Distribution and Developmental Regulation of AMPA Receptor Subunit Proteins in Rat Retina

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PURPOSE. To learn more about a possible functional role of α-amino-3-hydroxy-5-methyl-4-isoxasole-propionate (AMPA) receptors in retinal development, the spatial distribution and temporal regulation of all AMPA receptor subunit proteins was studied in rats.

METHODS. Immunohistochemistry was performed on retinal sections between embryonic days (E)20 and E21 and the adult stage by using specific antibodies against AMPA subunits GluR1 to 4.

RESULTS. All AMPA subunits were expressed in the ganglion cell layer from E21 on. In the inner plexiform layer (IPL), discernible bands of labeling appeared at distinct retinal ages for the different subunits. GluR1 immunoreactivity (IR) was concentrated in two broad bands by postnatal day (P)3, whereas three bands were visible beginning on P9. Two bands were located in a region of the IPL where off-cells terminate, and one band was found in the innermost part of the IPL where on-cells terminate. In contrast, two bands of GluR2/3 and GluR4 IR in the IPL were only discernible beginning on P14 and seemed to be located between the bands of GluR1-IR. GluR2/3 and GluR4 were observed both in horizontal cells and in the outer plexiform layer from early developmental stages on. GluR1 was not found in the outer retina, indicating that horizontal and bipolar cell processes in the rat express AMPA receptors composed of subunits GluR2 to 4. Double-labeling experiments with cell-specific markers revealed the expression of subunits GluR1 to 4 in cholinergic and All amacrine cells.

CONCLUSIONS. AMPA receptors are expressed before synapse formation, indicating a role not only in fast signal transmission but also in the establishment of inner retinal circuits. The differences in spatial and temporal subunit expression suggest that different retinal cell types selectively express distinct types of AMPA receptors during development of the rat retina. (Invest Ophthalmol Vis Sci. 2000;41:3600–3606)

Glutamate is not only the main excitatory neurotransmitter of the vertical signal pathway in the adult vertebrate retina but seems also to play an important role in the establishment of specific inner retinal circuits during retinal development. However, virtually nothing is known about the involvement of the different types of glutamate receptors in such developmental regulations within the mammalian retina.

Three main subtypes of ionotropic glutamate receptor have been characterized by pharmacologic studies and have been named according to their selective agonists: N-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methyl-4-isoxasole-propionate (AMPA; see Hollmann and Heinemann for a review). The AMPA receptor, which is involved in fast glutamatergic transmission in the adult mammalian central nervous system (CNS) is made up of four subunits termed GluR1, GluR2, GluR3, and GluR4, each of which contains two major splice variants, flip and flop. Each subunit can form a functional homomeric receptor when expressed in oocytes, although it is generally presumed that in vivo AMPA receptors are heteromeric and are composed of at least two different subunits. It is known that the distinct functional properties of AMPA receptors are due to differences in subunit composition; for instance, AMPA receptors containing the GluR2 subunit confer low calcium permeability. Thus, changes in response to glutamate during neuronal development can occur through changes in channel properties due to alterations in subunit composition and/or the transient appearance of specific glutamate receptor subtypes. Because synaptic receptor heterogeneity is a key factor underlying different functional properties in neurons, it is important to determine which receptor subunits are expressed in a given neuronal type at different stages of development.

The expression of AMPA receptor subunits in the mammalian retina has been studied mainly by in situ hybridization. Only a few immunohistochemical studies exist, and all were performed in the adult vertebrate retina. From these studies we know that the high transcript levels of AMPA receptor mRNAs observed in the adult vertebrate retina are indeed reflected by their high expression in protein. This

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underscores the important role of AMPA receptors in mediating light-induced synaptic transmission.16,17

However, not much is known about developmental aspects of AMPA receptor expression in the mammalian retina. The present study is the first to immunocytochemically analyze the temporal regulation and distribution of AMPA receptor subunits to elucidate when AMPA receptors are first expressed and whether there are distinct patterns of expression that may change during retinal development because of different functions AMPA receptors may have during the establishment of retinal circuitry.

METHODS

Tissue Preparation

All experiments were in compliance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Retinas from Brown Norway rats at different developmental stages between embryonic day (E)21, and the adult stages were used. The day of birth was designated as postnatal day (P)0. Rats were killed with CO2 and decapitated. Their eyes were removed, the anterior poles were dissected, and the eyecups were immersed for 15 to 30 minutes in 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) at 4°C. After washing in PB, tissues were cryoprotected by immersion in 40% methanol, and sections were incubated for 1 hour in 0.3% Triton X-100 in phosphate-buffered saline (PBST) to reduce background staining. The primary antibodies were diluted in PBST containing 10% normal goat serum (NGS; Sigma, Munich, Germany) and 0.3% Triton X-100 in phosphate-buffered saline (PBST) to reduce background staining. The primary antibodies were diluted in PBST containing 10% NGS and incubated for 3 hours at room temperature or overnight at 4°C. After washing with PBST, the samples were incubated for 1 hour with the biotin-conjugated secondary antibody (dilution 1:200; Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) in PBST with 5% NGS. After rinsing in PBST, retinal sections were processed with an avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector) and stained with diaminobenzidine reaction products. For double-labeling immunofluorescence, the preblocking step and primary antibody incubation were performed in 20% NGS plus 2% bovine serum albumin (BSA; Sigma). The secondary antibodies were conjugated either to Cy5 or fluorescein isothiocyanate (FITC; both from Sigma; dilutions were 1:100 for FITC and 1:1000 for Cy5).

Antibodies

Polyclonal antibodies against GluR1, GluR2/3, and GluR4 (PharMingen, San Diego, CA) were raised against synthetic peptides corresponding to the C-terminal sequences in the intracellular domain of the rat GluR clones and were affinity purified by using the corresponding peptides.15,16 The antibody against subunit GluR1 does not cross-react with other AMPA subunits. The GluR2/3 antibody does not cross-react with GluR1. It recognizes the GluR4 splice variant but not GluR4. The antibody against subunit GluR4 does not cross-react with GluR1, GluR2, or GluR3, or GluR4c. Each antibody used recognizes both flip and flop slice variants. Partial antibodies were used at the following concentrations: anti-GluR1 and anti-GluR4, 4 μg/ml, and anti-GluR2/3, 2 μg/ml. Cholinergic amacrine cells were immunodetected using a specific monoclonal antibody against choline acetyltransferase (clone 1E6; dilution 1:250; Chemicon), and all amacrine cells were visualized with a monoclonal antibody against parvalbumin (clone PA-235 diluted 1:200; Sigma). Control retina sections were processed as described earlier, except that the first antisera were omitted, resulting in no staining.

RESULTS

AMPA subunits GluR1 to 4 were present in the retina at all developmental ages analyzed but showed different spatial distribution and temporal expression patterns. AMPA subunits were found in different cell types of the inner nuclear layer (INL), in most cells of the ganglion cell layer (GCL), and in both the outer and inner plexiform layers (OPL, IPL).

GluR1 Subunit Expression

GluR1 immunoreactivity was found in most cells of the GCL and in cells in the inner part of the neuroblast layer (NBL) in early developmental stages (Fig. 1, P3, arrows). No staining was observed in the outer part of the NBL where differentiating horizontal cells are localized. Later in development, GluR1 immunoreactivity was never observed in horizontal cells or the OPL. This is in line with what has been observed in the adult rat retina12 but not in the adult cat retina where GluR1 was expressed in cone bipolar cell dendrites in the OPL.14

In the INL, subpopulations of amacrine cells showed GluR1 immunoreactivity at all postnatal stages (Fig. 1, thick arrows). One of these subpopulations was characterized by a thick primary process, which is indicative for AII amacrine cells (Fig. 1, inset; P20). Their predominant glutamatergic input comes from rod bipolar cells, and previous physiological evidence has suggested that AII amacrine cells express both non-NMDA and NMDA receptors.19,20 Expression of GluR1 in all amacrine cells was confirmed by a double-labeling study in which parvalbumin was used as a marker for all cells (Fig. 4A, arrowhead). Similarly, cholinergic amacrine cells visualized with an antibody against choline acetyltransferase expressed the GluR1 subunit (Fig. 4B, arrowhead). In addition to these amacrine cell populations, a few labeled cells were occasionally observed in the middle of the INL (Fig. 1, arrowheads).

Interestingly, GluR1 immunoreactivity in the IPL was confined to distinct bands as early as P3 (Fig. 1, thin horizontal arrows), which is before synapse formation in this layer and long before the first visual input (around P16 in our rat strain). At P9, three bands of GluR1 immunoreactivity were clearly discernible. Two of them were located in the outer half of the IPL, where processes of the off pathway terminate, whereas the third band was close to the GCL and appeared to be related to the on pathway.
GluR2/3 Subunit Expression

The staining pattern of GluR2 was indistinguishable from that of GluR3 with the antibody used in the present study. However, previous immunohistochemical studies in the adult vertebrate retina\textsuperscript{12} suggested that distribution patterns of GluR2 and GluR3 are similar and that GluR2 rather than GluR3 is predominantly expressed in the rat retina during development as well as in adulthood.

GluR2/3 expression was found in most cells of the GCL and in various subpopulations of amacrine cells in the inner part of the INL at all developmental stages (Fig. 2, thick arrows). The double-labeling study demonstrated that, as for GluR1, all amacrine cells (Fig. 4C, arrowhead) and cholinergic amacrine cells (Fig. 4D, arrowhead) express the AMPA subunits GluR2/3. In contrast to GluR1, staining for GluR2/3 was also observed in horizontal cells beginning on P3 (Fig. 2, inset, arrowheads). Moreover, labeling in the OPL appeared around P9, thus indicating a synaptic localization of GluR2/3 at the beginning of the second postnatal week. These results are in agreement with previous in situ hybridizations\textsuperscript{8–10} and immunohistochemical studies\textsuperscript{12–15} which also found GluR2/3 expression in the outer part of the INL and the OPL of adult mammalian retinas.

FIGURE 1. Localization of AMPA subunits GluR1 in the rat retina at different developmental stages. GluR1 was expressed in many neurons in the GCL and in subpopulations of amacrine cells (thick arrows) beginning in the early developmental stages. Inset, P20: Two putative AII amacrine cells immunoreactive for GluR1. Two broad bands of GluR1 expression in the IPL were already present at P3 (horizontal arrows), one close to the INL, the other close to the GCL. At P9, three bands of GluR1 immunoreactivity were visible: two located in the outer half of the IPL and the third in the innermost part of the IPL. This stratification within distinct synaptic layers did not alter after light input (see P20). Left: Control sections for each developmental stage. Scale bar, 20 μm. NBL, neuroblastic layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

FIGURE 2. Localization of AMPA subunits GluR2/3 in the rat retina at different developmental stages. Similar to GluR1, GluR2/3 was expressed in many cells in the GCL and in numerous amacrine cells (thick arrows) beginning in the early developmental stages. In contrast to GluR1, horizontal cell bodies (arrowheads) were immunoreactive for GluR2/3 beginning on P3 (inset). Labeling of the OPL was visible beginning on P9. Two bands of GluR2/3 immunoreactivity were discernible in the IPL only beginning on P14 (horizontal arrows, P20). Left: Control sections for each developmental stage. Scale bar, 20 μm. See legend to Figure 1 for abbreviations.
In contrast to GluR1, no discernible bands of GluR2/3 immunoreactivity in the IPL were observed in the first postnatal week (compare Fig. 2 with Fig. 1; P3 and P9). Only later in development did GluR2/3 immunoreactivity concentrate in two bands (Fig. 2, P20, horizontal arrows), indicating that the synaptic expression of GluR1 precedes that of GluR2/3 by approximately 2 weeks. One band of GluR2/3 immunoreactivity was located in the middle of the IPL and one above, in a region of the IPL where processes of off-cells stratify. Qin and Pourcho found a similar expression pattern of GluR2/3 in the IPL of the adult cat retina. The bands of GluR2/3 immunoreactivity appeared to be located between those of GluR1 expression, because the upper band was not as close to the INL as the GluR1 band, and the lower band was oriented more toward the middle of the IPL. However, analyses on the ultrastructural level are necessary to determine the exact location of GluR subunits in the IPL. In contrast to GluR1, no band of GluR2/3 immunoreactivity was observed in the inner part of the IPL where on-cells terminate.

**GluR4 Subunit Expression**

GluR4 expression was found in most cells of the GCL and in subpopulations of amacrine cells in the inner part of the INL at all developmental stages (Fig. 3, thick arrows and stars). Expression of GluR4 in all amacrine cells (Fig. 4E, arrowhead) and cholinergic amacrine cells (Fig. 4F, arrowhead) was verified by double-labeling. Similarly to GluR2/3, a clear GluR4 staining was observed in horizontal cells (Fig. 3, arrowheads) and in the OPL beginning on P3. GluR4 expression in the outer part of the INL and in the OPL of the adult retina has previously been demonstrated by in situ hybridization and immunohistochemistry, and our present results show that this specific pattern of expression is already present at early developmental stages.

Similar to GluR2/3, two bands of GluR4 immunoreactivity were discernible in the IPL beginning on P14 (Fig. 3, P20, thin horizontal arrows) and seemed to overlap those of GluR2/3.

**DISCUSSION**

In this immunocytochemical study we investigated the expression of AMPA subunits GluR1 to 4 in the developing rat retina and showed that all subunits were expressed beginning on E20 or E21. The early expression long before eye opening suggests that AMPA receptors are not only involved in the transmission of light signals but may also play an important role in the development of inner retinal circuits and establishment of synaptic connections.

AMPA receptor subunits show distinct distribution patterns in both synaptic layers and among the various retinal cell types, indicating that different types of glutamate receptors exist at all levels of retinal information processing and that they may be differentially distributed in the on and off pathways. Moreover, the developmental alterations and differences in the distribution of the different subunits suggest that the functional properties of AMPA receptors in distinct cell types may change with retinal age. It has to be pointed out, however, that our immunocytochemical data can give no information on how the subunits are combined in functional receptors and that electrophysiological analysis as well as ultrastructural examinations will be necessary to clarify this point. Nonetheless, our finding of multiple subunits expressed in individual cells such as cholinergic amacrine cells supports the assumption made by other investigators that AMPA subunits in vivo are heteromeric.

**Glutamate Receptor Expression in the Outer Retina**

Previous in situ hybridization studies performed in the adult rat have demonstrated labeling for GluR2/3 and GluR4 mRNA in the outer part of the INL. In the current study, we have...
shown that GluR2/3 and GluR4 subunits are present in horizontal cells and that they are expressed in early postnatal stages. Moreover, the staining observed in the OPL indicates a synaptic localization of these subunits and therefore suggests that input to second-order neurons in the rat retina may be mediated by different types of AMPA receptors in addition to NMDA receptors.23

In the middle part of the INL where bipolar cell bodies are located, only a few cells were occasionally immunoreactive for GluR1, whereas staining for GluR2/3 and GluR4 was never above background. This is comparable to a recent immunohistochemical study in cat in which cell bodies and primary dendrites of bipolar cells were only weakly labeled.15 Those investigators, however, reported strong staining of subunits GluR2/3 and/or GluR4 in invaginating and basal dendrites of bipolar cells in the OPL and concluded that all bipolar cell classes express at least one GluR subunit. This observation, together with the finding that AMPA receptor-mediated currents can be elicited in bipolar cells of adult rats,24 suggests that in rat, GluR2/3 and GluR4 subunits may be located not only on processes of horizontal cells but also on bipolar cell dendrites. However, ultrastructural examinations are needed to determine the exact localization of the different GluR subunits in the OPL.

Glutamate Receptor Expression in the Inner Retina

Up to 30 different types of amacrine cells exist in the mammalian retina, and our data indicate that every ionotropic glutamate receptor subunit expressed in the rat retina is also expressed by amacrine cells. Because of considerable differences...
in the distribution of glutamate receptor subunits, we suggest that a differential expression of glutamate receptor subtypes is important for generating functional heterogeneity among amacrine cells.

Our results indicate that all amacrine cells express AMPA receptors, which is in agreement with previous electrophysiological findings indicating the presence of functional non-NMDA receptors on all amacrine cells.19 Electrophysiological evidence for the expression of both NMDA and non-NMDA receptors in some cholinergic amacrine cells25,26 is also supported by our results, because we found expression of all AMPA subunits in cholinergic amacrine cells in the INL. Moreover, virtually all cells in the GCL expressed AMPA receptor subunits, and some displaced amacrine cells in the GCL are reportedly cholinergic.27

We found that the IPL is immunoreactive for all AMPA subunits but with differences in temporal expression and localization within the different sublayers. This suggests that there is a rather precise pattern of stratification of the IPL with respect to the expression of specific glutamate receptor subunits. GluR1 labeling had already concentrated in two bands at P3, indicating that this subunit may play an important role in establishing early synaptic connections in the inner retina. In contrast, although a first expression of GluR2 to 4 subunits in the IPL was found around P9, distinct bands of labeling only appeared at approximately P14 which is shortly before eye opening (~P16). Thus, the main alterations in the expression of AMPA receptor subunits GluR2 to 4 take place within the period in which bipolar cells form synapses with postsynaptic dendrites.28,29 Because all ionotropic glutamate receptor subunits studied so far have been found exclusively in processes postsynaptic to bipolar cell ribbon synapses,14,30,31 different types of glutamate receptors may be involved in the establishment of distinct synaptic connections and may specify the roles of the different types of bipolar cells in the adult rat retina. This is supported by our findings that different subunits seemed to colocalize in distinct bands within the IPL.

All AMPA receptor subunits were expressed in a large number of cells located in the GCL, a finding that is in line with previous in situ hybridization studies.8–10 Individual ganglion cells have been shown to coexpress multiple subunits of the AMPA receptor by in situ hybridization on serial semithin sections10 and single-cell RT-PCR.32 In addition, differences in the Ca2+ permeability of AMPA- or kainate-induced whole cell currents were found in rat retinal ganglion cells,33 giving rise to the possibility that AMPA receptor subunits coassemble in heteromeric receptors with distinct functional properties.

In conclusion, our data suggest that different types of AMPA receptors are expressed in different cell types, both in the outer and inner retina. Because alterations in the expression of AMPA subunits occur almost exclusively within the first two postnatal weeks, we propose that they are related to processes of synapse formation in the retina. At the time of eye opening the expression patterns are already very similar to those observed in adult retinas indicating that visual experience prompts no further developmental changes.

References