Effect of photoreceptor degeneration on RNA splicing and expression of AMPA receptors

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Purpose: Glutamate is the most important neurotransmitter for excitatory synapses, and its ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is widely expressed in the vertebrate retina. AMPA receptors are hetero-oligomers composed of subsets of four distinct subunits termed GluR1-4. This study was conducted to examine the developmental progression of the flip-to-flop alternative splicing switch of AMPA receptors in wild-type and rd mouse retina.

Methods: The flop:flip ratio and expression levels of GluR1-4 from postnatal day 8 (P8) to P40 were calculated using quantitative real-time polymerase chain reaction (PCR) analysis and immunoblot analysis. The time course of photoreceptor degeneration in rd mice was histologically analyzed.

Results: In wild-type mouse retina, the flop:flip ratio in GluR1, but not GluR2-4, dramatically increased between P16 and P20. In rd mice, photoreceptor degeneration progressed from P10 to P20. GluR1 flop:flip ratio in rd mice was normal compared with wild-type mice before P16, however, the dramatic increase between P16 and P20 was completely suppressed. The suppression in the later phase of retinal degeneration was specific to GluR1 and was not observed in GluR2-4. Moreover, the expression levels of GluR1, GluR3, and GluR4 were increased in rd mice.

Conclusions: These results suggest that the inherited form of photoreceptor degeneration in rd mice contributes to the regulation of the flip-to-flop exon switch in GluR1 and the expression levels of AMPA receptors.

In the vertebrate retina, synapses connecting photoreceptors to bipolar and horizontal cells and those connecting bipolar cells to ganglion and amacrine cells are thought to utilize glutamate as a neurotransmitter [1] and its concentration is regulated by glutamate transporters [2,3]. Both ionotropic and metabotropic glutamate receptors are involved in retinal glutamatergic synaptic transmission. In the ionotropic receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) types subserve distinct functions. Among them, the AMPA-prefering glutamate receptors mediate fast excitatory postsynaptic currents [4]. AMPA receptors are hetero-oligomeric complexes composed of various combinations of four subunits termed GluR1, GluR2, GluR3, and GluR4. Each subunit exists in “flip” and “flop” isoforms that are generated by alternative mRNA splicing [5]. The two isoforms differ between 8-10 residues in a 38 amino acid cassette that forms part of the large extracellular loop of the receptor molecules [6-8]. Receptors containing flip subunits exhibit significantly slower desensitization kinetics and a greater steady-state component in their response to glutamate relative to those containing flop subunits [5,9-11]. In addition, flip-to-flop alternative splicing is developmentally regulated in a cell-specific manner [12]; prenatal AMPA receptors are comprised mostly of the flip form, with the flop form appearing postnatally in the rat brain [13]. These findings suggest that the flip-flop region plays a critical role in AMPA receptor function in some synapses.

In the previous study, we demonstrated that the flop:flip ratio in GluR1 and GluR2 dramatically increases during postnatal development in rat retina [14]. Although AMPA receptors are absent from photoreceptors, continuous dark conditions and light-induced retinal degeneration suppress developmental progression of flip-to-flop alternative mRNA splicing in the retinal GluR1. These results are consistent with the previous findings that photoreceptor degeneration is characterized by marked cellular changes in the inner retinal layers [15,16]. However, the study was carried out using a semiquantitative method. In addition, recent studies have suggested a possibility that the development and function of flip-to-flop alternative splicing might be different in animal models of light-induced or inherited forms of photoreceptor degeneration [15]. In the present study, we examined the AMPA receptor alternative mRNA splicing in mouse retina utilizing quantitative real-time PCR analysis. In addition, to determine the effect of inherited form of photoreceptor degeneration on the process, we employed rd mice, the best characterized animal model of retinitis pigmentosa. In this model, most rod photoreceptors die by three weeks after birth due to recessive mutation of the gene coding for the rod cGMP phosphodiesterase β-subunit [17].
METHODS

Animals: Experiments were performed using rd (C57BL/6 background) mice in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Light intensity inside the cages ranged from 100 to 200 lux under 12 h:12 h light-dark cycle. Heterozygous rd (rd/+; wild-type) and rd (rd/rd) mice. Genomic DNA was isolated from mouse tails, and the rd alleles were identified by the PCR analyses, by following directions described previously [18].

Histology: Wild-type and rd mice at postnatal day 10 (P10), P13, and P20 were deeply anesthetized with diethylether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.5% picric acid at room temperature. The eyes were removed and postfixed overnight in the same fixative and then embedded in paraffin. Histological sections of 7 μm thick were made along the vertical meridian, mounted, and stained with hematoxylin and eosin. At least three animals were used at each time point.

Amplification of GluR1-4 cDNAs: The retinas were carefully removed from the eyes at P8, P10, P13, P16, P20, and P40. Total retinal RNA from three or four animals at each point was extracted with Isogen (Nippon Gene, Tokyo, Japan), and resultant RNA was reverse-transcribed with Revertra ace (Toyobo, Osaka, Japan) to obtain cDNA. cDNA products from P20 retina of wild-type mice were used as the template for PCR amplification in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl₂, 0.2 mM dNTPs, 50 nM each set of oligonucleotide primers, 2.5 U Taq polymerase in 100 µL volume. The PCR quality and specificity was verified by electrophoresis of amplified fragments.

Restriction enzyme digestion of each PCR product was performed at 37 °C for 1 h. The oligonucleotide primers used for PCR amplification and quantitative real-time PCR analysis are shown in Table 1. These oligonucleotide primers encompassed the regions of maximum nucleotide sequence dissimilarity between the flip and flop exons, as well as between the four receptor sequences. Nucleotide sequences encoding the flip-containing polypeptides are available in the EMBL/GenBank database under accession numbers X57497 (GluR1), AB111957 (GluR2), NM_016886 (GluR3), and NM_019691 (GluR4), and the corresponding flop versions under BC056397 (GluR1), NM_013540 (GluR2), and AB022913 (GluR4). GluR3 flop version was retrieved from the Mouse Genome Informatics Resource (Mouse Genome Informatics version 3.22, The Jackson Laboratory, Bar Harbor, Maine).

Quantitative real-time PCR analysis: Quantitative real-time PCR analysis was performed with the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer’s protocol. Thermo cycling of each reaction was performed with each primer at a concentration of 50 nM. The following protocol was used: denaturation program (95 °C for 3 min), followed by the amplification and quantification program (95 °C for 15 s and 60 °C for 30 s) repeated for 45 cycles, with one cycle of a finishing program (72 °C for 1 min). The PCR quality and specificity was verified by melting curve analysis. A standard curve of cycle thresholds using serial dilutions of cDNA samples were used to calculate the relative abundance. The difference in the initial amount of total RNA between the samples was normalized in every assay using a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression as an internal standard (forward primer, 5'-TAC ACC ACC AAC TGC TTA G-3'; reverse primer, 5'-GGA TGC AGG GAT GTG GTT C-3').

Immunoblot analysis: Retinas were homogenized in 200 µl of ice-cold 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and the protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration in the tissue lysates was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Three micrograms of the protein were separated on 8% sodium dodecyl-sulfate-polyacrylamide gel and subsequently transferred to an Immobilon-P membrane (Millipore, Billerica, MA). After blocked with 5% skim milk, the membrane was incubated with anti-GluR2 or anti-GluR4 antibody (Chemicon, Temecula, CA: 1:500) and visualized using an ECL plus immunoblotting detection system (Amersham Bioscience, Piscataway, NJ). After detection of GluR4, proteins fixed on the membrane were stained with Coomassie brilliant blue (CBB).

Statistics: Data are presented as mean±SEM except as noted. When statistical analysis was performed, Student’s t-test was used to estimate the significance of the results. Statistical significance was accepted at p<0.05.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequences (5’-3’)</th>
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<tbody>
<tr>
<td>GluR1 long-F</td>
<td>AGAAGGCGAGGATCAGACAAACCAG</td>
</tr>
<tr>
<td>GluR1 short-F</td>
<td>ACGCATGAAAGTTGGAGGTTAATCT</td>
</tr>
<tr>
<td>GluR1 flip-R</td>
<td>AGCTGTCTTGCCTTACGTCCGGA</td>
</tr>
<tr>
<td>GluR1 flop-R</td>
<td>AGCTGTCTTGCCTTGGATGACC</td>
</tr>
<tr>
<td>GluR2 long-F</td>
<td>AGATGGAAAGGACACCAAAAGTAG</td>
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<tr>
<td>GluR2 short-F</td>
<td>AGACCATGAAAGTGCCGAGCAAC</td>
</tr>
<tr>
<td>GluR2 flip-R</td>
<td>AGCTGTCTTGCCTTACGTCCGGA</td>
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<tr>
<td>GluR2 flop-R</td>
<td>AGCTGTCTTGCCTTGGATGACC</td>
</tr>
<tr>
<td>GluR3 long-F</td>
<td>AGCTGCGTACCCAAAAGCCCTCC</td>
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<tr>
<td>GluR3 short-F</td>
<td>ATACGATGAAAGTGGTGGAAATC</td>
</tr>
<tr>
<td>GluR3 flip-R</td>
<td>AGCTGTCTTGCCTTACGTCCGGA</td>
</tr>
<tr>
<td>GluR3 flop-R</td>
<td>AGCTGCGTACCCAAAAGCCCTCC</td>
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<tr>
<td>GluR4 long-F</td>
<td>GGATGGAAAGGACACCAAAAGTAG</td>
</tr>
<tr>
<td>GluR4 short-F</td>
<td>AGACCATGAAAGTGCCGAGCAAC</td>
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<tr>
<td>GluR4 flip-R</td>
<td>AGCTGTCTTGCCTTGGATGACC</td>
</tr>
<tr>
<td>GluR4 flop-R</td>
<td>AGCTGCGTACCCAAAAGCCCTCC</td>
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F represents forward primer; R represents reverse primer.
RESULTS
Photoreceptor degeneration in rd mouse retina: Retinal structure in rd mice was indistinguishable from that of wild-type mice at P10 (Figure 1A,B). However, rd mice at P13 showed continuous thinning of the outer nuclear layer (ONL) that is composed of photoreceptor nuclei (Figure 1D), and resulted in a single row of photoreceptors at P20 (Figure 1F) in contrast to wild-type mice (Figure 1C,E). These observations demonstrate that photoreceptor-specific degeneration occurs between P10 and P20 in rd mice.

Identification of alternative splicing variants: To reveal the existence of alternatively spliced variants of AMPA receptor subunits, we performed RT-PCR using total RNA isolated from P20 wild-type mouse retina. We prepared two sets of primers specific for individual GluR1-4 flip and flop isoforms, respectively (Table 1). PCR products using “long” forward primers are 685 bp (long in Figure 2C) while 200 bp for “short” forward primers (thick lines; short in Figure 2C) in each form. Digestion of the “long” products with restriction enzymes produced fragments of the following sizes: 572 and 113 bp for GluR1-flip cut by XspI whereas the GluR1-flop was uncut; 568 and 117 bp for GluR2- and GluR4-flop cut by HincII whereas the GluR2- and GluR4-flip were uncut; 447 and 238 bp for GluR3-flip, and 447, 121, and 117 bp for GluR3-flop cut by HincII (Figure 2A). Digestion of the “short” products with restriction enzymes produced fragments of the following sizes: 87 and 113 bp for GluR1 flip cut by XspI whereas the GluR1 flop was uncut; 83 and 117 bp for GluR2-4 flop cut by HincII whereas the GluR2-4 flip were uncut (Figure 2B). The size of each fragment was as expected with respect to the
enzyme restriction sites (Figure 2C). From these findings, we concluded that both flip and flop isoforms of all four GluR subunit mRNAs are expressed in P20 mouse retina.

**AMPA receptor mRNA splicing in mouse retina:** We next examined the relative expression levels of all eight splicing forms by quantitative real-time PCR analysis using “short” forward primers (Figure 2A). According to the results, we calculated the flop:flip ratio of each AMPA receptor subunit in wild-type and rd mice from P8 to P40 (Figure 3). The initial flop:flip ratio was around 2.0 in all four GluR subtypes. Interestingly, GluR1 (Figure 3A) flop:flip ratio in wild-type mice (closed circles) dramatically increased to about three fold be-

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**Figure 2.** DNA fragments specific for flip and flop sequences. A, B: PCR products using “long” forward primers (Table 1) were 685 bp while those using “short” forward primers were 200 bp. The PCR products amplified using “long” and “short” forward primers were digested with restriction endonucleases and then electrophoresed on a 3% agarose gel (A and B, respectively). The digested fragments were visualized after staining with ethidium bromide. C: Schematic representation of the specific GluR-flip and -flop fragments were amplified using “long” forward primers and “short” forward primers (thick lines). The restriction endonuclease cleavage patterns were used to confirm specific amplification. X represents XspI; H represents HincII.
Figure 3. GluR flop:flip ratio in the postnatal mice retina. The ratio was calculated by quantitative real-time RT-PCR analysis. The flop:flip ratio in GluR1 was dramatically increased between P16 and P40 in wild-type mice (A). Note the significantly low GluR1 flop:flip ratio in rd mice as compared to wild-type mice at P20 and P40. Each data point represents the mean±SEM of the values obtained from three or four independent experiments. *p<0.05.

Figure 4. GluR1-4 mRNA expression in the postnatal mice retina. The relative mRNA expression levels in GluR1-4 were calculated by quantitative real-time RT-PCR analysis. GluR1, GluR3, and GluR4 mRNA levels in rd mice were significantly increased as compared with wild-type mice. Each data point represents the mean±SEM of the values obtained from three or four independent experiments. *p<0.02, **p<0.001.
between P16 and P20, and to about 12 fold at P40 (Figure 3A). On the other hand, the ratios for GluR2-4 did not change significantly and remained relatively static from P8 to P40 in both wild-type and rd mice (open circles).

**AMPA receptor expression levels in mouse retina:** We next examined relative expression levels of total (flip+flop) mRNA in each subunit, as that of wild-type mice at P8 being 100% (Figure 4). In wild-type mice, GluR1 and GluR4 mRNA expression levels were decreased gradually (Figure 4A,D). However, in rd mice, they were rather increased and significantly higher than those of wild-type mice at P20 in GluR1 and after P16 in GluR4. GluR3 mRNA expression levels in rd mice were also upregulated after P20 (Figure 4C). On the other hand, GluR2 mRNA expression levels in rd mice were similar to those of wild-type mice at all the time points we examined (Figure 4B). We also examined GluR2 (96 kDa) and GluR4 (102 kDa) protein expression levels from P8 to P40. Consistent with the data of quantitative PCR analysis, GluR4 (Figure 5B), but not GluR2 (Figure 5A), protein expression in rd mice was clearly higher than that of wild-type mice after P14. To confirm whether same amount of proteins were loaded, proteins fixed on the membrane were stained with CBB after detection of GluR4 (Figure 5C). These results suggest that protein expression levels in the retina are consistent with the mRNA expression levels in both wild-type and rd mice.

**DISCUSSION**

In this study, we used a quantitative real-time PCR analysis to examine the development of AMPA receptor alternative mRNA splicing in mouse retina, and demonstrated a dynamic molecular change in GluR1 during postnatal retinal development. In mouse retina, GluR1 immunoreactivity is detected in...
ganglion cells, horizontal cells, and OFF-cone bipolar cells [19]. After the onset of vision, vertical synaptic connections consisting of photoreceptors, bipolar cells, and ganglion cells become the principal pathway by which visual information is transmitted from the retina to the visual cortex through the optic nerve. Thus, in GluR1-positive cells, the alteration of AMPA receptor kinetics by flip-to-flop alternative splicing may have physiological significance in relation to the development of the visual system. We previously demonstrated that flop:flip ratios in both GluR1 and GluR2 are dramatically increased during rat retinal development [16]. In rat retina, amacrine and ganglion cells express GluR1 predominantly, bipolar cells express GluR2 predominantly, horizontal cells express GluR3 predominantly, and Müller glia express GluR4 predominantly [20,21]. Such different expression patterns may lead to different developmental regulation of AMPA receptors in rat and mouse retinas.

On the other hand, our results demonstrated that photoreceptor degeneration suppresses GluR1-specific flip-to-flop alternative splicing in rd mouse (Figure 3A) as well as light-damaged rat retina [14]. We previously examined rat retina after light-induced photoreceptor degeneration and demonstrated that light-induced c-fos gene expression in the GCL is at least partially controlled through a cone-specific pathway [22]. Interestingly, in the outer plexiform layer, GluR1 is mainly expressed on neural processes associated with the terminals of cone photoreceptors, whereas GluR2-4 are distributed to both cone and rod photoreceptors [19]. Considering GluR1 expression pattern in mouse retina, the suppression in GluR1 flop:flip ratio may reflect a synaptic reorganization of a cone pathway in rd retina, in which most rod photoreceptors are absent. Recent studies have shown that AMPA receptor desensitization protects neurons against the excitotoxic effects of AMPA receptor activation, as demonstrated by the fact that pharmacological reduction of desensitization enhances AMPA receptor-mediated neuronal death [23-25]. Since receptors containing flip subunits exhibit significantly slower desensitization kinetics relative to those containing flop subunits [5-11], suppression of flip-to-flop mRNA splicing in GluR1 may be one of the internal defense systems in rd mouse regulation of the function of GluR subunits is specified not only by flip-to-flop alternative splicing, but also by mRNA editing in the R/G site, which is located immediately before the flip-flop sequence. The R/G-site editing is thought to influence desensitization behavior with flop desensitizing faster [26], and this occurs in GluR2-4 mRNAs, but not in GluR1 mRNA. Further studies are needed to determine whether the uniqueness of the GluR1 is responsible for the specific effect of retinal degeneration on GluR1 mRNA splicing.

We also found that GluR1, GluR3 and GluR4 mRNA expression levels in rd/rd mice were significantly higher than those of wild-type mice during and after the period of photoreceptor degeneration (Figure 4). Previous studies have reported that GluR1 protein expression was increased in rd/rd mouse retina, another model of photoreceptor degeneration [27], and in rat retina dark-reared for 1 week [28]. Taken together, our present findings suggest that rod photoreceptor-mediated visual input may be involved in the regulation of both RNA expression levels and splicing of AMPA receptors. Conversely, further investigations of events leading to the modification of AMPA receptor mRNAs may uncover the detailed mechanism of retinal development and degeneration. We plan to examine these issues in various animal models of retinitis pigmentosa and macular degeneration.

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