Cell-specific Expression of N-Methyl-D-Aspartate Receptor Subunits in Müller Glia and Neurons from the Chick Retina

Mónica Lamas, Irene Lee-Rivera, and Ana María López-Colomé

**Purpose.** Functional N-methyl-D-aspartate (NMDA) receptors (NMDARs) in Müller glia may influence glutamate neurotransmission through feedback loops between glia and neurons. The physiologic properties of NMDARs derive from the subunit composition of the tetrameric receptor. We characterized glycine binding to the NMDAR coagonist site in Müller cell membranes and determined NMDAR subunit expression in chick Müller glia compared with retinal neurons to make predictions about the heteromeric assembly of NMDARs.

**Methods.** Kinetic and pharmacologic properties of the glycine coagonist site were determined by radiolabeled ligand binding to membrane preparations from chick Müller glia and retinal neurons in primary culture. The molecular composition of NMDARs was analyzed by RT-PCR amplification and Western blotting.

**Results.** The NMDAR coagonist site in Müller cell membranes has 5-fold lower affinity for glycine and 30-fold lower affinity for D-serine compared with values obtained for synaptic membranes from whole retina and with reported values in brain tissue. NR1 subunit N-terminal and C-terminal splice-variant expression also differs in Müller cells and retinal neurons.

**Conclusions.** Pharmacologic characteristics of NMDAR coagonist-site differ in Müller glia and neurons from the retina, in agreement with the distinct subunit expression profile found. Whereas NRMDs in Müller glia contain exclusively exon 5 that lacks NR1 subunits, receptors in distinct subtypes of neurons may contain NR1 with or without exon 5, suggesting a cell-specific assembly of the NMDAR complex. Structural differences in NMDARs could underlie the differential participation of neurons and glia in the physiologic control of glutamate transmission in the retina.

**Glutamate** is the main excitatory neurotransmitter in the vertebrate retina, mediating transmission from photoreceptors to horizontal and bipolar cells in the outer retina and from bipolar to ganglion and amacrine cells in the inner retina. Glutamate activates two major types of receptors. Ionotropic glutamate receptors are oligomeric ligand-gated cation channels that respond to N-methyl-D-aspartate (NMDA), D-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), or kainate (KA), whereas metabotropic receptors are seven transmembrane G-protein-coupled receptors linked to the activation of second-messenger pathways.

Molecular cloning and functional expression studies have shown NMDA receptors (NMDARs) assemble as heteromers consisting of four subunits, two NR1 subunits that form the ion channel, and one or more NR2 (A-D) subunits that confer distinct properties to the receptor, such as peak open probability, sensitivity to glutamate, response kinetics, and possibly NR3 (A-B) subunits. The activation of NMDARs by glutamate requires the binding of glycine, as an obligatory coagonist, to a strychnine-insensitive site located within the NR1 subunit, whereas the glutamate recognition sites are formed by NR2 subunit interaction. Whether the glycine site is saturated in vivo is still controversial, though increased glycine levels at NMDAR-containing synapses has been shown to potentiate neuronal damage in some pathologic conditions. The physiologic properties of NMDARs derive from the differential assembly of subunits and is increased by the generation of eight variants of NR1 by alternative splicing that arise from the insertion or deletion of exons 5 and 21 or from the use of an alternative exon/intron acceptor site in exon 22.

NMDARs have been shown to play an important role in retinal synaptic transmission, particularly at the inner plexiform layer (IPL). In situ hybridization studies in mammalian retina have shown the expression of NMDAR subunits NR1, NR2A, NR2B, and NR2C mainly in the IPL. Although some discrepancy exists with mRNA localization, a wide distribution of NR1 subunit protein in both plexiform layers of the mammalian retina and a more restricted distribution of NR2 subunit proteins in specific neuronal populations has been demonstrated using immunocytochemistry.

Evidence for glycine participation in excitatory transmission in the retina reveals that NMDARs in synaptic membranes from plexiform layers of the retina have pharmacologic properties different from those previously described in brain neurons, mainly regarding the effect of antagonists and polyamines at the coagonist, glycine-binding site. Glycine binding to this site in retinal membranes is not modified by the specific antagonists 7-chlorokynurenate (7CK) and 5-nitro-6,7-dichloro-1,4-dihydronaphoxaline-2,3-dione (ACEA-1021), as opposed to their action in the brain. Such properties might relate to differences in NR1 posttranscriptional processing or in the heteromeric assembly of subunits in the retina. In support of this notion, novel NR1 splice isoforms and a specific subunit expression profile have been identified in the chick retina, though the cellular location of these subunits in neurons and glia is still unknown.

In addition to well-known maintenance functions, the participation of glial cells in neurotransmission by the modulation of neuronal activity has been documented and shown to be

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From the Instituto de Fisiología Celular, Departamento de Neurociencias, Universidad Nacional Autónoma de México (UNAM).

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Corresponding author: Ana María López-Colomé, Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apdo Postal 70–253, México D.F. 04510; acolome@ifc.unam.mx.

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essential for the normal functioning of the nervous system. Glial cells respond to neurotransmitters released by neurons through the activation of specific receptors and intracellular pathways, which directly or indirectly modify neuronal activity. Müller radial glia processes enwrap synaptic contacts at both plexiform layers of the retina and are exposed to synaptically released Glu. Indeed, Glu has been shown to modify gene expression in Müller cells through NMDAR-mediated activation of transcription factor pathways, and to evoke the release of neuroactive compounds that feed back onto neurons. Among these compounds, recent studies have identified an exclusively glial putative neurotransmitter, t-serine, which is released on Glu stimulation, mimics the activity of glycine at the strychnine-insensitive site of NMDARs, and could act as an endogenous glutamate coagonist. In the vertebrate retina, t-serine is present in Müller cells and astrocytes and has also been shown to contribute to the physiologic activation of NMDARs in retinal neurons.

In the present study, we analyzed the characteristics of glycine and t-serine binding to the coagonist site of NMDARs and the expression of NMDAR subunits in chick cultured Müller cells compared with retinal neurons. Our results demonstrate that the properties of the coagonist site of NMDARs and the expression of NR1 subunit splice isoforms differ in neurons and glia from the retina, suggesting a distinct oligomeric structure and regulation of NMDA glutamate receptors in these cell types.

METHODS

Cell Culture

Primary cultures of Müller glia were obtained from retinas of 7-day-old chick embryos, as described previously. Briefly, tissue was dissociated in 0.25% trypsin, filtered, and resuspended in minimum essential medium (MEM; Gibco BRL, Grand Island, NY) containing 0.05% glucose, 25 mM NaHCO₃, 0.0125% gentamicin, penicillin, and streptomycin, 0.025% neomycin, and 10% fetal bovine serum (FBS), and was seeded at a density of 6 × 10⁶ cells/cm² flask. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. Confluent cultures were used for all experiments. Primary cultures of retinal neurons were obtained following the same procedure as for Müller cells, except that cells were seeded at low density (1 × 10⁶ cells/well) onto poly-D-ornithine-coated dishes, medium was changed to OptiMEM (Invitrogen, Carlsbad, CA) supplemented with 4% FBS, and cells were maintained until 70% confluent. The purity of the cultures was assessed using glial fibrillary acidic protein (GFAP) and neuron-specific enolase antibodies. Human embryonic kidney 293 cells (ATCC CRL1573; American Type Culture Collection, Rockville, MD) were grown in the same conditions as described for Müller cells. All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Tissue culture reagents and plastics were from Gibco BRL, and all other chemicals and reagents were from Sigma Chemical Co. (St Louis, MO).

Membrane Preparation

Cultured cells were harvested, homogenized in 25 vol of 5 mM HEPES/4.5 mM Tris buffer (HTB) pH 7.4, kept on ice for 15 minutes to allow complete osmotic disruption, and subsequently centrifuged at 45,000g for 20 minutes at 4°C. The pellets were stored at −20°C for 48 hours, thawed, and washed once in HTB buffer, twice in the same buffer containing 1 mM EDTA, and twice more without EDTA. Pellets were stored at −70°C for at least 1 week. Before assay, membranes were washed another four times with HTB.

Synaptosomal fractions from the outer (P1) and the inner (P2) plexiform layers were isolated as previously described, from retinas homogenized in 0.32 M sucrose containing 10⁻⁴ M MgSO₄. Membranes were obtained as described for Müller cells. Protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA).

Binding Assay

Membrane protein (50–100 µg) was incubated for 2 hours at room temperature, with the indicated concentrations of [³H]glycine, in a final volume of 175 µL. Non-specific binding was defined in the presence of 1 mM unlabeled glycine. When tested, 100 µM spermine was added during the incubation period. Dose-response curves were obtained by incubation of the membranes with 100 nM [³H]glycine, in the presence of increasing concentrations of unlabeled glycine, t-serine, or 7-chlorokynurenate (7CK), as indicated. Reaction
was stopped by centrifugation at 90,000 rpm in an air-driven microcentrifuge (Beckman). Pellets were washed four times in HTB, dissolved in 2% SDS, and counted for radioactivity in a liquid scintillation counter (LS6000 SC; Beckman Instruments, Palo Alto, CA). Data were analyzed by nonlinear regression (PRISM4; GraphPad Software, San Diego, CA) and were expressed as the mean ± SEM of three independent experiments performed in triplicate.

Association curves were constructed by measuring the binding of 
$[3H]$ glycine (20, 60, and 100 nM; Sp. Act. 30–60 Ci/mmol) or 
$[3H]$ D-serine (20 and 60 nM; Sp. Act.16.4 Ci/mmol; Perkin Elmer Life Sciences, Shelton, CT) at time points from 2 to 120 minutes. Nonspecific binding was defined in the presence of 1 mM unlabeled glycine or D-serine. pH sensitivity was determined using the following buffers: 5 mM HEPES/4.5 mM Tris-HCl, pH 4.2, pH 6.4, pH 7.4; 50 mM HEPES/KOH, pH 8; 50 mM Tris-HCl, pH 9.

### Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from cultured Müller cells using Trizol reagent (Invitrogen) following the manufacturer’s instructions. First-strand cDNA templates were synthesized from 2 μg total RNA using random hexamers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) according to standard procedures. PCR was carried out using NR1 primers designed for differentiating specific NR1 isoforms, as previously described.9

### Protein Analysis

Membrane preparations from cultured Müller cells, cultured neurons, and whole cell extracts from brain, cerebellum, and the HEK293 cell line were homogenized in Laemmli buffer48 and boiled for 5 minutes. Western blot analysis was performed on PVDF (Hybond; Amersham, Piscataway, NJ) membranes using standard procedures, and the filters were hybridized for 1 hour at room temperature to polyclonal antibodies recognizing the NMDAR subunits NR2A, NR2C, NR2D (Santa Cruz Biotechnology, Santa Cruz, CA), and NR2B (Zymed Laboratories, South San Francisco, CA). Bound antibody was visualized using the enhanced chemiluminescence detection system (ECL Plus; Amersham) in combination with a peroxidase-conjugated antibody. Coomassie staining was used for protein loading control.

## RESULTS

### Glycine Binding to the NMDAR Coagonist Site in Membranes from Müller Cells

The specific binding of $[3H]$ glycine to membranes from cultured Müller cells was analyzed in the presence of saturating concentrations of strychnine (500 μM) to prevent an interaction with inhibitory glycine receptors. Binding was found to be saturable (Fig. 1A), temperature independent (data not shown), and modulated by pH, in agreement with the presence of pH-sensitive domains in NMDARs from Müller glia, as shown previously for neuronal NMDARs.49 Maximum specific binding was obtained at pH 7.4 (Fig. 1B), whereas no significant change in nonspecific binding was observed. The best-fit values for the equilibrium dissociation constant ($K_d$) and maximum binding ($B_{max}$) were $632 ± 198$ nM and 43.58 ± 7.02 pmol/mg protein, respectively. Comparative data depicted in Table 1 show that the calculated values for kinetic and association constants in Müller cells differ significantly from those previously found for synaptosomal membranes from retinal inner and outer plexiform layers.27

### D-Serine Interaction with NMDAR Coagonist Site in Müller Cell Membranes

As found for glycine binding (Fig. 1), $[3H]$ D-serine bound to NMDARs in Müller cell membranes in a pH-sensitive manner (Fig. 2A). Association curves in Figure 2B show that D-serine binding is saturable and time dependent. Reaction equilibrium was reached at 75 minutes; hence, subsequent determinations were performed in this condition.

The dose-response analysis of $[3H]$ glycine displacement by unlabeled glycine and D-serine (Fig. 2C) revealed that glycine is equally potent in membranes from Müller cells (IC$_{50}$ = 1.6 μM), cultured neurons (IC$_{50}$ = 1.4 μM), and synaptosomal fractions from the inner plexiform layer (P2 IC$_{50}$ = 2.2 μM) and the outer plexiform layer (P1 IC$_{50}$ = 5 μM) of the mature retina. Although the potency of D-serine differed slightly in Müller cells (IC$_{50}$ = 24 μM), neurons (IC$_{50}$ = 44 μM), and synaptosomal fractions P1 (IC$_{50}$ = 30 μM) and P2 (IC$_{50}$ = 57 μM), statistical analysis using an F-test (PRISM 4; GraphPad) revealed this variation to be nonsignificant (not shown). In all the cell preparations tested, glycine was more potent than D-serine for displacing bound $[3H]$ glycine. Hill analysis of the inhibition curves showed that the Hill coefficient (nH) for glycine and D-serine was not significantly different from 1, which confirms that both agents bind to a single receptor site.

### Effect of Spermine and 7-Chlorokynurenic Acid on Glycine Binding to Müller Cell Membranes

Previous studies demonstrated that the pharmacologic properties of $[3H]$ glycine binding to the NMDAR coagonist site in synaptic membranes from the chick retina$^{20–29}$ differ from those in brain membranes.$^{50–52}$ Saturation analysis in Figure 3A shows that strychnine-insensitive $[3H]$ glycine binding to Müller cell membranes is not affected by spermine (100 μM). The best-fit calculated values for $K_b$ (620 ± 122 nM) and $B_{max}$ (58.82 ± 4 pmol/mg protein) in the presence of spermine do not differ significantly from control values in the absence of this polyamine, in contrast with its inhibitory effect in neuronal synaptosomal membranes from the retina.$^{20–27}$ (Fig. 3A).

**Table 1. Calculated Constants for $[3H]$ Glycine Binding to Retinal Membranes**

<table>
<thead>
<tr>
<th></th>
<th>Müller Cells</th>
<th>Retinal P1 Fraction</th>
<th>Retinal P2 Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (nM)</td>
<td>632 ± 198</td>
<td>139 ± 17.92*</td>
<td>133 ± 11.67*</td>
</tr>
<tr>
<td>$B_{max}$ (pmol/mg protein)</td>
<td>43.58 ± 7.02</td>
<td>2.76 ± 0.25*</td>
<td>4.11 ± 0.47*</td>
</tr>
<tr>
<td>$K_{obs}$ 20 nM ($10^{-3}$/min)</td>
<td>25 ± 2</td>
<td>7.51 ± 2*</td>
<td>9.30 ± 1.98*</td>
</tr>
<tr>
<td>$K_{obs}$ 60 nM ($10^{-3}$/min)</td>
<td>29 ± 5</td>
<td>14.45 ± 2.6*</td>
<td>14.19 ± 0.99*</td>
</tr>
<tr>
<td>$K_{obs}$ 100 nM ($10^{-3}$/min)</td>
<td>41 ± 10</td>
<td>13.45 ± 0.52*</td>
<td>15.31 ± 0.03*</td>
</tr>
</tbody>
</table>

Values were calculated from saturation curves or time-course experiments performed as described for Figure 1. Data are expressed as the mean ± SEM of three independent experiments performed in triplicate.

*Data from Rodriguez-Conteras et al$^{46}$ and Calderon and López-Colomé.27 P1 indicates synaptosomal fraction from the OPL; P2, synaptosomal fraction from the IPL.
The ability of the glycine-site antagonist 7CK to inhibit [3H] glycine binding to Müller cell membranes was tested. As shown in Figure 3B, nonlinear regression analysis of the dose-response curves for [3H] glycine inhibition by glycine and 7CK yielded IC_{50} values of 1.6 μM and 200 μM, respectively, in agreement with previous binding studies and functional assays in retinal neurons. Densitometric analysis of the electrophoretic gels was plotted as the relative expression of NR1 isoform referred to a housekeeping gene (for actin).

The relative expression of NR1 C-terminal splice-isomers in Müller cells and retinal neurons was analyzed by RT-PCR and compared with the whole retina using two sets of primers specifically designed to amplify the regions spanning from exon 20 to exon 22 and to discriminate between C-terminal variants containing only exon 22 (C2 cassette) and those that also contain either exon 21 or the novel retinal exon C3. In cultured retinal neurons, amplification using primers 2868F and 3390R (the region from exon 19/C0 cassette to exon 22/C2 cassette) generated 3 fragments: a 550-bp product corresponding to variants containing only exon 22 (C2 cassette) and those that also contain either exon 21 or the novel retinal exon C3. 

**Molecular Analysis of NMDA Receptor Subunit Expression in Cultured Müller Cells**

**NR1 Subunit.** In neuronal NMDARs, alternative splice-exon 5 of the NR1 subunit carries the glycine coagonist binding site and is involved in the regulation of channel properties by spermine, pH, and zinc. The NR1 N-terminal splice variants in Müller cells were compared to those of retinal neurons and synaptosomal fractions from the retina. Membranes were incubated with 100 nM [3H] glycine in the presence of increasing concentrations of either unlabeled glycine or D-serine. IC_{50} values were determined using a software program (PRISM4; GraphPad). All data are the mean ± SEM of three independent experiments performed in triplicate.

**FIGURE 2.** [3H] D-serine interaction with NMDAR coagonist site in Müller cell membranes. (A) Effect of pH on specific [3H] D-serine binding. Membranes were incubated with 60 nM [3H] D-serine; nonspecific binding was defined in the presence of 1 mM glycine. (B) Association curves for [3H] D-serine at 60 (▼) and 100 nM (●) concentration. (C) Inhibition of [3H] glycine binding to Müller cells, retinal neurons, and synaptosomal fractions from the retina. Membranes were incubated with 60 nM [3H] glycine in the presence of increasing concentrations of either unlabeled glycine (●) or D-serine (▲). IC_{50} values were determined using a software program (PRISM4; GraphPad). All data are the mean ± SEM of three independent experiments performed in triplicate.

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were performed in the presence of 500 μM strychnine. Data are expressed as the mean ± SEM of three experiments performed in triplicate.

whole retina31 (Fig. 5, lane 3) are specifically expressed by neurons.

NR2 Subunits. Molecular analysis of NR2 subunit mRNA (not shown) and protein expression (Fig. 6) showed that NR2A, NR2B, NR2C, and NR2D are all expressed by Müller cells and retinal neurons in culture.

DISCUSSION

Glycine is a well-established modulator of excitatory neurotransmission, acting as an obligatory coagonist of glutamate at NMDARs.11,12 Examination of the effect of the glycine-site NMDAR antagonists kynurenate and ACEA-1021 and of polyamines on glycine binding to membranes from the whole retina and from retinal synaptic fractions26–28 showed that glycine binding to the coagonist site in chick retina is inhibited by spermine27 and insensitive to antagonists such as 7CK,26–29 contrary to the effects described for brain tissue and for recombining NMDARs.30–52 These findings are in agreement with functional data showing that, in contrast to cortical tissue, glycine does not potentiate NMDA-induced toxicity in the chick retina and, in fact, may protect retinal neurons from this type of damage.29,53 Furthermore, ischemic damage in the retina is not attenuated by 7Cl-thiokynurenate, whereas it is known to significantly reduce neuronal loss in the brain.54 The molecular mechanisms underlying these observations likely relate to the molecular composition of NMDARs in the brain55 and the retina.55,45 particularly given that NR1 splice variants specific for the retina have been recently identified.31 The properties of the NMDAR coagonist site in the retina could be ascribed to neurons or to glial cells because both cell types carry functional receptors.35,58

Results from the present work show that the kinetic and pharmacologic properties of glycine interaction with the NMDAR coagonist site differ in Müller cells and retinal neurons, correlating with the cell-specific expression of N-terminal NR1 splice variants found (Fig. 4). We showed that glycine (Fig. 1) and the putative ligand for the glycine-site, d-serine (Fig. 2), competitively inhibit [3H] glycine binding to NMDARs in Müller cells with 5-fold and 10- to 30-fold lower affinity, respectively, compared with the values previously obtained by us for synaptic (neuronal) membranes from chick retin26,27 (see Table 1). These results are in contrast to findings in rat brain membranes56 and heterologous expression systems,57 which show that d-serine binds with identical or even higher affinity than glycine to NMDARs and is functionally 100 times more effective than glycine at inducing NMDAR-mediated potentiation of spontaneous synaptic currents.56–58 Because glycine and d-serine interact with the same site of NMDARs in Müller glia, increases in the extracellular concentration of glycine or d-serine could play a functional role in retinal excitability through a feedback regulation of NMDAR activity in Müller glia, in addition to their possible effect on neuronal receptors.53–55 Indeed, in pathologic conditions such
as retinal ischemia, in which an important elevation of extracellular glutamate is observed, glial cells are depolarized and might release glycine through reverse transporter activity,15 which, in turn, could induce neuronal excitotoxic damage by activating NMDARs on neurons and glia. The release of D-serine from Müller cells on physiologic stimulation has not yet been demonstrated. However, Bergmann cells from the cerebellum, the only other type of radial glia present in the mature central nervous system, have recently been shown to release D-serine on glutamate stimulation, which could also be true of Müller glia.

Our present results demonstrate that the specific glycine-site antagonist 7CK has no effect on glycine binding to membranes from neurons or Müller glia (Fig. 3B), whereas it has been shown to bind with high affinity to postsynaptic densities from rat cerebral cortex and to NR1 fragments containing the glycine site and to inhibit NMDA-induced responses in cortical slices. These findings may explain the lack of effect of kynurenicates in protecting retinal neurons from glutamate-induced excitotoxicity.29,54

Polyamines, particularly spermine, have been shown to stimulate glycine binding to NMDARs in brain membranes through an increase in affinity, in contrast with the clear inhibition of this interaction observed in membranes from the whole retina.27

Spermine-evoked potentiation and pH sensitivity of NR1 homomeric receptors are determined by the absence of the N-terminal exon 5 (N1 cassette). Site-directed mutagenesis of the 6 amino acids contained in N-terminal exon 5 demonstrated that NR1 lacking this insert (NR1a) is modulated by polyamines, whereas NR1 containing this exon (NR1b) is not. Our results show that, although Müller cells express exclusively exon 5-lacking NR1 subunits, glycine binding is not modified by spermine (Fig. 3A). However, the interaction of polyamines with NMDARs also relates to the type of NR2 subunit(s) included in the heteromeric complex.55,65,66 Receptors containing NR1a/NR2C have been shown to be insensitive to glycine-independent and to glycine-dependent stimulation by spermine and to voltage-dependent inhibition by polyamines but to preserve pH sensitivity.49 NR2C-containing receptors also exhibit lower sensitivity to Mg2+/H+-block than NR2A/B-containing receptors and may be activated in glial cells, which exhibit more negative membrane potential than neurons.68 We showed that NR2A-D NMDAR subunits are present in Müller cells and neurons (Fig. 6). Given that NR2C is expressed at significant levels only in the cerebellum and the retina,23 the prevalence of a spermine-insensitive NMDAR com-
bination, including NR1a/NR2C in Müller cells, and NR1b/NR2C in retinal neurons can be postulated. It is important to point out that the exclusive expression of exon 5-lacking NR1 isoforms in Müller cells and the expression of NR1 isoforms containing and lacking this exon in neurons could relate to the existence of multiple subpopulations of neurons compared with a homogeneous population of Müller cells in the retina. This assumption is supported by recent work demonstrating a differential response to NMDA by neurochemically identified subpopulations of neurons at the IPL, which could be attributed to the NR1 splice variants forming the channel, still unidentified in specific subtypes of retinal neurons, and to the inclusion of different NR2 receptor subunit combinations.

In addition to the expression of distinct NR1 N-terminal splice variants in neurons and glia (Fig. 4), NR1 C-terminal splice isoforms containing the retinal C-terminal cassette C231 is exclusively expressed in neurons (Fig. 5) and, hence, could represent a novel target for developmental regulation of glutamate neurotransmission by signal transduction pathways because it is transiently expressed at the period of synapse consolidation in the retina and it contains a consensus sequence for casein kinase II phosphorylation. Based on the distinct properties of the glycine coagonist site and the differential expression of NMDAR subunits and splice variants in Müller cells and retinal neurons, our study suggests that NMDAR regulation differs in both cell types. To our knowledge, the present work constitutes the first attempt to compare NMDARs in neurons and glia from the retina, contributing relevant evidence for the elucidation of the specific properties of NMDARs in this tissue and their possible role in normal and pathologic excitatory neurotransmission. Further understanding of these differences could shed light on the general mechanisms through which glia integrates neuronal inputs and participates in the modulation of synaptic activity in this tissue.

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References


64. Durand GM, Bennet MV, Zukin RS. Splice variants of the NMDA receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. Proc Natl Acad Sci USA. 1993;90:6731–6735.


