

NMDA receptors in cultured radial glia

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Received 13 January 1997

Abstract The expression of the NMDA subtype of glutamate receptors was investigated by Western blot analysis and RT-PCR in cultured chick Bergmann and Müller glial cells. Using subunit-specific antibodies directed to the carboxy terminus of the rat NMDAR2A/B we detected the expression of the NMDAR2 subunit in both kinds of culture. The functional subunit of the NMDA receptor, NMDAR1, was detected by means of RT-PCR. These results, together with our previous functional characterization of NMDA receptors in radial glia, provide conclusive evidence for the expression of functional NMDA receptor/channels in Bergmann and Müller glia cells. Our findings strengthen the notion of a modulatory role of glial cells in synaptic transmission.

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Key words: Glutamate receptor; Radial glia; Bergmann glia; Müller glia; NMDA receptor

1. Introduction

Glutamate is the major excitatory neurotransmitter in the vertebrate brain [1]. Consequently, it is implicated in all aspects of synaptic transmission including synaptic plasticity, neurodegenerative disorders and neurotoxicity [2]. Glutamate receptors are classified into two groups: ionotropic and metabotropic receptors. Several sequences that encode metabotropic and ionotropic glutamate receptors have been cloned and their properties studied in heterologous expression systems [3].

Ionotropic receptors are subdivided into two distinct types: receptors for NMDA (*N*-methyl-D-aspartate), and non-NMDA receptors. The biophysical properties of the NMDA receptor include high Ca^{2+} permeability, voltage-dependent Mg^{2+} block and allosteric modulation. Molecular and functional evidence suggests the existence of heteromeric NMDA receptors. To date, two gene families that encode NMDA receptor subunits have been identified in rat brain. One family comprises the NMDAR1 gene and the other the NMDAR2 genes (NMDAR2A–D). Functional NMDA receptors are composed of the NMDAR1 subunit and any one of the four NMDAR2 subunits [3].

Bergmann and Müller glia cells express functional glutamate receptors on their membranes [4,5]. These receptors are linked to Ca^{2+} entry, protein kinase C (PKC) translocation to the membrane, phosphoinositide hydrolysis and increase in DNA binding activity of the activator protein 1 (AP1) [6–8]. Radial glial cells play an important role during development supporting neuronal migration and laminar patterning [9]. Unlike other radial glia cells, Bergmann and Mül-

ler glia are not converted into astrocytes after birth [9] and their role in the adult brain is not clear, but it has been proposed that they could modulate neuronal excitability [10]. In this context, since both cell types surround glutamatergic synapses, the characterization of glutamate receptors in these cells has been the focus of our research. The identity of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/low affinity kainate (KA) receptors has been investigated in radial glia, but the molecular identity of NMDA receptors has not been established yet [11]. Although NMDA receptors in chick radial glia have been functionally characterized [4,8], no expression of the cloned sequences encoding NMDA receptors has been reported in these cells [10]. Recently *in situ* hybridization studies have shown the expression of the NMDA 2B mRNA receptor subunit in rat Bergmann glia [12]. In the present work, we examined the pattern of expression of the NMDAR1 and NMDAR2A or B subunits in both cell types, to define whether a common set of NMDA receptors is expressed in radial glia.

2. Materials and methods

Primary cultures of cerebellar Bergmann glia cells were prepared from 14-day-old chick embryos, whereas for Müller glia cultures 7-day-old chick embryos were used. Both cultures were established as described [4,5]. Total RNA from rat brain, rat liver and from the cultures was isolated according to the method of Chomczynski and Sacchi [13]. Specific oligonucleotides were synthesized according to the published sequence of the duck NMDAR1 subunit [16] and correspond to positions:

Sense	(2143) 5'-TGGCTGCTGGTGGGGCTGTCTG-3' (2164)
Antisense	(3293) 5'-CCTGGGGCGGTGGGGATGATGT-3' (3314)
Nested	(2431) 5'-GCCGGGGGTCGTTGATGCCTGT-3' (2452)

For RT-PCR reactions, first strand cDNA was synthesized using 2 μg of total RNA and 200 U of MMLV reverse transcriptase (Gibco). PCR conditions were 3 μl of cDNA reaction (approx. 0.5 μg), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.1% gelatin, 80 μM each of dATP, dGTP, dCTP, dTTP, 10 pmol of each primer, and 2.5 U of Taq DNA polymerase (Boehringer). Amplification of chick NMDAR1 was done by 35 PCR cycles (95°C 1 min, 62.3°C 25 s, 72°C 1 min). Nested PCR was performed under the same conditions except that the annealing temperature was 61.1°C for 30 s, and that 30 cycles were done. PCR products and plasmids containing NMDAR1 and mGluR1 sequences were resolved by 1% agarose gel electrophoresis, transferred to nylon membranes and hybridized with the PCR nested fragment labeled with digoxigenin-UTP. The blot was hybridized at 50°C and washed twice in 2 \times SSPE 0.1% SDS, followed by two washes in 0.1 \times SSPE 0.1% SDS for 20 min at 60°C. Detection was done according to the manufacturer's instructions (Boehringer) using CDP-start as substrate.

For Western blots, a crude synaptosomal fraction was obtained [14]. Briefly, the cells were harvested and collected by centrifugation for 5 min at 2500 $\times g$. The pellet was homogenized in 10 volumes of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1 mM PMSF, aprotinin 1.8 $\mu\text{g}/\text{ml}$ and leupeptin 1 $\mu\text{g}/\text{ml}$, and centrifuged at 1500 $\times g$ for 5 min. The supernatant was centrifuged at 10 000 $\times g$ for 15 min to obtain the

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P2 fraction. Approximately 80 µg of the preparation was electrophoresed into SDS-polyacrylamide gels and transferred to BioBlot-NC nitrocellulose membranes (Costar). The subunits of the NMDA receptors NMDAR1 and NMDAR2A/B were detected using polyclonal antibodies raised against a synthetic peptides corresponding to the C-terminus of rat NMDAR1 or NMDAR2A/B (Chemicon International Inc.). Immunoreactivity was detected using the ECL chemiluminescence method (Amersham).

3. Results

Chick cerebellar Bergmann glia and retinal Müller cells were chosen for this study for two reasons. First, these two cell types have been successfully cultured in a system lacking neuronal cells, and can be easily obtained in sufficient quantities for RNA and protein analysis. Second, the precise localization of these cells within the central nervous system, i.e. in intimate contact with glutamatergic synapses, provides two unique experimental systems with which the contribution of glial receptors to neuronal electrical activity has been started to be elucidated [5–8], making it imperative to establish the molecular identity of the glutamate receptors involved.

Glia and neurons share the same subtypes of glutamate receptors except for NMDA receptors, about which there is controversy whether they are expressed in glia [10]. In radial glia cultured cells, NMDA elicits changes in IP₃ metabolism [4], binding activity of AP1 [7,8] and PKC translocation to the membrane [4]. In order to detect the expression of NMDA subunits in these cells we first used an immunological approach with commercial anti-NMDAR1 and NMDAR2A/B antibodies. The NMDAR1 subunit could not be detected in Müller or Bergmann glia. In contrast an immunopositive band of 180 kDa was obtained in Bergmann glia cultures using an anti-NMDAR2 A/B antibody (Fig. 1). Note that a faint band of this molecular weight was observed in Müller glia cultures. Since with the anti-NMDAR1 antibody no signal was detected even in P2 fractions of chick brain, we decided to use another strategy to detect the NMDAR1 subunit. For this purpose we synthesized a set of primers that amplify the published sequence of the duck NMDAR1 subunit [15]. These oligonucleotides amplify a fragment of 1172 bp. When total RNA from the cultures was analyzed by RT-PCR with these primers a sharp band of the predicted size was obtained (Fig. 2). To rule out the possibility that under our working conditions DNA was present in our RNA preparations and

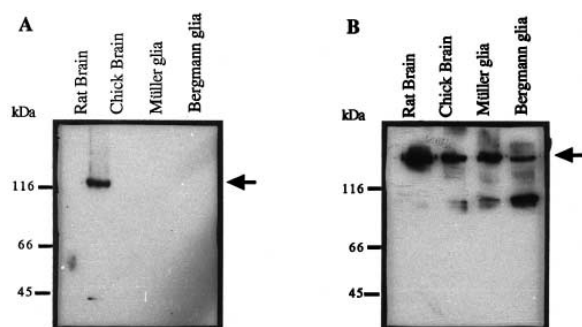


Fig. 1. Western blot analysis of NMDAR1 and NMDAR2A/B polypeptides. Crude synaptosomal fractions were fractionated by 7.5% SDS-PAGE, transferred and analyzed with anti-NMDAR antibodies. A: Immunopositive polypeptides to anti-rat NMDAR1 antibodies. B: Immunopositive polypeptides to anti-rat NMDAR2A/B antibodies.

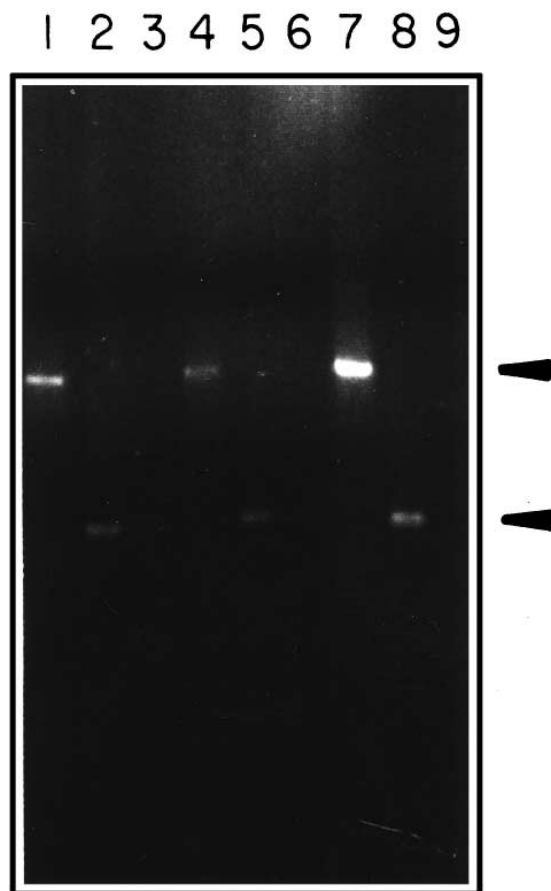


Fig. 2. Expression of the NMDAR1 subunit mRNA in radial glia. cDNA was synthesized from total RNA isolated from Bergmann glial cells (lanes 1–3), Müller glial cells (lanes 4–6) and chick cerebellum (lanes 7–9), and used in PCR reaction. Lanes 1, 4 and 7 are the PCR products using the first set of primers, lanes 2, 5 and 8 are the products of nested PCR using as template the product of the first reaction. Lanes 3, 6 and 9 are controls of PCR reactions using RNA instead of cDNA as template.

could thus serve as template in PCR reactions, we used our RNA preparations directly in the PCR reactions and as expected, no amplification was obtained (Fig. 2). Furthermore, the specific nature of the amplified product was challenged in a nested PCR reaction. The nested primer corresponds to nucleotides 1987–2008 (antisense), these primers reamplify an expected fragment of 328 bp (Fig. 2). To confirm the identity of the 328 bp fragment, restriction analysis was done. Treatment of the PCR fragment with *Bst*NI and *Not*II results in fragments that are of the predicted sizes according to the NMDAR1 duck sequence (Fig. 3). Finally, the 328 bp fragment was used as a probe in Southern blotting, and as depicted in Fig. 4, the nested fragment specifically hybridizes with the rat NMDAR1 plasmid [16] but not with the mGluR1 plasmid [17].

4. Discussion

In this report we describe the expression of the essential subunit of NMDA receptors and of a NMDAR2 subunit (NMDAR2A or NMDA2B) in cultured Bergmann and Müller cells. Although radial glia has been implicated in the migration patterns of neurons toward their defined adult posi-

tions, it has recently become evident that glial glutamate regulates the opening of receptor channels [4,5], activates second messenger cascades [4,6], causes the release of neuroactive substances [18], and induces the binding to DNA of transcription factors [7,8]. Glutamate and glutamate receptors confer on radial glia the ability to receive and emit signals, suggesting a role for these cells in the processing of information in the central nervous system [10]. In order to obtain a better understanding of their putative role in this process it is of relevance to establish the pattern of expression of glutamate receptors.

The NMDAR1 subunit is required for the ensemble of functional NMDA receptors [19]. Using subunit-specific antibodies, NMDAR1 has been detected on rat astrocytes of the visual cortex [20] and recently in human Müller cells [21]. In situ hybridization experiments have revealed transcripts encoding the NMDA2B subunit in Bergmann glia cells [12]. NMDA-induced currents have been observed in mouse Bergmann glia [22], rat hippocampal and cortical astrocytes and in freshly dissociated Müller cells [21]. Previous work in our laboratories has shown that chick radial glial cells express functional NMDA receptors linked to Ca^{2+} entry, PKC activation, IP_3 turnover and AP-1 DNA binding [4,8]. Nevertheless, no molecular identity of these receptors was available. To address this problem, we first tried to detect the different subunits with anti-subunit antibodies. As shown in Fig. 1, no signal was revealed with anti-NMDAR1 antibodies, besides the one of the positive control (rat brain). A plausible explanation for the absence of the NMDAR1 subunit even in chick brain is that the duck and the rat sequences diverge in the carboxy terminus, therefore the antigen is most probably absent in the chick NMDAR1 subunit. We used another antibody to detect a member of the NMDAR2 subfamily. This

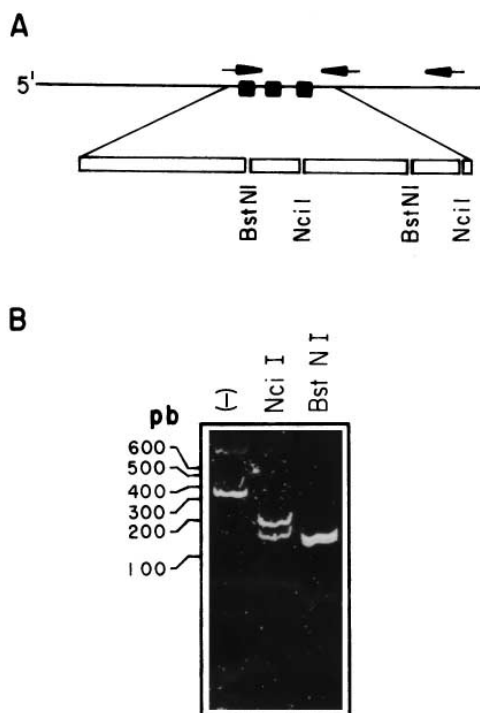


Fig. 3. Identity of the 328 bp nested fragment by restriction analysis. A: Schematic representation of the localization of primers the used for NMDA amplification and restriction map of the nested fragment. B: Products of the restriction analysis.

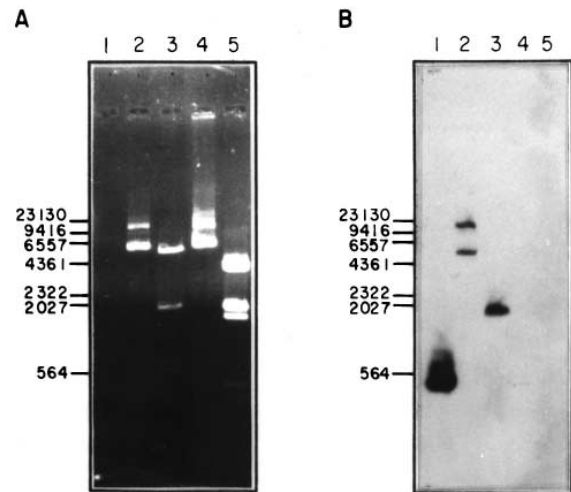


Fig. 4. The 328 bp nested fragment hybridizes with the NMDAR1 sequence but not with the mGluR1. A: Ethidium bromide staining of the gel prior to DNA transfer. B: Southern blot probed with DIG-labeled nested fragment. Lane 1: 328 bp nested fragment; lane 2: rat NMDAR1 plasmid; lane 3: NMDAR1 plasmid restricted with *Pst*I; lane 4: rat mGluR1 plasmid; lane 5: mGluR1 plasmid restricted with *Pst*I. Note that restriction of both plasmids with *Pst*I resulted in excision of the insert.

antibody reacts against NMDAR2A and NMDAR2B subunits. When the membranes were exposed to anti-NMDAR2A/B antibodies a clear immunopositive band of the expected size was observed in Bergmann glia and in Müller glia cells. At this stage we cannot identify the molecular nature of this polypeptide, but the fact that the mRNA of the NMDAR2B subunit has been reported to be expressed in rat Bergmann glia favors the idea that the detected subunit is NMDAR2B.

Using a RT-PCR strategy we were able to detect the NMDAR1 subunit with the primers listed above. We amplified the expected 1172 bp fragment. One could argue that the primers are rich in GC, but a low 3' end stability is predicted when the sequence is analyzed with the OLIGO 4.1 program (National Biosciences Inc.). The oligonucleotides were selected according to this program and are suitable for PCR in stringent conditions.

In order to confirm that this fragment corresponds to the NMDAR1 sequence, we performed a nested PCR. Again, we were able to amplify a fragment of the expected size (328 bp) (Fig. 2). When this fragment was restricted with *Bst*I and *Nci*I the bands obtained were according to the restriction map of the duck NMDAR1 clone. *Bst*I produced 145, 121 and 46 bp fragments, while the fragments expected with *Nci*I are 187 and 137 bp in size. Finally, when we labeled the 328 bp fragment and used it as a probe in Southern blot experiments, we detected that this fragment hybridizes with the rat NMDAR1 sequence, but not with the mGluR1 or the vector sequences.

The similarity between avian and mammalian sequences of glutamate receptors is around 90% [23,24], which at the nucleotide level become 85% [23]. The experiments described in this paper provide conclusive evidence for the expression of NMDA receptors in cultured chick radial glia cells.

Experiments currently in progress in our laboratory are aimed at establishing the signal transduction pathways triggered by NMDA receptors, activation that might be involved in gene expression regulation in radial glia.

Acknowledgements: This work was supported by grants from CONACYT-México to A.O. and A.M.L.-C. We are grateful to Prof. Shigetada Nakanishi for supplying the NMDA and mGluR1 cDNA clones. We also thank the Unidad de Análisis de Biosecuencias y Estructuras of the Departamento de Genética y Biología Molecular CINVESTAV-IPN for using their facilities. The authors acknowledge the technical assistance of Edith López Hernández and Clara Hernández-Kelly.

References

- [1] Watkins, J.C. and Evans, R.H. (1981) *Annu. Rev. Pharmacol. Toxicol.* 21, 165–204.
- [2] Nakanishi, S. (1992) *Science* 258, 597–603.
- [3] Hollmann, M. and Heinemann, S. (1994) *Annu. Rev. Neurosci.* 17, 31–108.
- [4] López-Colomé, A.M., Ortega, A. and Romo-de-Vivar, M. (1993) *Glia* 9, 127–135.
- [5] Ortega, A., Eshhar, N. and Teichberg, V.I. (1991) *Neuroscience* 35, 399–345.
- [6] Cid, M.E. and Ortega, A. (1993) *Eur. J. Pharmacol. Mol. Pharmacol.* 245, 51–54.
- [7] Sanchez, G. and Ortega, A. (1994) *NeuroReport* 5, 1209–1212.
- [8] López-Colomé, A.M., Murbartian, J. and Ortega, A. (1995) *J. Neurosci. Res.* 41, 179–184.
- [9] Cameron, R. and Rakic, P. (1991) *Glia* 4, 124–137.
- [10] Hansson, E. and Rönnebeck, L. (1995) *FASEB J.* 9, 343–350.
- [11] López, T., López-Colomé, A.M. and Ortega, A. (1994) *NeuroReport* 5, 504–506.
- [12] Luque, J. and Richards, G. (1995) *Glia* 13, 228–232.
- [13] Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- [14] Retz, K., Young, A. and Coyle, J. (1982) *Eur. J. Pharmacol.* 79, 319–322.
- [15] Kurosawa, N., Kondo, K., Kimura, N., Ikeda, T. and Tsukada, Y. (1994) *Neurochem. Res.* 19, 575–580.
- [16] Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1991) *Nature* 354, 31–37.
- [17] Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. and Nakanishi, S. (1991) *Nature* 349, 760–765.
- [18] Martin, D. (1992) *Glia* 5, 81–94.
- [19] Monyer, H., Sprengel, R., Schoepfer, R., Herg, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B. and Seeburg, P. (1992) *Science* 256, 1217–1221.
- [20] Steinhäuser, C. and Gallo, V. (1996) *Trends Neurosci.* 19, 339–345.
- [21] Puro, D., Yuan, J. and Nikolaus, S. (1996) *Vis. Neurosci.* 113, 319–326.
- [22] Müller T., Grosche, J., Ohlemeyer, C. and Kettenmann, H. (1993) *NeuroReport* 4, 671–674.
- [23] Ottinger, H.P., Gerfin-Moser, A., Del Principe, F., Dutly, F. and Streit, P. (1995) *J. Neurochem.* 64, 2413–2426.
- [24] Paperna, T., Lamed, Y. and Teichberg, V.I. (1996) *Mol. Brain. Res.* 36, 101–113.