~20 nucleotides in length.

Using antibody from one patient and $tRNA_1^{Ala}$, a purified slow RNA, we isolated the fragments protected from digestion by either ribonuclease T_1 or A. Further digestion to completion with these enzymes followed by a two-dimensional electrophoretic separation of the resultant oligonucleotides (*10*) yielded simple "fingerprint" patterns on autoradiography. The oligonucleotides were isolated and characterized by redigestion, and their locations in the molecule are shown in Fig. 1A, lines a and b. The protected region includes the anticodon stem and loop. The same result was obtained with $tRNA_2^{Ala}$, another member of the slow family, and with $tRNA_3^{Ala}$, which belongs to the fast family and contains two pairs of base changes in the anticodon stem. This implies that the stem sequence is not critical for antigenicity but leaves open the possibility that a duplexed stem structure is required.

In the naked state, the anticodon loop is exposed and susceptible to nuclease attack, so its presence in the protected fragment is particularly striking. To confirm that the antibody blocks digestion in this region and to obtain more detailed definition of the binding site, we performed "footprint" experiments to reveal nuclease-sensitive sites that are shielded by antibody. Immunoprecipitated $tRNA^{Ala}$ was labeled at its 3′ end, and individual species were isolated by gel electrophoresis (*9*). The $tRNA_2^{Ala}$ species was subjected to mild ribonuclease digestion in the presence or absence of PL-12 antibody and analyzed by gel electrophoresis (Fig. 2). The antibody blocked digestion of bonds in the anticodon loop by the single-strand–specific ribonucleases T_1, *Bacillus cereus*, and T_2 (which are specific for G residues, for pyrimidines, and are not base-specific, respectively). Similarly, digestion by the double-strand–specific ribonuclease V_1 of bonds in the stem on the 5′ side of the

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