Distinct Perisynaptic and Synaptic Localization of NMDA and AMPA Receptors on Ganglion Cells in Rat Retina

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ABSTRACT
At most excitatory synapses, AMPA and NMDA receptors (AMPARs and NMDARs) occupy the postsynaptic density (PSD) and contribute to miniature excitatory postsynaptic currents (mEPSCs) elicited by single transmitter quanta. Juxtaposition of AMPARs and NMDARs may be crucial for certain types of synaptic plasticity, although extrasynaptic NMDARs may also contribute. AMPARs and NMDARs also contribute to evoked EPSCs in retinal ganglion cells (RGCs), but mEPSCs are mediated solely by AMPARs. Previous work indicates that an NMDAR component emerges in mEPSCs when glutamate uptake is reduced, suggesting that NMDARs are located near the release site but perhaps not directly beneath in the PSD. Consistent with this idea, NMDARs on RGCs encounter a lower glutamate concentration during synaptic transmission than do AMPARs. To understand better the roles of NMDARs in RGC function, we used immunohistochemical and electron microscopic techniques to determine the precise subsynaptic localization of NMDARs in RGC dendrites. RGC dendrites were labeled retrogradely with cholera toxin B subunit (CTB) injected into the superior colliculus (SC) and identified using postembedding immunogold methods. Colabeling with antibodies directed toward AMPARs and/or NMDARs, we found that nearly all AMPARs are located within the PSD, while most NMDARs are located perisynaptically, 100–300 nm from the PSD. This morphological evidence for exclusively perisynaptic NMDAR localizations suggests a distinct role for NMDARs in RGC function. J. Comp. Neurol. 498:810–820, 2006. Published 2006 Wiley-Liss, Inc.†

Indexing terms: NMDA; AMPA; synaptic and perisynaptic distribution; postembedding immunogold; retinal ganglion cell

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS), including the retina (Slaughter and Miller, 1983; Copenhagen and Jahr, 1989; Brandstätter et al., 1998). At most mature excitatory synapses, AMPA and NMDA receptors (AMPARs and NMDARs) contribute to evoked and miniature excitatory postsynaptic currents (mEPSCs) (Bekkers and Stevens, 1989; McBain and Dingledine, 1992; Silver et al., 1992; but see Clark and Cull-Candy, 2002), and immunogold electron microscopy (EM) studies indicate that NMDARs and AMPARs are colocalized in the postsynaptic density (PSD) (Kharazia and Weinberg, 1997; Bernard and Bolam, 1998; Kharazia and Weinberg, 1999; Nusser, 2000). Extrasynaptic NMDARs also may participate in synaptic transmission and plasticity (Massey et al., 2004; Scimemi et al., 2004), but their localization pattern has not been analyzed by immunogold EM.

Retinal ganglion cells (RGCs) receive excitatory glutamatergic input from bipolar cells (Wässle and Boycott, 1991). Although evoked EPSCs in RGCs are mediated by AMPARs and NMDARs (Mittman et al., 1990; Diamond and Copenhagen, 1993; Lukasiewicz et al., 1997; Matsui et al., 1998; Higgs and Lukasiewicz, 1999; Matsui et al.,

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PERISYNAPTIC NMDARS ON RETINAL GANGLION CELLS

1999; Chen and Diamond, 2002), spontaneous mEPSCs on RGCs are mediated solely by AMPARs both in mammalian and amphibian retina (rat: Chen and Diamond, 2002; salamander: Taylor et al., 1995; newt: Matsui et al., 1998). NMDARs encounter a lower glutamate concentration during evoked EPSCs than do AMPARs and an NMDAR component emerges in mEPSCs when glutamate uptake is reduced (Chen and Diamond, 2002), suggesting that NMDARs may be located extrasynaptically on RGC dendrites (see also Matsui et al., 1998). This is in apparent contrast to preembedding EM studies in mammalian retina indicating that NMDAR subunits are present in postsynaptic elements at cone bipolar cell ribbon synapses (Hartveit et al., 1994; Fletcher et al., 2000; Pourcho et al., 2001). These studies did not determine the identity of the immunopositive postsynaptic process, however, and the preembedding immunoperoxidase method, which allows reaction product to diffuse throughout the immunopositive process, precludes distinction between localization to the PSD versus the immediately surrounding extrasynaptic membrane (Ottersen and Landsend, 1997; Nusser, 2000; Breit and Nicoll, 2003). In the case of AMPARs, physiological and anatomical data are consistent with a synaptic localization on RGCs (Mittman et al., 1990; Taylor et al., 1995; Qin and Pourcho, 1996; Lukasiewicz et al., 1997; Qin and Pourcho, 1999; Jacoby and Wu, 2001; Chen and Diamond, 2002), although subsynaptic localization of AMPAR and the identity of immunopositive processes have not been examined with immunogold EM.

Here we examine the precise localization of NMDARs and AMPARs on dendritic membranes in rat retina with high-resolution postembedding immunogold EM while positively identifying RGC processes with cholera toxin subunit B (CTB) retrograde tracing methods. We find that the large majority of NMDARs on RGC membranes are located at perisynaptic sites, whereas most AMPARs are localized to the PSD. NMDAR density is highest 100–300 nm from the edge of the PSD, suggesting that the receptors are not scattered evenly throughout the extrasynaptic membrane but are instead targeted to specific perisynaptic locations. These results indicate a novel subsynaptic localization pattern for NMDARs at a central synapse and suggest that NMDARs may play a distinct role in the integration of synaptic input by RGCs.

MATERIALS AND METHODS

CTB injection

Care and handling of animals were in accordance with NIH Animal Care and Use Committee guidelines. Postnatal day (P)15 Sprague-Dawley rats, maintained on a 12:12-hour light-dark cycle, were anesthetized with pentobarbital and immobilized in a stereotaxic frame. The skin over the skull was incised and small holes were drilled in the skull at sites above the right and left superior colliculus (SC), and 1–1.2% CTB (List Biological Laboratories, Campbell, CA) was injected bilaterally (2 μL each site).

Tissue fixation

Five to seven days following the injection, animals were deeply anesthetized with halothane, decapitated, and both eyes were removed and hemisected. For LM immunofluorescence, eyecups were then fixed in 4% paraformaldehyde, eyes were removed and hemisected. For LM immunofluorescence and EM immunocytochemistry, retinas were isolated, immediately cut into 100–200 μm-thick strips, and subjected to pH-shift fixation (Sassoe-Pognetto and Ottersen, 2000, with minor modifications). Retina strips were fixed in 4% paraformaldehyde in 0.1 M PB at pH 6.0 for 20–30 minutes and then in 4% paraformaldehyde plus 0.01% glutaraldehyde at pH 10.5 for 10–20 minutes at RT. After several washes in PB with 0.15 mM CaCl₂ (pH 7.4 at 4°C), the tissue was cryoprotected with glycerol (60 minutes each in 10%, 20%, 30%, then overnight in 30%) in 0.1 M PB prior to freeze substitution and low-temperature embedding.

Primary antibodies

Antibodies directed toward NMDAR subunits (NR1 and NR2) and AMPAR subunits (GluR2/3 and GluR4) were purchased from Chemicon (Temecula, CA). Polyclonal anti-NR1 (Cat. no. AB1516) was raised in rabbit against a synthetic peptide (LQNQKDTVLPRRAIEREEGQLQLCSRHRES) corresponding to the C-terminus of the rat NR1 subunit and recognizes major splice variants 1a, 1b, 2a, and 2b. This antibody has been characterized previously in rat retina by Western blotting (recognizing a band at ~116 kD) and immunocytochemistry (Fletcher et al., 2000; Gründler et al., 2000). Polyclonal anti-NR2B (Cat. no. AB1557P) was raised in rabbit against an affinity-purified C-terminal fusion protein (amino acids 984–1104). This antibody has been characterized previously in rat and cat retina by Western blotting (recognizing a band at ~150 kD) and immunocytochemistry (Goebel et al., 1998; Gründler et al., 2000; Pourcho et al., 2001). Polyclonal anti-GluR2/3 (Cat. no. AB1506) was raised in rabbit against an affinity-purified peptide (EGYNCYGIESVKI from the carboxy terminus of rat GluR2) and recognizes both GluR2 and GluR3. Polyclonal anti-GluR4 (Cat. no. AB1506) was raised in rabbit against an affinity-purified peptide from the carboxy terminus (RQQSSLAVIASDL). These two AMPAR antibodies have been widely used in mammalian retina including rat and characterized previously by Western blotting (recognizing a band at 100–110 kD) and immunocytochemistry (Goebel et al., 1998; Hack et al., 2001, 2002; Gründert et al., 2002, 2003; Li et al., 2002). Polyclonal goat anti-CTB (List Biological; Cat. no. 104) has been characterized in rat retina by immunocytochemistry (Rivera and Lugo, 1998). In addition, the following antibodies have been used in studies of fish retina (Jan et al., 2001) and the CNS (Moga et al., 2003): polyclonal rabbit anti-PKC (Chemicon, to label rod bipolar cells); monoclonal mouse anti-GluR2 (Chemicon; Cat. no. MAB397), raised against a recombinant fusion protein TrpE-GluR2 (N-terminal portion, amino acids 175–430 of rat GluR2) and recognizing a band at ~102 kD; monoclonal mouse GluR3 (Chemicon, Cat. no. MAB5416), raised against a fusion protein (N-terminal portion, amino acids 245–451 of rat GluR3) and recognizing a band at ~110 kD.
LM immunofluorescence

LM immunofluorescence was performed as described previously (Zhang et al., 2003). Slides were rinsed in PBS, blocked in 5% normal donkey serum (NDS, Sigma, St. Louis, MO) in PBS for 1 hour, incubated overnight in primary antibodies (CTB diluted 1:4,000, or NR1 diluted 1:30, or NR2B diluted 1:60, or GluR2/3 diluted 1:50, or GluR4 diluted 1:50) in 2% NDS plus 1% bovine serum albumin (BSA, Sigma) with 0.3% Triton X-100 at RT for anti-NMDA and at 4°C for others. After rinsing, sections were incubated for 2 hours at RT in Cy3- or FITC-conjugated donkey antigoat IgG (1:400 and 1:100, respectively) for CTB and Cy3- or FITC-conjugated donkey antirabbit IgG (1:400 and 1:100, respectively) for NR1, NR2B, GluR2/3, and GluR4. All fluorescent secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Slides were rinsed and coverslipped with Vectashield (Vector, Burlingame, CA).

Whole-mounted retinas were incubated in anti-CTB for 5–7 days and the secondary antibody for 1 day at 4°C.

For double labeling, sections were incubated overnight in a mixture of primary antibodies (CTB 1:4,000 + PKC 1:100, or CTB 1:4,000 + NR1 1:30 or NR2B 1:60, or CTB 1:4,000 + GluR2/3 1:50 or GluR4 1:50) in 5% NDS plus 2% BSA with 0.3% Triton X-100. Sections were rinsed and then incubated for 2 hours at RT in a mixture of two secondary antibodies (FITC-conjugated donkey antigoat IgG and Cy3-conjugated donkey antirabbit IgG). Procedures for washing between and after antibody incubations were the same as for single labeling.

Specificity of immunostaining for anti-NR1, NR2B, GluR2/3, and GluR4 was confirmed with control experiments in which the antibodies were preadsorbed with the polypeptide fragments or, in the case of the NR2B antibody, the fusion protein used as the antigen (all from Chemicon: Cat. no. AG344 [NR1], Cat. no. AG262 [NR2B], Cat. no. AG305 [GluR2/3], Cat. no. AG306 [GluR4]). No fluorescence was detected with preadsorbed primary antibodies (Fig. 1), or when primary antibodies were omitted in the first incubation, and no cross-reactivity between unmatched secondary antibodies was detected (data not shown).

Immunoreactivity (IR) was visualized with a confocal laser scanning microscope (Zeiss LSM-510; Thornwood, NY) through 25× and 63× oil objectives. Brightness and contrast of the final images were adjusted in Adobe Photoshop 6.0 (San Jose, CA).

EM immunocytochemistry

Freeze substitution was performed as described (Petralia and Wenthold, 1999), with minor modifications. Following cryoprotection, retinal strips were plunge-frozen in liquid propane at –190°C in a Leica EM CPC (Leica, Austria). The tissue was then transferred into a freeze-substitution device (Leica EM APS) and treated with 0.5% uranyl acetate in 100% methanol at –90°C for 36 hours, after which the temperature was increased stepwise to –45°C. Samples were washed several times in precooled methanol and progressively infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Fort Washington, PA) (1:1 Lowicryl to methanol, 2 hours; 2:1 Lowicryl to methanol, 2 hours; 100% Lowicryl, 2 hour; 100% Lowicryl, overnight) at –45°C. Finally, the samples were polymerized (–45°C to 0°C) with ultraviolet light for 40 hours and then at RT overnight.

One μm semissections were oriented to achieve optimally transverse –70-nm-thick ultrasections, which were collected on Formvar-carbon-coated nickel-slot grids. Postembedding immunocytochemistry was performed as described (Yang et al., 2003; Zhang et al., 2004) with minor modifications. Grids were washed with distilled H2O followed by a Tris-buffered saline (TBS, 0.05 M Tris buffer, 0.7% NaCl, pH 7.6) wash, incubated in 5% BSA in TBS for 30 minutes, and then incubated in 30-μL drops of

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**Fig. 1.** Preadsorption with antigen blocks primary antibody immunoreactivity. A: Images of retinal vertical sections showing anti-NR1 labeling pattern in control (A1) and following preadsorption of the antibody with the peptide antigen (A2). Panel A3 shows a representative EM section incubated with preadsorbed primary antibody.

B: As in A, but with the anti-NR2B antibody. C: As in A, but with the anti-GluR2/3 antibody. D: As in A, but with the anti-GluR4 antibody. In EM images, presynaptic ribbons are indicated by arrowheads. Scale bars = 20 μm and 0.1 μm for light and electron micrographs, respectively.
antigoat CTB (1:3,000) in TBS-Triton (TBST, 0.01% Triton X-100, pH 7.6) with 2% BSA and 0.02 M NaN₃ overnight at RT. Grids were washed on three separate drops of TBS (pH 7.5) for 10, 10, and 20 minutes, followed by TBS (pH 8.2) for 5 minutes. Grids were then incubated for 2 hours on drops of donkey antigoat IgGs coupled to 15 or 18 nm gold particles (Electron Microscopy Sciences) in TBST (pH 8.2) with 2% BSA and 0.02 M NaN₃. Following washes in TBS (pH 7.6) for 5, 5, and 10 minutes, grids were washed in ultrapure water and dried. Grids were counterstained with 5% uranyl acetate and 0.3% lead citrate in distilled H₂O for 8 and 5 minutes, respectively.

In double-labeling experiments, grids were incubated overnight at RT either with a mixture of antigoat CTB (1:3,000) and antirabbit NMDARs (NR1 1:10 and NR2B 1:30) or a mixture of CTB (1:3,000) and AMPARs (GluR2/3 1:30 and GluR4 1:30), followed by a mixture of donkey antigoat IgG (1:20) coupled to 15 or 18 nm gold particles and donkey antirabbit IgG (1:20) coupled to 10 nm gold particles, respectively. In triple-labeling experiments, grids were first incubated with a mixture of CTB (1:3,000) and NMDARs (NR1 1:10 and NR2B 1:30), followed by antigoat AMPARs (GluR2 1:30 and GluR3 1:30), then by a mixture of IgGs coupled to 15, 10, and 5 nm gold particles (Electron Microscopy Sciences), respectively. Procedures for washing and counterstaining between and after antibodies incubation were the same as for single labeling.

Specificity of immunostaining at the EM level for antigoat IgG (1:3,000) and antirabbit NMDARs (NR1 1:10 and NR2B 1:30) was assessed when the secondary antibodies were applied alone, either for CTB single labeling or for double- or triple-labeling with NMDARs and/or AMPARs (data not shown). In addition, when one or two primary antibodies were eliminated and then the two or three appropriate secondary antibodies were applied in double or triple labelings, only gold specific for the remaining primary antibody was detected.

Grids were viewed on a JEOL 1200 EM and images were digitalized. Final figures were processed only for brightness and contrast and annotations were added in Adobe Photoshop 6.0.

**Data collection**

Ultrathin sections were prepared from six rats and sections containing inner plexiform layer (IPL) were chosen randomly for double labeling (CTB and NMDARs or AMPARs) and were photomontaged at 25,000× magnification. Each montage covered the full depth of the IPL, with a total area of 9,892 µm² for NMDARs and 8,959 µm² for AMPARs, respectively. RGC dendrites at cone bipolar terminals, labeled with antibodies to PKC (Kosaka et al., 1988; Vecino et al., 2002) that labeled the optic nerve bundles, and proximal dendrites were clearly distinguished (Fig. 2A–D), similar to reports in ground squirrels showing that three different sizes of ganglion cells were retrogradely labeled when CTB was injected bilaterally into the SC (Rivera and Lugo, 1998). In samples of 22 whole-mounted retinas, the average density of labeled RGCs was 3,214 ± 685 cells/mm², comparable to previous measures obtained with other retrograde tracers in rat retina (2,116–2,725 cells/mm²: Linden and Perry, 1983; Villegas-Perez et al., 1988; Vecino et al., 2002) that labeled ~90% of the ganglion cell population (Linden and Perry, 1983). The primary dendrites of CTB-labeled RGCs clearly extended into the IPL and their higher-order branches and distal dendrites formed two bands in the IPL (Fig. 2B,D). At higher magnification, two distinct bands were evident in the outer and middle thirds of the IPL, which may correspond to regions of input from OFF and ON cone bipolar terminals, respectively (Nelson et al., 1978; Peichl and Wässle, 1981; Amthor et al., 1989), whereas rod bipolar terminals, labeled with antibodies to PKC (Kosaka et al., 1998) occupied the inner third (Fig. 2F). This morpho-
logical view of IPL sublaminae, in which the division between sublamina a and b occurs at the outer third of the IPL, is similar to that described in cat (Nelson et al., 1978; Peichl and Wässle, 1981) but contrasts slightly with physiological experiments in rat suggesting that the a–b border occurs in the middle of the IPL (Euler et al., 1996). Additionally, a relatively small number of CTB-positive “displaced” RGCs appeared in the INL (Fig. 2C,E/F). Bunt et al. (1974; Buhl and Dann, 1988).

At the ultrastructural level, gold particles labeling CTB were present in the perikarya and proximal and distal dendrites of RGCs but were absent from the nucleus (Fig. 2G,H). CTB-positive RGC dendrites were found in sublamina a and b of the IPL and rarely constituted more than one of the two postsynaptic elements in the dyad synapse (Fig. 2I,J). The mean density of CTB-associated gold particles in RGC dendrites (15.45 ± 2.97 gold/μm²; n = 37) was 59 times higher than that in Müller cell processes (0.26 ± 0.04 gold/μm²; n = 35; P < 0.001), indicating that CTB gold labeling was specific to RGC processes.

NMDARs on RGCs are distributed perisynaptically

NMDARs were labeled with antibodies recognizing NR1 and NR2B subunits (Takumi 1999). IR at the LM level was similar to previous reports in rat retina (Fletcher et al., 2000; Kalloniatis et al., 2004). Intense, punctate labeling was evident in the IPL and outer plexiform layer (OPL), in addition to somatic labeling in the GCL and INL (Fig. 3A,C). Double labeling of NR1 or NR2B and CTB showed that all CTB-positive RGCs were NR1- or NR2B-positive (Fig. 3B,D). Not all NR1 or NR2B immunopositive puncta were colocalized with CTB-labeled ganglion cell dendrites, however, indicating that NMDARs also are expressed in other cell types. NR1 and NR2B IR also was evident in the outer plexiform layer.

At the EM level, antibodies toward NR1 and NR2B were combined (Nusser et al., 1998; Petralia et al., 1999; Petralia and Wenthold, 1999; Takumi et al., 1999; Racca et al., 2000) and applied to tissue that had been retrogradely labeled with CTB. The large majority (97%) of NMDAR-immunogold particles was located outside the PSD, distributed primarily along the extrasynaptic plasma membrane (Figs. 3E,F, 5A). Similar patterns were observed in sublamina a and b of the IPL (Fig. 3E,F); the density of gold particles in the two sublaminae were not significantly different (2.91 ± 0.41 [n = 24] vs. 3.54 ± 0.23 [n = 27], P > 0.1). Immunogold particles were detected mostly on the intracellular surface of the extrasynaptic plasma membrane; some particles were present in the postsynaptic cytoplasm of RGC dendrites and somata (data not shown), consistent with LM results (Fig. 3A–D). In the single instance in which both postsynaptic elements at the dyad were CTB-positive RGC processes, both expressed NMDARs perisynaptically (data not shown). In 51 identified RGC dendrites at cone dyad, 92% (47) expressed NMDARs perisynaptically and 8% (4) exhibited both synaptic and extrasynaptic expression; 80% of the other CTB-negative postsynaptic process (presumably amacrine cells) were immunonegative (Fig. 3F), consistent with previous reports (Fletcher et al., 2000; Pourcho et al., 2001).

The tangential distribution of NMDAR gold particles was analyzed within and outside the synapse. Particles within the synapse were located in the lateral margin of
affixed RGC dendrites and somata (yellow).

E,F: on the perisynaptic plasma membrane (F). Scale bar

CTB-labeled RGC dendrite in which small gold particles (arrow) were

lamina, two ribbons (arrowheads) each making a dyad with the same

sublamina, a cluster of small gold particles (arrow) in the perisynaptic

ticles) and CTB (large particles). At a cone dyad in the OFF

membrane of RGC dendritic profiles (1592

480 nm of the PSD edge (Fig. 5A).

synaptically, 76% of the gold particles were located within

the PSD but were not seen in the middle (Fig. 5B). Extrasynaptically, 76% of the gold particles were located within

480 nm of the PSD edge (Fig. 5A).

The mean length of the extrasynaptic plasma membrane of RGC dendritic profiles (1592 ± 96 nm, n = 51) was much greater than that of the PSD (180 ± 8 nm, n = 51), which would favor detection of extrasynaptic receptors. However, the mean density of extrasynaptic NMDAR gold particles (2.98 ± 0.24 per μm) was almost four times greater than that in the PSD (0.77 ± 0.38 per μm, P < 0.001; Table 1). To compare density in the PSD and at varying distances from the synapse, the distance along the membrane between each membrane-associated extrasynaptic particle and the edge of the PSD was measured and collected into 180-nm bins (Fig. 5C). Extrasynaptic NMDAR density within 720 nm of the edge of the synapse was 3–11 times higher than in the PSD (Fig. 5C, P < 0.001). Immunopositive processes usually contained nume-

rous perisynaptic gold particles (Fig. 5D), enhancing the precision of density calculations.

AMPARs on RGCs are localized primarily within the PSD

AMPARs were labeled with antibodies recognizing the GluR2/3 and GluR4 subunits (Takumi et al., 1999). IR at the LM level was evident primarily in the OPL and IPL; GluR4 signal in the IPL segregated clearly into two distinct bands, but no such stratification was observed with GluR2/3 (Fig. 4A–D), similar to previous studies in rat retina (Peng et al., 1995; Hack et al., 2002). These antibodies were combined to enhance the signal for EM immunocytochemistry. At the EM level, 77% of AMPAR immunogold particles in RGC dendritic profiles were located within the PSD (Figs. 4E,F, 5E). A consistent postsynaptic labeling pattern was observed throughout the IPL, and the density of AMPAR immunogold was not significantly different in sublamina a and b (25.35 ± 5.89 [n = 21] vs. 26.67 ± 8.87 [n = 22], P > 0.5). 23% of AMPAR immunogold was associated with the extrasynaptic plasma membrane (Fig. 4F) or in the RGC dendritic cytoplasm. 43 of 48 identified RGC dendrites (90%) expressed AMPARs synaptically; the remaining profiles had no gold in the PSD but exhibited IR either extrasynaptically or in the cytoplasm. Generally, when the postsynaptic RGC profile was AMPAR-positive, the other postsynaptic process in the dyad was negative (Fig. 4E,F), consistent with previous reports (Qin and Pourcho, 1996, 1999; Brandstätter, 2002).

The tangential distribution of gold particles within the synapse indicated that AMPA-IR was directed toward the center of the PSD (Fig. 5F). Gold particles at extrasynaptic membranes were observed only rarely (Fig. 5E). The mean length of the PSD for AMPAR-positive RGC dendrites was 181 ± 9 nm (n = 43), nearly identical to the PSDs in NMDAR-positive RGC dendritic profiles. The mean particle density of AMPARs in the postsynaptic membrane (15.8 ± 1.8 gold/μm²) was significantly greater than that in extrasynaptic membrane (0.55 ± 0.15 gold/μm², P < 0.0001; Table 1). AMPAR immunogold density within the PSD was 7–66 times higher than in the perisynaptic membrane (Fig. 5G).

**TABLE 1. Comparison of Immunogold Densities (Mean ± SE, Gold/μm²) of NMDARs and AMPARs in Various Cell Membranes**

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<thead>
<tr>
<th>Location</th>
<th>NMDA</th>
<th>AMPA</th>
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<tr>
<td>I. The PSD of RGC</td>
<td>0.77 ± 0.38 (51)*</td>
<td>15.76 ± 1.78 (43)</td>
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<tr>
<td>II. Extrasynaptic membrane of</td>
<td>2.98 ± 0.24 (51)</td>
<td>0.55 ± 0.15 (43)</td>
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<tr>
<td>RGC</td>
<td>2.98 ± 0.24 (51)</td>
<td>0.55 ± 0.15 (43)</td>
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<tr>
<td>III. Mitochondrial outer membrane</td>
<td>0.06 ± 0.02 (47)</td>
<td>0.08 ± 0.02 (43)</td>
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<td>IV. Muller cell membrane</td>
<td>0.07 ± 0.02 (44)</td>
<td>0.11 ± 0.03 (43)</td>
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<td>Ratios of signal to noise (/ test)</td>
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<td>I/III</td>
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<td>II/IV</td>
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*The number of samples. ** P < 0.1 *** P < 0.005 **** P < 0.0001 (Two-sided t test)
Simultaneous labeling of NMDARs and AMPARs in CTB-positive RGCs

In a subset of experiments, tissue was triple-labeled for CTB, AMPARs, and NMDARs. Of 50 identified RGC dendrites at cone dyads, 40 (80%) were simultaneously positive for synaptic AMPARs and extrasynaptic NMDARs.

Fig. 4. AMPAR immunoreactivity in the rat retina. A,C: GluR2/3 and GluR4 immunoreactivity in the IPL, OPL, and in somata within the GCL and INL. B,D: GluR2/3- and GluR4-positive puncta colocalized with CTB-labeled RGC dendrites and somata (yellow). E,F: Electron micrographs showing double immunogold labeling of AMPARs (small golds) and CTB (large golds) in the OFF and ON sublamina, respectively; small gold particles are clustered (large arrows) in the PSD of RGC processes. F: A rare example of an AMPA-positive RGC process expressing AMPARs perisynaptically (small arrows). Scale bars = 20 μm in A–D; 0.1 μm in E,F.

Fig. 5. Quantitative comparison of NMDARs and AMPARs localization in RGC dendrites. A,E: Histograms show the tangential distribution of immunogolds for NMDARs (n = 51, A) and AMPARs (n = 48, E) within and outside the PSD. The perisynaptic region was divided into 60 nm bins. B,F: Histograms show the tangential distribution of immunogold particles labeling NMDARs (n = 4 synapses, B) and AMPARs (n = 48 synapses, F) within the PSD. C,G: Histograms show the density of immunogold particles labeling NMDARs (n = 51, C) and AMPARs (n = 48, G). The perisynaptic region was divided into 180 nm bins. D,H: Histograms showing the number of gold particles detected at NMDAR- and AMPAR-immunopositive synapses, respectively.

Simultaneous labeling of NMDARs and AMPARs in CTB-positive RGCs

In a subset of experiments, tissue was triple-labeled for CTB, AMPARs, and NMDARs. Of 50 identified RGC dendrites at cone dyads, 40 (80%) were simultaneously positive for synaptic AMPARs and extrasynaptic NMDARs.
Fig. 6. Simultaneous labeling of NMDARs, AMPARs, and RGCs. Electron micrographs showing triple immunogold labeling of AMPARs (5 nm gold), NMDARs (10 nm gold), and CTB (18 nm gold). A,B: AMPAR gold particles (small arrows) were located in the PSD while NMDAR gold particles (large arrows) were perisynaptic on individual CTB-positive RGC dendrites in the ON and OFF sublamina, respectively. C: A rare example of NMDARs and AMPARs colocalized within the PSD. Scale bar = 0.1 μm.

(Fig. 6A,B), five (10%) were AMPAR-negative synaptically but NMDAR-positive extrasynaptically, and five (10%) were NMDAR-negative synaptically. Three of the five profiles in this last group were AMPAR-positive synaptically (Fig. 6C), indicating that NMDARs and AMPARs are colocalled in the PSD of only a very small fraction (6%) of RGC dendrites.

DISCUSSION

The results presented here demonstrate strikingly different subsynaptic localization patterns of AMPARs and NMDARs on RGC dendrites in rat retina. Most AMPARs are located within the PSD, similar to most central synapses, but nearly all NMDARs are located extrasynaptically. NMDARs are not merely scattered evenly throughout the extrasynaptic membrane, however; rather, they are located primarily 100–300 nm from the edge of the PSD, suggesting that they are anchored at specific locations by submembrane receptor scaffolding proteins in RGC dendrites. The contrasting localization patterns of NMDARs and AMPARs do not reflect distinct synapse populations because they appear at individual synapses that are immunopositive for both receptor types. Moreover, similar patterns occur in sublaminae a and b of the IPL, indicating that synapses onto ON and OFF RGCs express AMPARs synaptically and NMDARs perisynaptically.

Methodological considerations

A range of different fixation methods and times were tested in an effort to strike an optimal balance between ultrastructural integrity and antigenicity. Gentle fixation and short incubation times were necessary because NMDA receptor antigenicity decreases markedly with strong, prolonged fixation (Fletcher et al., 2000). In our hands, existing fixation protocols used for postembedding NMDAR EM (Petralia and Wenthold 1999; Petralia et al., 1999), when applied to retina, yielded undetectable NMDAR IR, although tissue preservation was improved relative to that shown here.

Most functional NMDARs comprise two NR1 and two NR2 subunits (Dingledine, 1999); many functional properties and targeting characteristics appear to be determined by the specific complement of NR2 (A–D) subunits (Cull-Candy and Leszkiewicz, 2004). For example, extrasynaptic NMDARs contain primarily the NR2B subunit, while NR2A-containing receptors are located primarily in the PSD (Tovar and Westbrook, 1999; Hartveit et al., 1994). Preliminary data indicates that NR2B-containing receptors contribute to light-evoked EPSCs (T. Kalbaugh and S. Chen, unpubl.), prompting us to use a cocktail of NR1 and NR2B antibodies. Antibodies to both subunits intensely stained the IPL and colocalled with CTB IR (Fig. 3B,D), suggesting that the NR1 and NR2B antibodies labeled RGC NMDARs with similar efficacy. Preliminary data indicate a similar, primarily perisynaptic pattern of NMDAR expression (although less intense labeling) when the NR1 antibody is used alone (Zhang and Diamond, unpubl.). It remains possible, however, that the small fraction (3%) of NMDARs in the postsynaptic density reflects NR2A-containing receptors, as suggested previously (Hartveit et al., 1994). Although immunofluorescent puncta for NR2A and NR2B subunits have been shown to be clustered at different synaptic “hot spots” in the IPL (Fletcher et al., 2000), further ultrastructural studies are required to determine the specific subsynaptic distribution of different NR2 subunits (NR2A-D) in RGC dendrites.

Perisynaptic location of NMDARs limits their activation during quantal events

The perisynaptic distribution of NMDARs observed here contributes strong morphological support to physiological
evidence that NMDARs on RGC dendrites are located a greater distance from the release site than are AMPARs. NMDARs are not activated by glutamate released from a single vesicle (Taylor et al., 1995; Matsui et al., 1998; Chen and Diamond, 2002), unless glutamate uptake is reduced (Chen and Diamond, 2002), and NMDARs encounter a lower synaptic glutamate concentration during evoked responses than do AMPARs (Chen and Diamond, 2002). Taken together, these previous physiological data and the morphological results presented here suggest that transmitter released from a single vesicle is not sufficient to activate NMDARs located perisynaptically, several hundred nm from the release site. The transmitter concentration reaching the NMDARs appears to be diminished by dilution into the larger volume of the perisynaptic space and by rapid removal of free transmitter by glutamate transporters. During an evoked response, however, NMDARs are activated more easily by higher levels of transmitter, presumably generated by multivesicular release from individual ribbon synapses (Singer et al., 2004) and/or glutamate spillover between synapses.

Glutamate transporters buffer synaptically released glutamate on a very rapid time scale (Diamond and Jahr, 1997; Diamond, 2005), but during a synaptic event extrasynaptic glial transporters only slightly affect the glutamate concentration waveform within the synaptic cleft (Diamond and Jahr, 1997). The large effect of uptake on NMDAR activation at RGC synapses (Chen and Diamond, 2002), then, indicates a different arrangement at the synapses studied here, one in which transporters may be positioned between the release site and the NMDARs and therefore able to intercept a considerable fraction of synthetically released glutamate before it reaches the receptors. The density of NMDARs peaks at about 200 nm from the edge of the PSD (Fig. 5A); transporters could, therefore, limit NMDAR activation if they were located within the synaptic cleft (i.e., the PSD or the apposed presynaptic membrane) or in the immediately perisynaptic region. As this area appears to contain only neuronal pre- and postsynaptic membranes, it seems likely that neuronal glutamate transporters limit the activation of postsynaptic NMDARs. The subsynaptic localization of glutamate transporters and the specific transporter subtype(s) acting at these synapses remains to be determined.

**AMPARs are located within the PSD**

The results presented here demonstrate that AMPARs are localized predominantly within the PSD, consistent with physiological evidence that they mediate synaptic activation of RGCs (Lukasiewicz et al., 1997; Jacoby and Wu, 2001; Chen and Diamond, 2002). Moreover, AMPARs are expressed at the highest density in the middle of the PSD (Figs. 4, 5F), ensuring their rapid activation by synthetically released glutamate and, perhaps, minimizing molecular interactions with perisynaptic NMDARs and associated proteins. Perisynaptic AMPARs were observed only very rarely (Figs. 4F, 5E) and may have reflected receptors in transit to or from the synaptic cleft (Bredt and Nicoll, 2003). The nearly complete spatial segregation of AMPARs and NMDARs suggests that any synaptic plasticity in RGC dendrites (Hosoya et al., 2005) may be distinct from NMDAR-dependent forms of plasticity studied elsewhere in the brain (Malenka and Bear, 2004).

**Receptor localization is similar at synapses onto ON and OFF RGCs**

ON and OFF RGCs play apparently inverse processing roles in the retinal network and receive synaptic input in distinct layers of the IPL. Morphological and molecular differences in synapses onto ON and OFF RGCs, however, have not been reported. Both RGC types receive excitatory input mediated by NMDARs and AMPARs (Mittman et al., 1990; Cohen et al., 1994), and ON and OFF parasol RGCs in primate retina express glutamate receptors at similar densities (Lin et al., 2002). At the immunogold EM level, we observed no significant difference in the subsynaptic localization patterns and densities of either AMPARs or NMDARs in the ON and OFF sublaminae of the IPL. It remains to be investigated whether receptors in ON and OFF RGCs comprise distinct subunit combinations that may underlie different synaptic processing tasks (e.g., Duprat et al., 2003; Perez-Otano and Ehlers, 2004).

**Comparison with previous work**

The punctate pattern of NMDAR antibody labeling observed here at the LM level (Fig. 3) is similar to that reported previously (Hartveit et al., 1994; Fletcher et al., 2000; Pourcho et al., 2001), but our finding that NMDARs are largely excluded from the PSD in RGC dendrites contradicts previous preembedding EM studies (Hartveit et al., 1994; Pourcho et al., 2001). The discrepancies between our and previous results are likely due to methodological differences. First, preembedding immunoperoxidase methods (Hartveit et al., 1994; Fletcher et al., 2000; Pourcho et al., 2001) offer significantly greater sensitivity, albeit with less spatial resolution, than the postembedding immunogold technique used here. The peroxidase reaction product can diffuse within the immunopositive process and may bind nonspecifically to structures in the PSD (Baude et al., 1995; Ottersen et al., 1998), making it difficult to determine quantitatively the precise subsynaptic localization of receptors (Ottersen and Landsend, 1997). The postembedding immunogold method reduces such artifacts because antibodies are applied directly to the surface of thin sections and the resin restricts label diffusion, permitting higher spatial resolution (Ottersen and Landsend, 1997; Nusser et al., 1998; Takumi et al., 1999; Nusser, 2000). Consequently, significant differences between pre- and postembedding approaches have been observed in the retina (Haverkamp et al., 2001) and elsewhere in the CNS (Baude et al., 1993, 1995; Nusser et al., 1994; Bernard et al., 1997; Bernard and Bolam, 1998). Second, in previous studies of synaptic glutamate receptor localization in the IPL (Hartveit et al., 1994; Qin and Pourcho, 1996, 1999; Fletcher et al., 2000; Pourcho et al., 2001; Hack et al., 2002), the specific cellular identity of the immunopositive postsynaptic process was not positively determined. NMDAR-immunopositive processes, therefore, may have belonged to amacrine cells, a subset of which receive synaptic inputs mediated by NMDARs (Dixon and Copenhagen, 1992; Goebel et al., 1998; Fletcher et al., 2000; Gründler et al., 2000). Here, retrograde labeling of RGCs by CTB provided a reliable marker of RGC process in the IPL (Fig. 2), enabling receptor localization to be examined on identified RGC processes.
Molecular mechanisms underlying perisynaptic NMDAR localization

At most excitatory synapses in the brain, glutamate receptors and associated signaling proteins are organized in the perisynaptic membrane by a family of membrane-associated guanylate kinases (MAGUKs) characterized by PDZ interaction domains (Kim and Sheng, 2004). Although NMDARs are most often associated with PSD-95 (Kennedy, 1997), the two proteins exhibit distinct developmental profiles (Sans et al., 2000) and NMDARs cluster in the absence of PSD-95 (Migaud et al., 1998), suggesting that other MAGUKs also may anchor NMDARs in the membrane. In the hippocampus another MAGUK, SAP-102, is expressed early in development (Sans et al., 2000), when many NMDARs are extrasynaptic (Perez-Otano and Ehlers, 2004), making it an attractive candidate for anchoring perisynaptic NMDARs in RGCs. Immunofluorescence microscopy reveals punctate expression of PSD-93, PSD-95, and SAP-102 in the IPL of rat retina (Rodriguez et al., 1998; Fletcher et al., 2000), although perisynaptic colocalization of NMDARs and specific MAGUKs remains to be determined.

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