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Combinatorial RNA splicing alters the surface charge on the NMDA receptor

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Transcripts encoding four NMDA receptor subunits, generated from the NMDAR1 gene by alternative RNA splicing, have been demonstrated in adult rat brain. RNA transcripts derived from cDNAs encoding each form direct the formatio.. of functional NMDA receptors in *Xenopus* oocytes. The two amino acid cassettes of 21 and 37 amino acids found in the splice variants increase the positive extracellular surface charge on the subunits and may thereby modulate the functional properties of the receptor.

Receptor; Channel; Excitatory amino acid; Glutamate; RNA splicing

1. INTRODUCTION

The NMDA receptor mediates the influx of Ca^{2+} and monovalent cations at postsynaptic sites in response to glutamate, the major excitatory transmitter in mammalian brain [1–3]. The NMDA receptor has been implicated in neuronal modulation, including long-term potentiation in the hippocampus [4], and in neuronal pathology, including cell death after cerebral ischemia [5]. The endogenous ligands of the receptor are various and, besides glutamate, include glycine, which is required for channel opening, and Mg^{2+}, which blocks the channel in a voltage-dependent manner [6]. A second divalent antagonist, Zn^{2+}, blocks at a second site [6]. Exogenous modulators of channel activity include neurotoxins [5] and psychoactive agents, such as phencyclidine [6] and ethanol [7]. Recently, a rat cDNA was reported (NMDAR1), which encodes a 105.5 kDa polypeptide capable of forming homo-oligomeric glutamate-activated channels in oocytes that exhibit many of the characteristics expected of a bona fide NMDA receptor [8]. We now demonstrate the existence of three additional receptor subunits generated from the same gene as NMDAR1 by alternative RNA splicing.

The nucleotide sequences presented here have been submitted to the EMBL/GenBank database under the accession number X65227.

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2. MATERIALS AND METHODS

2.1. *Sequence analysis of NMDAR1 cDNAs*

NMDAR1 cDNAs were obtained from rat brain poly(A)$^+$ RNA by RT-PCR. The 5′ coding region was made using the 5′ primer HB248 (5′ GCGCGGATCCAAGCTTAGCTCATGAGCACCATGCACCTGCTG) and the 3′ primer HB252 (5′ CTGGTATCCTCGAGGTTTCTCTG). The 5′ primer contains *Bam*HI and *Hin*dIII sites for subcloning and encompasses the initiation codon and five untranslated nucleotides [8]. The 3′ primer spans the *Xho*I site at nucleotide 1230. The 3′ coding region was made using the 5′ primer HB251 (5′ GAGAAACCTCGAGGATACCAG) and the 3′ primer HB250 (5′ GCGCGGATCCTCAGCTCTCCCTATGACGGG). The 5′ primer spans the *Xho*I site at nucleotide 1230. The 3′ primer contains a *Bam*HI site for subcloning and encompasses the termination codon of NMDAR1 and one untranslated nucleotide.

Rat brain poly(A)$^+$ RNA (100 ng; Clontech) was mixed with pdN_6 random primers (100 pmol; Pharmacia) and denatured at 65°C for 5 min. Reverse transcription (200 U MMLV transcriptase; Gibco-BRL) was performed at 42°C for 1 h in 20 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, containing 16 U RNasin (Pharmacia). The resulting cDNA was heat denatured at 94°C for 2 min in 100 μl of PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100) containing 100 pmol of each specific primer, 200 μM dNTPs and 2.5 U of Taq polymerase (Promega), prior to amplification using the following program: 95°C, 0.8 min; 55°C, 1.5 min; and 72°C, 3 min. After 40 rounds of amplification an additional 10 min were allowed for extension at 72°C.

For sequencing, the PCR products were cut with *Hin*dIII and *Xho*I (5′ half) or *Xho*I and *Bam*HI (3′ half) and ligated into Bluescript KS(−). The sequences of all four inserts were determined on one strand by dideoxy chain termination with modified T7 DNA polymerase (Sequenase, USB) and a series of strategically placed primers. In this paper the first nucleotide of the initiation codon is assigned position 1 and the NMDAR1-LL coding sequence is numbered continuously 1 through 2877. The sequences were identical to that reported [8] except for a 63 nucleotide insertion (cassette 1) at positions 571–633 in the L version of the 5′ coding sequence and a 111-nucleotide deletion at positions 2653–2763 (cassette 2) in the S version of the

3′ coding sequence. The sequence of cassette 1 was determined on both strands in three independent clones.

2.2. *NMDAR1 transcripts in adult rat brain*

Transcript levels were quantitated by RT-PCR as described above. It was essential to eliminate DNA heteroduplexes [9] by diluting the PCR products 10- to 100-fold and carrying out three further rounds of amplification. In the experiment shown, the amplification products were transferred to a nylon membrane and hybridized at high stringency to a ^{32}P-labeled NMDAR1-SS probe generated by random priming [10].

2.3. *In vitro synthesis of RNA transcripts*

Full-length NMDAR1-LL, NMDAR1-LS, NMDAR1-SL, and NMDAR1-SS cDNAs were assembled in the transcription vector pSP64(polyA)-N. 5′ PCR products (Fig. 1) were cut with *Hin*dIII and *Xho*I and 3′ PCR products with *Xho*I and *Bam*HI before a three-component ligation with *Bam*HI/*Hin*dIII cut pSP64(polyA)-N. The vector is a derivative of pSP64(polyA) (Promega) in which a *Not*I site has been introduced at the *Eco*RI site immediately 3′ of the polyA sequence. Polyadenylated RNA transcripts were synthesized in vitro from *Not*I-linearized template using SP6 RNA polymerase in the presence of m^{7}G(5′)ppp(5′)G.

2.4. *Electrophysiological measurements*

Stage VI *Xenopus* oocytes were defolliculated with collagenase (Sigma type 1A), injected with 10 ng of RNA (0.2 ng/nl) and maintained in culture at 18°C for at least two days. Recordings were made under two-electrode voltage-clamp at a holding potential of −80 mV and filtered at 100 Hz. All drugs were dissolved in ND-96 medium, lacking Mg^{2+} and supplemented with 10 μM glycine, and perfused through the recording chamber (0.3 ml) for 1 min at 3 to 4 ml/min. NMDA was presented at 100 μM. 10 μM D-APV, 100 μM Mg^{2+} and 100 μM Zn^{2+} were each delivered simultaneously with 100 μM NMDA. Between drug presentations, the chamber was flushed with the modified ND-96 medium. The initial spike was variable and may reflect the magnitude of a Ca^{2+}-activated chloride current endogenous to the oocyte.

3. RESULTS AND DISCUSSION

Primers based on the NMDAR1 sequence [8] were used to generate cDNAs from rat brain poly(A)$^+$ RNA by reverse transcription followed by the polymerase chain reaction (RT-PCR). The two halves of the NMDAR1 coding sequence were amplified separately and both yielded doublets upon agarose gel electrophoresis and ethidium staining (data not shown). DNA sequencing revealed that both components of both doublets are derived from the NMDAR1 gene. The more slowly migrating cDNAs arise from a 63 nucleotide insert at the 5′ end of the coding region (cassette 1) and a 111 nucleotide insert near the 3′ end (cassette 2). These additional sequences most probably arise through alternative RNA splicing utilizing exon cassettes or alternative donor or acceptor sites [11]. Intron retention [11] is unlikely for several reasons including the lack of consensus splice signals positioned to yield transcripts from which the faster migrating cDNAs would be derived.

The data suggested that there are four possible NMDAR1 transcripts: NMDAR1-LL, NMDAR1-SL, NMDAR1-LS, and NMDAR1-SS (Fig. 1), where NMDAR1-SL is that originally characterized by Moriyoshi and colleagues [8]. Because the same pair of PCR primers can be used to amplify the L (long) and S (short) forms of the 5′ half of the coding region, the RT-PCR data is semiquantitative and shows that in adult rat brain transcripts lacking cassette 1 are about 4 times more abundant than transcripts that contain them (Fig. 2). Similarly, transcripts containing cassette 2 are about 1.5 times more abundant than those that lack them (Fig. 2). If cassette 1 and cassette 2 (Fig. 1) arise from independent splicing events, these results imply that NMDAR1 subunit mRNAs should be present in rat brain RNA at ratios of LL/SL/LS/SS of about 3:12:2:8. RT-PCR of the entire coding region with the primers HB248 and HB250 revealed that all four splice

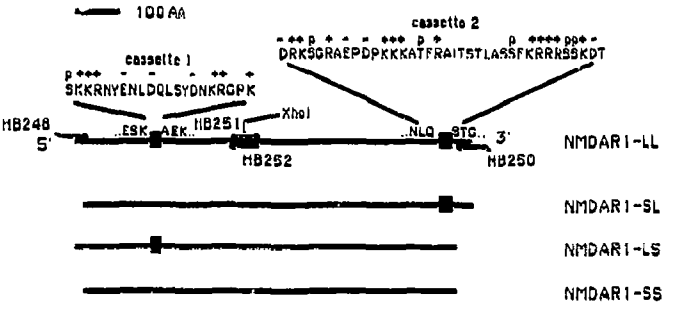

Fig. 1. Schematic showing the predicted structures of the four polypeptides derived from the NMDAR1 splice forms. The positions of the PCR primers used to amplify segments of the transcripts are indicated by arrows. The DNA sequences of the cDNAs were identical to that reported for NMDAR1-SL [8] except for a 63 nucleotide insertion (cassette 1) at positions 571–633 in the L version of the 5′ coding sequence and a 111 nucleotide deletion at positions 2653–2763 (cassette 2) in the S version of the 3′ coding sequence. Key: p, potential phosphorylation site within a cassette assigned using the program FEATURES (GCG Package, Version 7.0); +, positively charged residue; −, negatively charged residue.

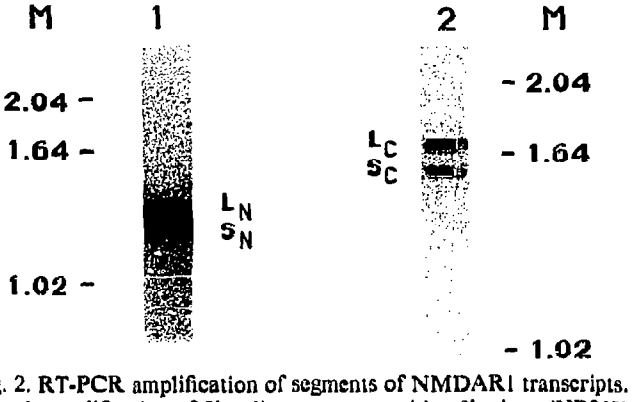

Fig. 2. RT-PCR amplification of segments of NMDAR1 transcripts. Lane 1: amplification of 5′ coding sequences with a 5′ primer (HB248) encompassing the initiation codon and a 3′ primer (HB252) encompassing the *Xho*I site at nucleotide 1230; lane 2: amplification of 3′ coding sequences using a 5′ primer (HB251) encompassing the *Xho*I site at nucleotide 1230 and a 3′ primer (HB250) encompassing the termination codon; M, markers. Assignments: L_N, amplified DNA encoding the 5′ coding region and containing cassette 1; S_N, amplified DNA encoding the 5′ coding region without cassette 1; L_C, amplified DNA encoding the 3′ coding region and containing cassette 2; S_C, amplified DNA encoding the 3′ coding region without cassette 2.

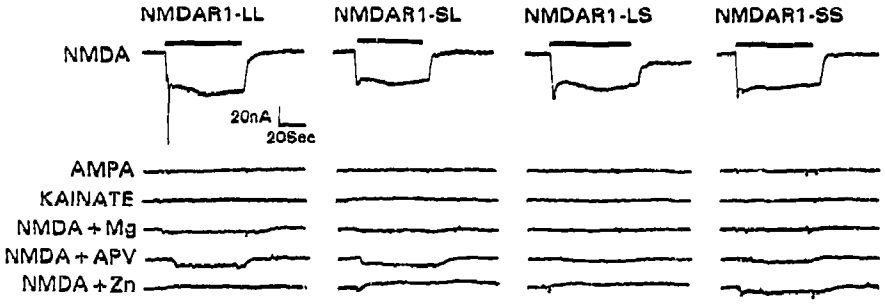

Fig. 3. Electrophysiological and pharmacological characterization of NMDAR1 splice forms expressed in *Xenopus* oocytes. The membrane potential was held at −80 mV. The duration of drug application is indicated by a bar. Although desensitization was often evident, the traces shown are from oocytes that did not exhibit desensitization during exposure to NMDA.

forms are indeed expressed in approximately the predicted ratios (data not shown).

In vitro synthesized RNA transcripts encoding the splice variants were injected into *Xenopus* oocytes and the pharmacological profiles of the resulting, presumably homo-oligomeric channels were examined. All four showed characteristics of functional NMDA receptors. Inward current was activated by 100 μM NMDA but not by α-amino-3-hydroxy-5-methyl-4-isooxazolepropionate (AMPA) and kainate, which activate non-NMDA glutamate receptors (Fig. 3). The effect of NMDA was blocked by: (1) 10 μM D-(−)2-amino-5-phosphonovalerate (D-AVP), a competitive antagonist; (2) 100 μM Mg^{2+}, or (3) 100 μM Zn^{2+} (Fig. 3 and Table I). Receptors with similar characteristics were obtained after simultaneous injection of all four RNA splice forms (Table I).

A working model for the NMDA and the related AMPA and kainate receptors [8,12,13], based on hydrophilicity plots and sequence homology with the better characterized nicotinic acetylcholine (ACh) receptor [14], places both amino acid cassette 1 and cassette 2 on the extracellular surface of the cell membrane (Fig. 4). A second model for ligand-gated channels [14–16] also places the two cassettes on the outside of the cell. One striking feature of the proposed models is the asymmetric distribution of side-chain charges with respect to the lipid bilayer and the significant accentuation of this asymmetry by the addition of either or both amino acid cassettes (Fig. 4). The net increase in positive charge produced by the two cassettes is 11 units (or 55 for a functional pentamer by analogy with the ACh receptor [14]. The net positive charge density and polarity of the NMDAR1 subunits are far higher than those previously

Table I

Pharmacological profile of NMDAR1 variants

	100 μM NMDA (nA)	50 μM AMPA	500 μM Kainate	10 μM Glutamate	NMDA+ 100 μM Mg^{2+}	NMDA+ 10 μM D-APV	NMDA+ 100 μM Zn^{2+}
		Percent of NMDA response					
NMDAR1-LL (n=17)	31.4 ± 4.9	0.0 ± 0.0	0.0 ± 0.0	194.3 ± 15.3	7.3 ± 0.9	11.5 ± 3.4	7.4 ± 5.8
NMDAR1-SL (n=12)	15.6 ± 3.4	0.0 ± 0.0	0.0 ± 0.0	190.0 ± 19.5	6.2 ± 3.7	16.1 ± 6.1	0.0 ± 0.0
NMDAR1-LS (n=7)	20.6 ± 3.9	0.0 ± 0.0	0.0 ± 0.0	191.1 ± 15.2	17.2 ± 2.8	19.8 ± 1.5	0.0 ± 0.0
NMDAR1-SS (n=14)	14.4 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	151.8 ± 14.9	3.9 ± 3.8	36.2 ± 10.0	7.3 ± 7.3
SS+SL+LS+LL (n=4)	21.8 ± 5.6	0.0 ± 0.0	0.0 ± 0.0	183.1 ± 5.3	8.0 ± 3.4	14.3 ± 1.4	0.0 ± 0.0

Steady-state currents elicited by 100 μM NMDA in the absence and presence of NMDA receptor antagonists, and in the presence of the non-NMDA glutamate agonists, AMPA and kainate. NMDA responses are in nA, while all other values are percentages of the NMDA response. Values in parentheses represent the number of oocytes tested for NMDA response. All other values listed represent measurements from three to seventeen oocytes. Currents elicited by NMDA in NMDAR1-LL were significantly (P<0.05) larger than those seen in NMDAR1-SS and NMDAR1-SL.

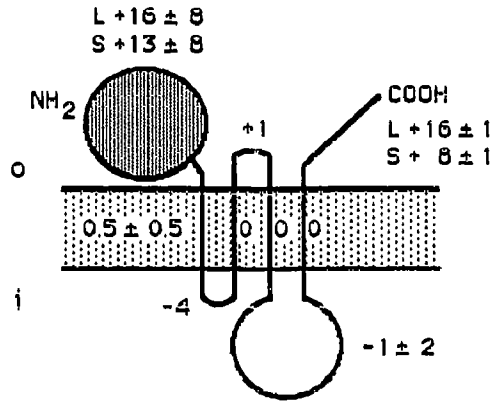

Fig. 4. Arrangement of the NMDAR1 subunits based on a structural model derived from hydrophilicity plots and studies of the nicotinic acetylcholine receptor [8,12,13]. The extracellular (o) and intracellular (i) sides of the bilayer are marked. The net charges on the extramembraneous and transmembrane segments are shown. The L forms of the N- and C-terminal extracellular domains contain the 21 and 37 amino acid cassettes (Fig. 1), while the S forms lack them. Glu, Asp and the C terminus were assigned charges of −1, Lys, Arg and the N-terminus charges of +1, and His a titratable charge of 0 to +1 (i.e. 0.5 ± 0.5) [17]. In contrast to the nicotinic ACh and $GABA_A$ receptors [17], the overall external charge due to the polypeptide side chains of NMDAR1 is of the same sign as the inwardly translocated ions. The external positive charge may be offset to some extent by negatively charged oligosaccharide appendages. However, the charge differences arising through alternative splicing would be unaffected, as the cassettes contain no potential glycosylation sites. The high density of histidine residues near the N terminus of the NMDAR1 subunit is unaffected by splicing but is expected to contribute to the pH dependence of the external charge and perhaps to Zn^{2+} binding.

noted for ACh and $GABA_A$ receptor subunits and for a voltage-gated Na channel [17].

Protein charge produced by amino acid side-chains [18], glycosylation [19] and phosphorylation [20] can influence channel properties through direct local field effects or through induced conformational changes. Therefore, in the case of the NMDA receptor, conductance, ion selectivety, the affinity and effects of charged ligands, and voltage-dependent properties such as Mg^{2+} block might be affected by RNA splicing. But, because the assignment of the transmembrane segments is not certain, it will be important to confirm the topology of the NMDAR1 subunits by experiment. If one or both of the cassettes were located inside the cell, potential phosphorylation sites [21] would be presented to the cytoplasm (Fig. 1). In this case, phosphorylation, as well as charge distribution, might be modulated by splicing.

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NOTE ADDED IN PROOF

A mouse NMDA receptor subunit corresponding to rat NMDAR1-SS has recently been reported by Yamazaki et al. [FEBS Letters (1992) 300, 39–45].