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Expression of NMDA receptor subunit 1 in the rat retina

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) belong to the ionotropic glutamate receptors, which play key roles in neuronal communication in the retina. NMDA receptors are tetrameric protein complexes usually comprising two obligatory NMDA receptor 1 (NR1) subunits and modulatory NMDA receptor 2/3 (NR2/3) subunits. Although the expression patterns of different NMDA receptor subunits have been extensively studied, in this study we focused on NR1 protein expression in the rat retina by immunofluorescence double labeling. We show that NR1 labeling is diffusely distributed in the outer plexiform layer (OPL) and throughout the whole inner plexiform layer (IPL). The NR1-immunoreactivity (IR) was displayed in a variety of cells in the inner nuclear layer (INL) and the ganglion cell layer (GCL). Interestingly, NR1 was expressed in both rod and cone bipolar cells identified by specific bipolar cell markers Chx10, protein kinase C (PKC) and recoverin. All the amacrine cells that we studied, including cholinergic, dopaminergic, GABAergic and glycinergic amacrine cells, were NR1-IR positive. In the ganglion cell layer, NR1-IR was expressed in all cells that were positive for the ganglion cell marker Brn3a. Our study suggests that the NR1 subunit is expressed more widely than was previously appreciated.

Introduction

Glutamate is the main excitatory neurotransmitter in the vertical signal pathway in the vertebrate retina. Glutamate receptors can be subdivided into two main groups. Ionotropic glutamate receptors are ligand-gated ion channels that respond to N-methyl-D-aspartate (NMDA), 2-amino-3-hydroxy-5-methyl-4-isoxasole-propionate (AMPA) or kainate, whereas metabotropic glutamate receptors act through second messenger systems (for reviews see: Hollmann and Heinemann, 1994). NMDA receptors play key roles in neuronal communication and synaptic plasticity (Köhr, 2006; Paoletti and Neyton, 2007; Paoletti, 2011) and are involved in numerous pathological processes (Gardoni et al., 2010; Javitt, 2010; Myers et al., 2011). Although NR1 subunits can assemble as homomeric ion channels, the current generated by such receptor homomers is only a small fraction of that generated by heteromeric assemblies of NR1 and at least one of the four NR2 subunits (NR2A–NR2D) or NR3 subunits (NR3A–NR3C) (Stephenson, 2001; Paoletti, 2011).

NR1 is the essential subunit for a functional NMDA receptor, ubiquitously expressed in the retina (Foldes et al., 1993). It has been shown that every neuron in the ganglion cell layer expresses the NR1 subunit regardless of developmental age in the rat retina. NR1 is also expressed in horizontal cells and subpopulations of amacrine cells in the INL (Brandstätter et al., 1994; Gründer et al., 2000). Several studies have demonstrated NR1 expression in the IPL, including both rod and cone bipolar cell pathways (Fletcher et al., 2000; Kalloniatis et al., 2004). In the primate and mouse retinas, NR1 is also expressed in the IPL and INL (Hughes, 1997; Lo et al., 1998; Grünert et al., 2002) although the identity of the NR1-positive cells has not been precisely determined. Despite these studies, it is still widely believed that AMPA receptors are the only class of ionotropic glutamate receptor that participates in synaptic transmission in the OPL of the mammalian retina. To revisit this question and examine the possibility that NMDA receptors also participate, we screened NR1 subunit expression in different retinal cell types by immunofluorescence double labeling in the rat. Here we report the presence of NR1 subunit labeling in rat bipolar cells, implying the potential for functional NMDA receptors on bipolar cells. Our results show that the NR1 subunit is widely distributed in multiple types of amacrine cells and bipolar cells, as well as in ganglion cells.

Abbreviations: AMPA, 2-amino-3-hydroxy-5-methyl-4-isoxasole propionate; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; MW, molecular weight; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; NR1, NMDA receptor 1; NR2,3, NMDA receptor 2,3; NR1-IR, NR1 immunoreactive; ONL, outer nuclear layer; OPL, outer plexiform layer; PB, phosphate buffer; PBS, phosphate buffered saline; PKC, protein kinase C; TH, tyrosine hydroxylase.

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Materials and methods

Tissue preparation

The rat retinas were prepared as previously described in detail (Ke and Zhong, 2007). A total of 12 adult male Sprague-Dawley rats (100–150 g) were used in this study. The experimental procedures described here were conducted in accordance with the NIH guidelines for animal experimentation. During this study all efforts were made to minimize the number of animals used and their suffering. The animals were deeply anesthetized with 20% urethane (10 ml/kg). The eyeballs were enucleated and hemisectioned and the posterior parts were immediately immersion-fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 20 min, chilled sequentially in 0.1 M PB with 10% (w/v) and 20% sucrose for 2 h each and in 0.1 M PB with 30% sucrose overnight at 4 °C. The fixed eyecups were embedded in OCT (Miles Inc., Elkhart, IN, USA) and frozen by liquid nitrogen, sectioned vertically at 14 μm thickness on a freezing microtome (Leica Microsystems, Nussloch, Germany) and mounted on gelatin chromium-coated slides.

Immunohistochemistry

Fluorescence immunocytochemical labeling procedure was described previously (Liu et al., 2010). The retinal sections were blocked in 0.1 M phosphate buffered-saline (PBS, pH 7.4) containing 6% normal donkey serum, 1% bovine serum albumin and 0.2% Triton X-100 for 2 h at room temperature (RT). For labeling NR1, specific mouse monoclonal antibody against NR1 (556308, 1:200 dilution, BD Pharmingen, BD Biosciences, San Diego, CA, USA) was used as the primary antibody. Single labeling experiments were conducted by incubating the sections with the antibody for 3 days at 4 °C in a medium containing 3% normal donkey serum, 0.5% bovine serum albumin and 0.2% Triton X-100 in 0.1 M PBS (DT-PBS). Binding sites were revealed by incubating with the secondary antibody (see below) in DT-PBS for 2 h at RT. For double labeling, the sections were incubated sequentially in a mixture of two primary and secondary antibodies. Antibodies used for labeling different bipolar cells were as follows: sheep anti-Chx10 polyclonal antibody (ab16141, 1:400 dilution, Abcam, Cambridge, UK) was used for labeling all bipolar cells; goat anti-protein kinase C α (PKC α) polyclonal antibody (sc-208-G, 1:2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for labeling rod bipolar cells (rod BCs); rabbit anti-recoverin polyclonal antibody (AB5431P, 1:2000 dilution, Chemicon, Temecula, CA, USA) was used for labeling types 2 and 8 cone bipolar cells. Antibodies used for labeling different amacrine cells were as follows: goat anti-choline acetyltransferase (ChAT) polyclonal antibody (AB144P, 1:800 dilution, Chemicon) (for cholinergic amacrine cells) and rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody (AB152, 1:400 dilution, Chemicon) (for dopaminergic amacrine cells); rabbit anti-GABA polyclonal antibody (A2052, 1:2000 dilution, Sigma-Aldrich, St. Louis, MO, USA) (for GABAergic amacrine cells) and rat anti-glycine antibody (1:1500 dilution, a kind gift from Dr. D. Pow, Brisbane, Australia) (for glycinergic amacrine cells). Goat anti-Brn-3a polyclonal antibody (sc-31984, 1:400 dilution, Santa Cruz Biotechnology) was used for labeling ganglion cells. Secondary antibodies used in this study were as follows: Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 555 donkey anti-mouse IgG (1:200 dilution, Jackson Immunoresearch Laboratories, West Grove, PA, USA) were used for staining NR1; Alexa Fluor 488 donkey anti-sheep IgG (1:200 dilution, Invitrogen, Carlsbad, CA, USA) for immunolabeling Chx10; Alexa Fluor 555 donkey anti-goat IgG (1:200 dilution, Jackson Immunoresearch Laboratories) for PKCα, ChAT and Brn3a; Alexa Fluor 555 donkey anti-rabbit IgG (1:200 dilution, Invitrogen) for recoverin, TH and GABA; DyLight 594-conjugated donkey anti-rat IgG (1:200 dilution, Jackson Immunoresearch Laboratories) for glycine. For controls of the double labeling, omission of one of the primary antibodies yielded only the immunoreactivity for the remaining antibody, while omission of both abolished any immunolabeling. Staining by a mixture of two secondary antibodies after incubation with one of the two primary antibodies showed no cross-reactivity of species-specific secondary antibodies.

Sections were scanned and photographed with a Leica SP2 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) using a 63 × oil-immersion objective lens. Single optical sections were taken through the preparation and recorded digitally. Double labeling was precisely evaluated by sequential scanning on single-layer optical sections to avoid any possible reconstruction stacking artifact. Images were resized and adjusted in Adobe Photoshop (Adobe Systems, San Jose, CA, USA) to reproduce the original histological data.

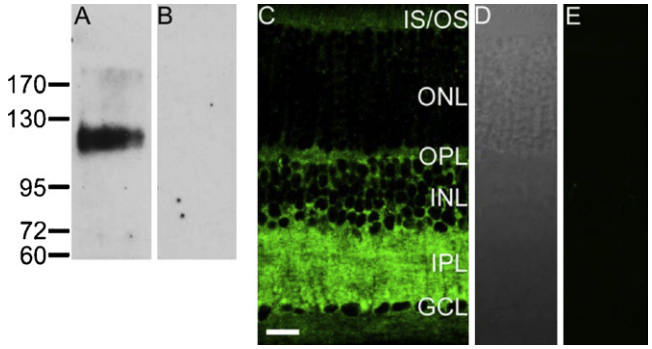

Fig. 1. Expression and localization of NR1 in the rat retina. (A) Western blot analysis of whole rat retina homogenates for the NR1 antibody revealing a single band of ~120 kDa. (B) No band was detected when the primary antibody was omitted. MW scale (kDa) is shown on the left of (A). (C) Confocal fluorescence micrograph of a vertical section of the rat retina, labeled by NR1 antibody. (D) Nomarski optics image of the retina. (E) No immunofluorescence labeling was present when the primary antibody was omitted. IS/OS, inner segment/outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μm.

Western blot analysis

The rat retinal extract samples (40 μg/each lane) were loaded, subjected to 8% SDS–PAGE and then transferred onto PVDF membranes. Non-specific binding was blocked for 2 h at RT in blocking buffer consisting of 20 mM Tris–HCl, PH 7.4, 137 mM NaCl, 0.1% Tween 20 and 5% non-fat milk. The samples were incubated with the anti-NR1 antibody (1:500 dilution, BD Pharmingen™, Franklin Lakes, NJ, USA) overnight at 4 °C. The blots were incubated with HRP-conjugated goat anti-mouse IgG (1:3500 dilution, Santa Cruz Biotechnology) for 2 h at RT, and finally visualized with enhanced chemiluminescence (Pierce Biotechnology Inc., Rockford, IL, USA). No band was detected when the NR1 antibody was omitted. To estimate the molecular weight (MW) of NR1, a prestained marker (Fermentas, Glen Burnie, MD, USA) was used.

Results

Specificity of the NR1 antibody

The anti-NR1 antibody detected a single band at a MW of ~120 kDa in rat retinal homogenates by Western blot analysis (Fig. 1A), which is in agreement with the MW of the NR1 in rat, cat and ferret brains (Catalano et al., 1997). No band was detected when the primary antibody was omitted (Fig. 1B).