N-Methyl-D-Aspartate Receptor Subunit NR3A in the Retina: Developmental Expression, Cellular Localization, and Functional Aspects

Nikolaus J. Sucher,1 Konrad Kohler,2 Lalitha Tenneti,5 Hon-Kit Wong,1 Tatiana Gründer,2 Sascha Fauser,2 Thomas Wheeler-Schilling,2 Nobuki Nakanishi,5 Stuart A. Lipton,3,5 and Elke Guenther2,4

PURPOSE. Recently, a novel N-methyl-D-aspartate receptor (NMDAR) subunit, NR3A, has been discovered in the brain and shown to decrease NMDAR activity by modulating the calcium permeability of the receptor channel. The insertion of NR3A within the NMDAR complex may thus alter NMDAR properties and play a crucial role during processes of neuronal development and degeneration. The present study is the first to investigate the expression and cellular localization of NR3A on the protein level in the retina and to elucidate its putative functional roles within the retinal circuitry.

METHODS. The expression of NR3A in the retina was analyzed by reverse transcription–polymerase chain reaction (RT-PCR), immunohistochemistry, and Western blot analysis. Functional aspects of NR3A in the retina were addressed by measuring the NMDA-induced increase in intracellular calcium, [Ca2+]i, in retinal cells prepared from wild-type (NR3A+/+)

RESULTS. NR3A protein expression was initially observed in the first postnatal week and was predominantly localized to cell bodies in the ganglion cell layer. In older animals, two bands of NR3A immunoreactivity were additionally observed in the inner plexiform layer. NMDA-evoked [Ca2+]i responses were found to be significantly greater in retinal cells in NR3A−/− mice than in wild-type retinas.

CONCLUSIONS. The data indicate that NR3A is specifically expressed in the inner retina and may modulate NMDAR-mediated calcium influx and thus [Ca2+]i levels in retinal ganglion cells and amacrine cells. (Invest Ophthalmol Vis Sci. 2003;44:4451–4456) DOI:10.1167/iovs.02-1259

The amino acid L-glutamate is thought to be the excitatory transmitter used by photoreceptor, bipolar, and ganglion cells in the vertical signaling pathway of the mammalian retina. Both metabotropic and ionotropic glutamate receptor types are present in the retina,1–7 and the cell-type–specific action of the universal transmitter glutamate is determined by the expression of particular types of glutamate receptors on the postsynaptic site.8,9

In addition to its role in excitatory signal transmission, glutamate, acting through its membrane receptors, also influences retinal development and plasticity. Recent evidence indicates that exposing the developing retina to glutamate receptor antagonists perturbs the neural circuitry mediating the antagonistic center-surround organization found in normal receptive fields.10 Activation of N-methyl-D-aspartate receptors (NMDARs) has been shown to play a role in structural plasticity in the retina and retinofugal projections.11–13 For example, exposure of the optic tectum of tadpoles to NMDA receptor (NMDAR) antagonists disrupts the topographic projection of retinal ganglion cell arbors.11–14 Conversely, exposure of the optic tectum to the receptor agonist, NMDA, increases the eye-specific segregation of these arbors.14

Increasing evidence indicates that, in addition to their fundamental role in information processing and development in the visual system, glutamate receptors produce neurotoxicity when excessively stimulated. A number of retinal diseases, such as glaucoma, vascular occlusion, optic neuropathy, and optic nerve trauma have been linked to glutamate excitotoxicity.15 Toxic effects on retinal ganglion cells are mediated predominantly through NMDARs16,17 and seem to be dependent on the Ca2+ permeability of these receptors.18,19

Functional neuronal NMDARs are thought to be heteromultimers that are composed of at least one NR1 subunit and one or more NR2 subunits (reviewed in Sucher et al.20). The recent discovery of an additional NR3A subunit in the brain suggests that the stoichiometry and functional diversity of NMDARs is more elaborate than previously thought.21–23 NR3A was shown to form stable complexes with NR1 and NR2 subunits in the brain, and NR3A knockout mice display increased NMDA-evoked currents. Cortical neurons in layer IV of the cerebral cortex of homozygous knockout (NR3A−/−) mice manifested a greater density of dendritic spines than those in wild-type (NR3A+/+) mice.24 These data suggest that NR3A may be involved in the development of synaptic elements by modulating NMDAR activity. NR3A is the first subunit that has been shown to decrease NMDAR activity, thus raising the possibility of its being neuroprotective.24

To date, nothing is known about the cellular expression of NR3A in the retina and its putative functional roles. The pharmacological and kinetic properties of an NMDAR channel strongly depend on the molecular composition of its receptor subunits, and this organization may change because of different functional requirements during neuronal differentiation in the retina. The present approach was thus designed to characterize the expression pattern of NR3A mRNA and protein in...
the developing and adult rodent retinas. Moreover, functional aspects of NR3A were investigated by calcium imaging of retinal cells from wild-type and NR3A−/− mice.

NR3A was expressed in the inner retina in amacrine cells and ganglion cells as well as in two distinct bands within in the inner plexiform layer, indicating a synaptic localization in both the on- and off-pathway. NMDA-evoked intracellular calcium, [Ca2+]i, responses were found to be significantly greater in retinal cells from NR3A−/− mice than in wild-type mice, indicating a modulation by NR3A of NMDA-mediated increases in [Ca2+]i, levels in retinal cells.

**Methods**

The treatment of animals in this study was in conformity with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the laws applicable in Germany, Hong Kong, and the United States, in accord with institutional review.

**Retinal Preparation**

Male pigmented rats (Brown Norway), C57BL/6 mice or, NR3A−/− mice backcrossed on C57BL/624 between embryonic day (E)16 and postnatal day (P)94 were anesthetized with ether and killed by decapitation. The eyes were enucleated and hemisected along the ora serrata.

**RNA Isolation and DNase Digestion**

For RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR), the retina was gently removed from the underlying tissue. The choroid was isolated without the adhering retinal pigmented epithelium. The anterior segment was discarded from the cornea and lens, and the iris/ciliary body was carefully removed. All isolated tissues were frozen immediately in liquid nitrogen and stored at −80°C until subsequent RT-PCR analysis. Tissues were lysed and homogenized under highly denaturing conditions. Samples were adjusted for the appropriate binding conditions of a silica gel-based membrane on spin columns (RNeasy; Qiagen, Hilden, Germany). The yield of total RNA was determined from the absorbance at 260 nm. To prevent DNA contamination, the samples were treated enzymatically with DNase-free DNase I (Roche Diagnostics, Mannheim, Germany), followed by phenol extraction and ethanol precipitation.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was reverse transcribed to cDNA in a mixture containing 10 mM Tris, 50 mM KCl (pH 8.3), 5 mM MgCl2, 1 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 0.08 A260 units random primer (dN)10, 50 U RNase inhibitor, and 20 U avian myeloblastosis virus (AMV) reverse transcriptase (all chemicals from Roche Diagnostics). The reaction mixture (10 μL) was incubated for 10 minutes at 25°C and then for 60 minutes at 42°C. After the 42°C incubation, the AMV reverse transcriptase was denatured by incubating the reaction at 99°C for 5 minutes before cooling to 4°C. Parallel control reactions containing all reagents except the reverse transcriptase were performed. The product of the reverse transcription reaction was amplified by PCR in a 50-μL volume containing 100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl2 (pH 9.2), 0.5 μM primer, and 2.5 μL DNA polymerase (AmpliTaq; Perkin Elmer, Weiterstadt, Germany). PCR was conducted in a thermal cycler (model 2400; Perkin Elmer) and consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, extension at 72°C for 1 minute, and annealing at 55°C for 1 minute, followed by extension at 72°C for 15 minutes.

**Oligonucleotides Used for PCR**

Primers were designed in the Heidelberg Unix Sequence Analysis Resources (HUSAR) primer computer program (German Cancer Research Centre, Heidelberg, Germany). The NR3A primer sequences were as follows: 5′-AGT CAT GGA CTC TGG AAT ATG G-3′ (sense primer) and 5′-CAT GTC TTC TGC TAG CTG CTG-3′ (antisense primer). The expected PCR product was a 307-bp fragment. PCR products were subcloned into the a plasmid (pCR-Script Amp S(+) ; Stratagene, Heidelberg, Germany) and sequenced with an automated gene sequencer (model 310; Prism 310; Perkin Elmer-ABI).

**Immunocytochemistry**

Retinas at different developmental stages between E16 and adult were used. After death, eyes were removed, and the anterior poles dissected. Eyecups were immersion fixed for 15 to 30 minutes, depending on the developmental ages of the retinas, at 4°C in 4% (wt/vol) paraformaldehyde and 0.1 M phosphate buffer (PB; pH 7.4). After the tissues were rinsed in PB, they were cryoprotected by immersion in 30% (wt/vol) sucrose in PB overnight at 4°C. Samples were then embedded in a tissue-freezing medium (Jung, Leica Instruments GmbH, Heidelberg, Germany), sectioned vertically to 12-μm slices with a cryostat, and collected on gelatin-coated slides.

Endogenous peroxidase was blocked for 20 minutes at room temperature in PB saline (PBS) containing 3% H2O2 and 40% methanol. The slides were rinsed in PBS and incubated for 2 hours in 0.1% PBS and 0.3% Triton X-100 (PBST) containing 10% normal goat serum (NGS). The NR3A antibody mAb723 was used at a dilution of 1:10 in PBST and 10% NGS and incubated at 4°C overnight. After the sections were rinsed with PBST, they were incubated with biotinylated goat anti-rabbit serum (Sigma-Aldrich, Deisenhofen, Germany), diluted 1:100 in PBST and 5% NGS for 2 hours at room temperature. Slides were then rinsed in PBS and incubated for 2 hours with avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Inc., Burlingame, CA). After the slides were rinsed with PB, they were incubated in diaminobenzidine (DAB) solution with nickel enhancement (1 mg/mL DAB, 0.2% glucose, 0.004% NH4Cl, 0.05% (NH4)2WO4, 1 μL/mL glucose oxidase in PB) and coverslipped with glycerol.

To examine the specificity of the monoclonal anti-NR3A antibody we performed immunohistochemical experiments in retinal sections that were obtained from NR3A knockout mice.

In addition, four retinal wholemounts were prepared at P10 and P50 to determine the number of labeled cells in the GCL. After dissection from the eyecup, four radial cuts were made to allow flattening of the retinas. For morphometric analysis, one peripheral and one central area (0.1 mm2) was selected for each retinal quadrant at 1 and 3 mm from the optic disc, with a total of eight areas per retina.

**Preparation of Retinal Cells**

Retinal cells were prepared from P5 and P6 NR3A wild-type (+/−), heterozygous (+/−), and homozygous (−/−) NR3A-deficient mice. NR3A genotyping was determined by RT-PCR.24 We have described the dissociation of retinal cells in detail previously.25,26 Briefly, retinas were dissected and enzymatically dissociated in Hanks’ balanced salt solution (HBSS) with papain. Retinal cells were plated onto poly-L-lysine-coated glass coverslips in 35-mm culture dishes that contained 2 mL of Eagles’ minimum essential medium supplemented with 16.5 mM glucose, 2 mM glutamine, and 5% fetal calf serum. Retinal cultures were incubated in a humidified atmosphere of 5% CO2 in air at 37°C.

[Ca2+]i Measurements

[Ca2+]i was analyzed with fura-2 acetoxymethyl ester (fura-2/AM), as described previously.27–29 For [Ca2+]i measurements, retinal cultures were used 3 to 18 hours after plating. Just before the experiment, the culture medium was exchanged for a physiological saline based on Hanks’ balanced salts. This saline consisted of (in mM); NaCl, 157.6; NaHCO3, 1; NaH2PO4, 0.34; KCl, 5.36; KH2PO4, 0.44; CaCl2, 2.5; HEPES, 5; and dextrose, 22.2, adjusted to pH 7.2 with 0.3 M NaOH. To enhance responses to NMDA, experiments were performed in nominally magnesium-free solutions. Fura-2/AM (10 μM) was added in HBSS at room temperature for 60 minutes, and then the cells were rinsed in HBSS. Fura-2 fluorescence was excited by a 75-W xenon light source.

The excitation wavelength was alternated between 350 ± 10 and
380 ± 10 nm, with emission at 500 nm. Images were collected using an intensified charge-coupled device (CCD) camera (model QX-100; Quantex, Sunnyvale, CA) mounted on a microscope (IM-35; Carl Zeiss Meditec, Oberkochen, Germany). Exposure time for each image was 540 ms. Digital images were obtained before and during NMDA (200 μM) application. For the determination of [Ca2+]i in a neuron, we used the region of interest feature of the QFM program of the camera’s image processing system (Quantex). We defined the region of interest as the largest square that could be placed by eye on the inner circumference of the neuron to be analyzed. After subtraction of the background, fluorescence ratios were calculated on a pixel-by-pixel basis and then displayed in histogram form as the mean ± SD. The mean of all pixels analyzed in this way was chosen to represent [Ca2+]i for that cell. Several determinations were obtained to ensure that slight movement of the region of interest did not significantly influence the [Ca2+]i data obtained by this method. To ensure that the concentration of NMDA applied to the culture under our conditions did not saturate the fura-2 dye, we showed that the addition of the Ca2+ ionophore ionomycin produced a much greater increase in neuronal [Ca2+]i.

**RESULTS**

**RT-PCR Analysis**

RT-PCR was performed to screen for expression of NR3A transcripts in different ocular tissues (Fig. 1). PCR products corresponding to NR3A transcripts were detected in the mouse and rat retinas at all developmental stages from P1 to adult (P94). In contrast, no NR3A mRNA expression was observed in non-neuronal ocular tissues (i.e., the choroid or ciliary body).

**Immunohistochemistry**

NR3A protein expression was investigated by immunohistochemistry with the anti-NR3A monoclonal antibody mAb7. Specific immunostaining of NR3A protein was first detected at approximately P3 to P4. Labeling was mainly confined to cell bodies in the GCL (Fig. 2). Morphometric analysis showed that virtually all cells in the GCL were immunoreactive at P10 (96% ± 3%) but only 75% ± 9% of the cells were labeled at P30.

**FIGURE 1.** RT-PCR analysis of NR3A mRNA expression in the retina, non-neuronal ocular tissue and control tissue. Ethidium-bromide-stained agarose gel electrophoresis of RT-PCR products in choroid (lane 1), P3 retina (lane 2), P21 retina (lane 3), P30 retina (lane 4), P45 retina (lane 5), adrenal gland (lane 6), ciliary body (lane 7), olfactory bulb (lane 8), positive plasmid control (lane 9), negative control (lane C), with a 100-bp ladder of molecular size markers (lane M).

**FIGURE 2.** Immunohistochemistry of NR3A protein at (A) P6, (B) P14, and (C) P30. (A–C) Black arrowheads: NR3A expression in cells of the ganglion cell layer; white arrowheads: labeling of presumed amacrine cells in the inner nuclear layer. Only after P14 were distinct bands of NR3A labeling observed in the inner plexiform layer (C, D; white arrows); (D) Distinct bands of NR3A immunoreactivity in the inner plexiform layer at higher magnification. (E) P30 control section in which the first antibody is omitted. Arrows: nonspecific labeling of blood vessels (see also C). (F) Absence of labeling in the NR3A−/− retina is a further control for the specificity of NR3A labeling by mAb7 in wild-type mice. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20 μm.
Because in the rodent retina displaced amacrine cells make up approximately 40% of the cells in the GCL, this result indicates that both ganglion cells and displaced amacrine cells express NR3A. Occasionally, labeled cells in the inner part of the INL, where amacrine cells are located, were observed (Fig. 2C, white arrowheads). Two distinct, faint bands of punctate immunoreactivity in the inner plexiform layer were observed in animals older than P14, indicating possible synaptic localization of NR3A on ganglion cell and amacrine cell dendrites (Figs. 2C, 2D; white arrows). Blood vessels were nonspecifically stained by the second antibody, as shown in a control section in which the first antibody was omitted (Fig. 2E, arrows; see also Fig. 2C). In addition, specificity of NR3A expression in the retina as identified by mAb7 was further demonstrated by the absence of immunoreactivity in NR3A<sup>−−</sup> mice (Fig. 2E; Das et al.<sup>24</sup>).

**NMDA-Induced Elevation of [Ca<sup>2+</sup>]i in Retinal Cells from NR3A Knockout Mice**

Functional aspects of NR3A were investigated by measuring NMDA-induced increases in [Ca<sup>2+</sup>]i in retinal cells prepared from NR3A<sup>−−</sup>, NR3A<sup>+/−</sup>, and NR3A<sup>/−</sup> mice.<sup>24</sup> Only amacrine cells and retinal ganglion cells have been observed to express functional NMDARs (Guenther et al., unpublished data, 2002) which is in agreement with our immunocytochemical data of NMDAR localization at young developmental stages.<sup>5</sup> Thus, increases in [Ca<sup>2+</sup>]i, induced by activation of NMDARs by exogenously applied NMDA can be assigned to these two retinal cell types. NMDA-evoked [Ca<sup>2+</sup>]i responses were significantly greater in retinal cells prepared from NR3A homozygous knockout mice compared with the [Ca<sup>2+</sup>]i responses in cells prepared from the wild-type mice (Fig. 3A). NMDA-induced [Ca<sup>2+</sup>]i responses were blocked by the NMDAR antagonist d-2-amino-5-phosphonovalerate (APV) in both wild-type and NR3A<sup>−−</sup> cells (data not shown). Retinal ganglion cells and amacrine cells have been shown to coexpress multiple heterologous non-NMDARs.<sup>6,27</sup> These non-NMDARs may be activated after NMDA-induced release of endogenous glutamate and thus may contribute to the [Ca<sup>2+</sup>]i elevation. Thus, to isolate specifically the NMDA-induced [Ca<sup>2+</sup>]i effects, the experiments were repeated in the presence of the non-NMDAR antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). NMDA-induced [Ca<sup>2+</sup>]i responses were significantly greater in retinal cells from NR3A<sup>−−</sup> mice than in wild-type mice (Fig. 3B), indicating that NR3A regulates NMDA-receptor function. To determine the possible effect of the NR3A subunit on non-NMDAR function, kainate-induced [Ca<sup>2+</sup>]i responses were studied in the presence of the NMDA-receptor antagonist APV. In an unexpected finding, kainate-induced [Ca<sup>2+</sup>]i responses were significantly smaller in the retinal cells from NR3A<sup>−−</sup> mice than in those from wild-type mice (Fig. 3C).

**DISCUSSION**

In the present study, the NMDAR subunit NR3A was present in the rodent retina from P3 on. Because other NR subunits (NR1, NR2A-D) have been observed from P1 on,<sup>3</sup> expression of functional NMDARs at this developmental age is highly likely and collected before and 14 seconds after addition of 200 μM NMDA (A), 200 μM NMDA plus 20 μM CNQX (B), or 50 μM kainate plus 400 μM APV (C). Data are the mean ± SEM of 70 to 200 cells from 5 to 12 animals (A), 70 to 100 cells from 8 animals (B), and 60 to 70 cells from 5 animals (C). Significant difference between wild-type and NR3A knockout mice (P < 0.001 by ANOVA followed by the Scheffé multiple comparisons of means).
corresponds well with our electrophysiological data (Schmid S, et al. IOVS 1998;39;ARVO Abstract 4554). After P14, a synaptic localization of NR3A was suggested by the distinct immunoreactivity in two bands within the inner plexiform layer. These bands appeared similar to NR1-immunoreactivity and correspond to the on- and off-sublaminae of the inner plexiform layer. No NR3A mRNA or protein expression was observed in non-neuronal ocular tissues.

Functional NMDAR channels in situ are thought to be formed by heteromeric expression of NR1 with one or more NR2 subunits. Each subunit combination may impart unique functional properties to the receptor-channel complex. The recent discovery of an additional NMDAR subunit, NR3A, suggests that the stoichiometry and functional diversity of NMDARs is more complex than previously thought. NR3A was initially cloned from retinal cDNA, and although the expression of NR3A in oocytes did not lead to the formation of homomeric functional channels, coexpression with NR1 and -2A or -2B led to a significant decrease in the current’s magnitude, suggesting that NR3A forms stable complexes with NR1 and NR2 subunits and modulates the receptor’s functional characteristics. Our new results show that the NMDA-induced increase in \( [\text{Ca}^{2+}]_i \) was significantly greater in retinal cells prepared from NR3A-knockout mice, suggesting that NR3A plays an important role in determining the function of the native receptors. Consistent with these results, cortical neurons from NR3A-knockout mice showed increased NMDA-evoked currents. It is interesting to note that the mean non-NMDA-mediated \( [\text{Ca}^{2+}]_i \) response was decreased in retinal cells of NR3A-knockout mice compared with those of wild-type mice. The mechanism and functional significance of this effect of NR3A on non-NMDARs or on downstream events, such as activation of voltage-dependent calcium channels, is not clear. Along similar lines, the NMDAR-mediated synaptic current was potentiated, whereas the non-NMDA component of synaptic current was reduced in NR2C knockout mice. In our studies, the kainate-induced \( [\text{Ca}^{2+}]_i \) responses exceeded 1 \( \mu M \) and therefore approached the maximum sensitivity of furin-2. Hence, no definitive statement about the absolute magnitude of the responses can be made. Nonetheless, the fact that kainate-induced responses were smaller in NR3A-knockout mice may indicate some type of compensation for the enlarged \( [\text{Ca}^{2+}]_i \) responses engendered by NMDAR activation in these mice.

Although, the presence of functional NMDARs in the RGCs has been well established, their physiological importance remains unclear; NMDARs were found to mediate only a minor component of the light response and NMDA currents seem to be downregulated in retinal ganglion cells after eye opening, depending on ambient light conditions (Albash G, et al. IOVS 2002;43;ARVO E-Abstract 2707). On the contrary, the toxic effects of glutamate appear to be predominantly mediated through NMDARs, and this may underlie several diseases including glaucoma.

In conclusion, the expression profile of the NR3A subunit described in the present study is consistent with earlier findings suggesting that NMDARs in ganglion cells and displaced amacrine cells contain the NR3A subunit. The increase in \( [\text{Ca}^{2+}]_i \) concentration of retinal cells after NR3A knockout is consistent with a possible role of NMDARs in retinal outgrowth, circuitry, and survival.

References


18. Stahn JS, Aizenman E, Lipton SA. Central mammalian neurons normally resistant to glutamate toxicity are made sensitive by elevated extracellular Ca²⁺: toxicity is blocked by the N-methyl-D-aspartate antagonist MK-801. Proc Natl Acad Sci USA. 1988:85; 6556–6560.
27. Zhang D, Sucher NJ, Lipton SA. Co-expression of AMPA/kainate
receptor-operated channels with high and low Ca\(^{2+}\) permeability
188.

28. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca\(^{2+}\) indi-
cators with greatly improved fluorescence properties. *J Biol

29. Sucher NJ, Lipton SA. Redox modulatory site of the NMDA recep-
tor-channel complex: regulation by oxidized glutathione. *J Neuro-

30. Meguro H, Mori H, Araki K, et al. Functional characterization of a hetero-

31. Stern P, Behe P, Schoepfer R, Colquhoun D. Single-channel con-
ductances of NMDA receptors expressed from cloned cDNAs:

32. Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH.
Developmental and regional expression in the rat brain and func-
540.

5025.

34. Aizenman E, Frosch MP, Lipton SA. Responses mediated by excitatory amino acid receptors in solitary retinal ganglion cells from rat. *J Physiol (Lond)*. 1988;396:75–91.

35. Cohen ED, Miller RF. The role of NMDA and non-NMDA excitatory