A Novel Isoform of Acetylcholinesterase Exacerbates Photoreceptors Death after Photic Stress

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PURPOSE. To study the involvement of stress-induced acetylcholinesterase (AChE) expression in light-induced retinal damage in albino rats.

METHODS. Adult albino rats were exposed for 24 hours to bright, damaging light. AChE expression was monitored by in situ hybridization, by histochemistry for AChE activity, and by immunocytochemistry. An orphan antisense agent (Monarsen; Ester Neurosciences, Ltd., Herzlia Pituach, Israel) was administered intraperitoneally to minimize light-induced AChE expression. The electroretinogram (ERG) was recorded to assess retinal function.

RESULTS. Twenty-four-hour exposure to bright light caused severe reduction in the ERG responses and augmented expression of mRNA for the “read-through” variant of AChE (AChE-R) in photoreceptor inner segments (IS), bipolar cells, and ganglion cells. AChE activity increased in IS. The expressed AChE protein was a novel variant, characterized by an extended N terminus (N-AChE). Systemic administration of the orphan antisense agent, Monarsen, reduced the photic induction of mRNA for AChE-R, and of the N-AChE protein. Rats exposed to bright, damaging light and treated daily with Monarsen exhibited reduced ERG responses, relatively thicker outer nuclear layer (ONL), and more ONL nuclei than did rats exposed to the same damaging light but treated daily with saline.

CONCLUSIONS. The findings indicate that the photic-induced novel variant of AChE (N-AChE-R) may be causally involved with retinal light damage and suggest the use of RNA targeting for limiting such damage. (Invest Ophthalmol Vis Sci. 2007;48:1290–1297) DOI:10.1167/iovs.06-0847

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these findings are relevant to the embryonic development of the retina and not to stress-induced malfunction in the adult retina. We have shown AChE mRNA expression in human adult photoreceptors—cells that are not involved in cholinergic synaptic activity—raising the possibility that AChE in photoreceptors exerts stress-related morphogenetic function(s). This hypothesis is particularly appealing, since the major excitatory neurotransmitter of the retina is L-glutamate, whereas ACh plays a limited role in visual information processing within the retina and is found mainly in amacrine and ganglion cells in the proximal part of the retina.

We tested the hypothesis that exposure to damaging light is a stressful event that induces the expression of AChE in the retina, with particular interest in the photoreceptors. We further identified the nature of the induced AChE variant and its role in retinal damage.

METHODS

Procedure

Adult male Sprague-Dawley albino rats (N = 60), weighing 200 to 300 g, were kept for at least 2 weeks in a 12-hour light–dark cycle with free access to food and water. Rats were exposed for 24 hours, starting at around 10 AM, at room temperature, to bright white light (260 foot-candles) in a specially designed light chamber, and then returned to the normal rearing conditions (12-hour light–dark cycle). To assess retinal function, we recorded the electroretinogram (ERG). For ERG recordings, rats were anesthetized by an intramuscular injection of ketamine hydrochloride (3.3 mg/kg), acepromazine maleate (6.7 g/kg), and xylazine (2 μg/kg). At the termination of the ERG follow-up period, rats were killed by an overdose of sodium pentobarbital, injected intraperitoneally, and their retinas were prepared for in situ hybridization (ISH), histochemical evaluation or immunocytochemistry. Eyes were enucleated and incubated (1-hour for cryosections and 24-hour for paraffin sections) in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde (4°C).

The rats were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with institutional guidelines.

Immunocytochemistry

Cryostat sections were incubated overnight, under agitation, with affinity-purified primary rabbit antiserum targeted against a synthetic peptide unique for rodent N-AChE at a dilution of 1:100 in 0.1 M PBS 1% Triton X-100. We also used an antibody for the core domain, common to all AChE variants (N-19; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were donkey anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC) at a 1:100 dilution and a donkey antigoat IgG coupled to Cy3 at a 1:100 dilution. Slides were viewed using a confocal microscope (Eclipse El600; Nikon, Tokyo, Japan; and Radiance 2000; Bio-Rad, Hercules, CA) scanning system.

Histochemical Staining for AChE Activity

Cryostat sections were stained for catalytically active AChE according to Karnovsky and Roots. Overnight incubation was done in a medium containing freshly prepared acetylthiocholine iodide (AChI) as a substrate in 0.1 M phosphate buffer (pH 6.0; 0.9 mg/mL), 0.1 M sodium citrate, 30 mM copper sulfate, distilled deionized water (DDW), and 5 mM potassium ferricyanide. The BChE inhibitor, iso-OMPA (a trisaccharide; Sigma-Aldrich, St. Louis, MO), was added (10^-5 M) to specifically localize AChE activity. The selective AChE inhibitor BW 284C51 (10^-5 M) was used as a negative control. Brownish staining, resulting from AChI hydrolysis, was examined under a light microscope (BH2; Olympus, Tokyo, Japan).

In Situ Hybridization

ISH was performed on paraffin-embedded sections, 5 μm thick, as previously described. Hybridization involved overnight incubation at 52°C in hybridization mix with a 5′-biotinylated, 2′-O-methylated AChE mRNA probe complementary to either the AChE-S (exon 1) rat isoform: (5402) 5′-CCGGGAGGAGGGGGUGGGGUUGGGGA UGGGAGAGAGUGGGGAGGUGCUGGGGUGGCU-3′ (5352); or the AChE-R (intron 4) rat isoform: (4397) 5′-CUAGGGAGAACAGAAGGGGGUGGGGUGGCU-3′ (5449). The numbers in parentheses denote nucleotide positions in the GenBank-deposited sequence (accession no. M55040; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Analysis

For quantitative analysis of staining in the ISH and histochemistry tests, we photographed slides and converted the micrograph to grayscale. Densities of retinal layers were quantified in arbitrary units by densitometry (TINA software ver. 2.10; Raytest, Straubenhardt, Germany). In each slide, we chose a rectangular window of width smaller than that of the narrowest layer to be measured, the photoreceptor inner...
segments (IS). The same window was used to measure density of the different retinal layers. Four random measurements were made in each retinal layer of a given retinal micrograph and averaged. The density of the outer nuclear layer (ONL) was set as the background, and all density measurements from other retinal layers were performed relative to this background. In this manner, we ensured that density measurements would be independent of technical factors such as thickness of section, duration of incubation, angle of slicing and others that may affect absolute density measurements but not relative measurements. Data from retinas of four rats undergoing the same experimental procedure were averaged. Because of the small sample size (n = 4), densitometry measurements were compared for statistical significance by using the Mann-Whitney nonparametric test.

Antisense Treatment
Monarsen (Ester Neurosciences, Ltd., Herzlia Pituach, Israel) antisense oligodeoxynucleotide directed against Rat AChE mRNA, a 20-mer oligodeoxynucleotide (5′-CTGCAATTTTTCGTGACC-3′) complementary to a sequence in exon 2 of rat AChE mRNA, was used as previously described. A single dose of Monarsen was found to have a transient action lasting between 24 and 72 hours, depending on the injected dose. Since development of light-induced retinal damage proceeds at a relatively slow rate, we decided to inject Monarsen on a daily basis throughout the entire duration of the follow-up (30 days). Intravital injection, the preferred route for retinal treatment, was an unrealistic solution for the treatment. Because the blood-retinal barrier had been shown to be disrupted after exposure to bright light, we decided to inject Monarsen intraperitoneally. Experimental rats were treated for 30 days with daily intraperitoneal injections of saline.

Electroretinogram
The ERG is the light-induced electrical activity of the retina that is used to assess retinal function. It is composed of two major components; the negative a-wave reflects light-induced electrical activity in the photoreceptors, whereas the positive b-wave is generated in post-receptor retinal neurons, mainly ON center bipolar cells.

Rats, kept overnight in total darkness, were anesthetized, as described earlier. The pupils were dilated (cyclopentolate hydrochloride 1%), and topical anesthetic drops (benoxinate HCl 0.4%) were used to prevent photic stress. Because the amplitude of the ERG waves depend on the intensities of the light stimuli, it is customary to assess retinal function quantitatively, by fitting the response–intensity data to a hyperbolic function:

\[ V/V_{\text{max}} = I/(I + \sigma) \]  

where \( V \) and \( V_{\text{max}} \) are the amplitudes of the ERG waves that are elicited respectively by a flash of intensity \( I \) and by a flash of saturating intensity, and the semisaturation constant \( \sigma \) is a measure of retinal sensitivity.

Results
ACH\textsubscript{E} gene expression is notably subject to complex stress-induced changes (Fig. 1). To assess the effects of photic stress on expression and activity of ACh\textsubscript{E}, we compared retinas of 15 albino rats, exposed to bright, damaging light for 24 hours to those of 15 unexposed control albino rats, raised in a 12-hour light–dark cycle. After exposure to the bright, damaging light, all 30 rats were kept in a regular 12-hour light–dark cycle with no further treatment for the entire 30-days of follow-up (experiment 1). Retinal micrographs from rats participating in this experiment clearly demonstrated light-induced modifications in ACh\textsubscript{E} expression and activity (Fig. 2). In the control retina, ISH (Fig. 2a) predictably indicated very low levels of expression of mRNA for the ACh\textsubscript{E}-R variant. After exposure to damaging light, expression of ACh\textsubscript{E}-R mRNA was augmented in the ganglion cells (GCs) and in retinal cells in the INL. However, the most striking effect was seen in the inner segments (IS) of the photoreceptors. This effect was noted as early as 1-day after exposure to damaging light, increased during the next 14 days (Figs. 2c, 2e), and was seen up to 30 days (data not shown), the longest period of follow-up. Densitometry measurements showed a mild, but statistically significant, increase in IS ACh\textsubscript{E}-R mRNA at 1 day after exposure, and a substantial, statistically significant, increase at 14 days after exposure to light (Table 1). Expression of ACh\textsubscript{E} mRNA, encoding the synaptic variant of ACh\textsubscript{E} was relatively unchanged after light exposure, compared with normal rat retinas (data not shown).

The augmented expression of ACh\textsubscript{E}-R mRNA was accompanied by increased cytochemical labeling for total ACh\textsubscript{E} catalytic activity (Fig. 2, right column), albeit at a different pattern. In the control retina, strong ACh\textsubscript{E} activity was seen in the proximal parts of the retina (IPL and ganglion cells) and very weak activity in the photoreceptor inner segments (Fig. 2b), reflecting the normal activity of the ACh\textsubscript{E}-S variant in the rat retina. It should be noted that the staining pattern in the proximal retina was rather diffuse and lacked the distinct layered staining, probably because we had to incubate the slices overnight to test for catalytic activity in the photoreceptor inner segments. With shorter incubation times (2–4 hours), the staining pattern in the proximal retina (data not shown), was very similar to that shown previously.

At 1 day and 14 days after exposure to bright, damaging light, ACh\textsubscript{E} activity increased in the photoreceptor inner segments (Figs. 2d, 2f). ACh\textsubscript{E} activity in the photoreceptors was refractory to iso-OMPA, an inhibitor of the ACh\textsubscript{E}homologous protein butyrylcholinesterase (BCh\textsubscript{E}), but was completely blocked by BW 284C51, a selective inhibitor of ACh\textsubscript{E} (data not shown), indicating that exposure to bright, damaging light increased ACh\textsubscript{E} but not BCh\textsubscript{E} activity in the photoreceptors. It was difficult to appreciate stress-induced changes in ACh\textsubscript{E} activity in the proximal part of the retina (INL and GC), because histochemical staining was already high in the control retinas, obscuring any stress-induced augmentation. Therefore, photic stress-related changes in ACh\textsubscript{E} activity were analyzed quantitatively only for the photoreceptor inner segments (IS in Table 1).

The light-exposure-induced ACh\textsubscript{E}-R accumulation in the photoreceptor inner segments could be an irrelevant byproduct of the damaging stress. Alternatively, it could have a detrimental role promoting light-induced photoreceptor death, or could be part of a recovery process. To distinguish between these possibilities, rats were treated daily by intraperitoneal injection of rat Monarsen, an antisense agent directed against the consensus domain in ACh\textsubscript{E} mRNA. Treatment initiated 1 day before light exposure and proceeded until the end of the follow-up period. Control rats in this experiment (experiment 2), were also exposed to the damaging, bright light, but were injected intraperitoneally daily with saline solution. We chose intraperitoneal injection for Monarsen administration, because daily intravital injection for 30 consecutive days was unrealistic, and because previous reports indicated a breakdown of the blood-retinal barrier after exposure to dam-
aging light, thus providing a simple route for delivering the antisense agent to the retina.

Figure 3 shows micrographs of retinas that were obtained 14 days after exposure to damaging light and underwent ISH for AChE-R mRNA (Figs. 3a, 3c), or histochemical staining for total AChE activity (Figs. 3b, 3d). Sections were derived from experimental rats treated with Monarsen (Figs. 3c, 3d), and from control rats treated with saline (Figs. 3a, 3b). It should be noted that in preliminary experiments, Monarsen treatment, either by intravitreal injection or by intraperitoneal injection, exerted no effects on the retinas of rats, which were raised under a 12-hour light–dark cycle and were not exposed to bright, damaging light (data not shown), similar to its null effects in the control brain. In contrast, averaged densitometry measurements from retinas of four rats of each group in this experiment (right of each micrograph) demonstrated that Monarsen, injected intraperitoneally, predictably crossed the blood–retinal barrier, reached the retina, and suppressed the light-induced expression of AChE-R mRNA in all retinal cells, having the strongest effect in the photoreceptor inner segments (Fig. 3c, arrowheads). Correspondingly, AChE activity in the photoreceptor inner segments was considerably weaker in the retina from rats treated with Monarsen (Fig. 3d, arrowheads) compared with those injected with saline (Fig. 3b, arrowheads). An important finding is that Monarsen did not alter AChE activity in the proximal retina (IPL and GC), sug-

**TABLE 1. The Effects of 24-hour Exposure to Bright, Damaging Light upon Expression of AChE-R mRNA**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experiment 1: Light Exposure with No Further Treatment</th>
<th>Experiment 2: 14 Days after Light Exposure with Daily Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ph IS (ISH)</td>
<td>1.0</td>
<td>296*</td>
</tr>
<tr>
<td></td>
<td>INL (ISH)</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>GC (ISH)</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>IS (Cytochemical staining)</td>
<td>1.0</td>
<td>2.0*</td>
</tr>
</tbody>
</table>

Effects were measured in photoreceptors inner segments (Ph IS), inner nuclear layer (INL) and ganglion cells (GC). AChE activity was measured in photoreceptors inner segments (IS). Experiment 1: Exposure to bright, damaging light and follow-up without any treatment. Experiment 2: Exposure to bright, damaging light followed by daily intraperitoneal injection of saline or Monarsen (antisense). Values indicate fold increase of staining relative to the corresponding layer in control rats that were not exposed to the bright, damaging light. Each value is the average of measurements in retinas from four different rats undergoing the same experimental procedure.

* Statistically significant ($P < 0.05$) difference when compared to control.
† Statistically significant ($P < 0.05$) difference when compared to rats treated daily with saline.
The control retina was positively stained for total AChE in the proximal margin of the INL (amacrine cells), in the IPL and in ganglion cells, whereas immunostaining of N-AChE was negligible (Fig. 4, top). A similar distribution of total AChE was found in the light-exposed rats, injected with saline (Fig. 4; second row, middle column). Exposure to light also induced intense labeling for N-AChE in the photoreceptor inner and outer segments (Fig. 4, second row, arrowheads), and in the distal portion of the INL (Fig. 4; second row, arrows). From the location of the stained cells in the INL and their structure, they appeared to be bipolar cells. Together with the ISH data (Figs. 2, 3), these findings suggest the accumulation of N-AChE-R in the light-exposed retina. In contrast, the retinal sections from rats, exposed to damaging light but treated with Monarsen, showed an immunostaining pattern similar to that of control rats (Fig. 4, lower row). Weak staining was seen at the photoreceptor level, and in the distal margin of the INL, suggesting that Monarsen prevented most, but not all, of the light-induced expression of N-AChE protein.

To challenge the functional role of AChE-R in light-induced retinal damage, we recorded the dark-adapted ERG before and at different time intervals after exposure to bright, damaging light. The ERG is an excellent test for our goal—that is, assessment of retinal function during an extended period of follow-up. The ERG reflects light-induced electrical activity of the entire retina. It is composed mainly of two waves: the negative a-wave reflects light-induced activity in the photoreceptors, and the positive b-wave is generated in the INL, mainly by ON-bipolar cells with contribution from Müller cells. After exposure, rats were treated daily with saline (n = 10) or Monarsen (n = 10), as described before. The ERG responses of all light-exposed rats were predictably attenuated, indicating substantial light-induced retinal damage. However, Monarsen-treated rats demonstrated ERG responses of considerably larger amplitude compared with saline-treated rats, as illustrated for two rats (one of each group) in Figure 5a. These responses were elicited in the dark-adapted state with the brightest light stimulus available in our photic stimulation system. Because the ERG responses depended on the light intensity of the stimulus used to elicit them, we recorded the ERG responses to light stimuli of different intensities and constructed the re-

**Figure 3.** The effects of Monarsen, an orphan AChE mRNA-targeted antisense agent, on light-induced expression of AChE-R mRNA and total activity of AChE. Rats were treated daily with intraperitoneal injection of saline or Monarsen and were killed 14 days after exposure (24 hours) to bright, damaging light. Average (±SEM) density values at the photoreceptors inner segments (IS), inner nuclear layer (INL), and ganglion cells (GC), obtained from retinas of four rats, are shown to the right of each representative micrograph. AChE-R mRNA and AChE activity were higher in the retinas of rats, exposed to bright, damaging light, and treated with saline (a, b), compared with light-exposed rats that had been treated with Monarsen (c, d). Most evident was the effect in the inner segments of the photoreceptors (arrowheads). *Statistically significant differences between Monarsen- and saline-treated rats (P < 0.05).
response–intensity curves for the a- and b-wave, as illustrated in Figures 5b and 5c for the two rats whose ERG responses are shown in Figure 5a. The response–intensity data were fitted to the hyperbolic function (equation 1) and the maximum response amplitude ($V_{\text{max}}$) of the a- and b-wave were derived.

ERG results, similar to those shown in Figure 5 were obtained from all the rats studied in this experiment. $V_{\text{max}}$ derived from the response–intensity data, was averaged according to the two treatment groups. Monarsen-treated rats showed a lesser degree of initial ERG deficits and better recovery of the a- and b-wave compared with saline-injected rats (Fig. 6). These differences were significant for the b-wave of all the rats throughout the follow-up period ($P < 0.001$).

An additional measure, commonly used, for quantitative assessment of light-induced retinal damage, is the thickness of the ONL. Because this parameter varies considerably as a function of retinal eccentricity (distance from the optic disc), along the vertical meridian, we measured ONL thickness in the superior part of the retina from control rats (four sections in each eye) and from rats treated with monarsen or saline.
rat) that were kept in a 12-hour light–dark cycle and were not exposed to damaging light yielded an average ONL thickness of 44.1 ± 1.7 μm. Similar measurements from saline-treated and Monarsen-treated rats (three rats in each group, four sections from each rat), that were prepared for histology 14 days after 24-hour exposure to bright, damaging light, gave the following average values, respectively: 22 ± 3.0 and 21.5 ± 3.0. Because the sections varied in total retinal thickness, we normalized ONL thickness to total retinal thickness. With this approach, the ONL's relative thickness in Monarsen-treated rats (0.217 ± 0.034) was significantly ($P < 0.05$) more than the ONL's relative thickness in saline-treated rats (0.160 ± 0.026). We also counted the number of nuclei in the ONL of these retinas by using a rectangle of 115 μm width and height that was slightly larger than the width of the ONL. We chose this approach instead of counting the number of nuclei rows, because we noticed multiple loci of missing nuclei spread in a mosaic pattern within the ONL of light-damaged retinas. We measured 103.3 ± 10.3 nuclei in control rats not exposed to the damaging bright light. This was significantly ($P < 0.001$) larger than the number of nuclei in light-damaged retinas regardless of the treatment that was given to the rats. The number of ONL nuclei in the Monarsen-treated rats (43.8 ± 7.6) was significantly ($P = 0.009$) larger than the number of ONL nuclei in the saline-treated rats (31.8 ± 4.4). Thus, the protective role of Monarsen against light-induced retinal damage was also evident from morphometric measurements.

**DISCUSSION**

Our findings demonstrate, for the first time, that a “read-through” isoform of AChE with an extended N terminus is induced by stress in the mammalian retina. The N-AChE protein(s) was also observed in other stress-respecting parts of the CNS; however, our current findings provide the first direct evidence on both its stress-induced accumulation in retinal photoreceptors and its causal involvement in the light-induced death of these cells. This conclusion is based on two observations. ISH indicated that photic stress induced the expression of mRNA coding for the “read-through” variant of AChE, whereas immunostaining indicated expression of a protein isoform of AChE carrying an extended N terminus. Together, these findings suggest that the N-terminally extended AChE-R variant (N-AChE-R protein) is expressed in the retinas of albino rats subjected to photic stress. However, we could not completely rule out the possibility that N-AChE-S was also induced by exposure to bright light. We showed that this novel isoform of AChE exerted antisense-suppressive neuronal cell death, as assessed from functional tests (ERG recordings) and morphometric measurements in the albino rat retina.

In the photic-stressed rats, Monarsen antisense treatment effectively suppressed the expression of N-AChE (Fig. 4) in the retina, while enhancing the recovery of treated retinas as evident from functional tests (Figs. 5, 6) and from morphometric measurements of ONL thickness. Qualitative ISH analysis indicated that the Monarsen treatment reduced the expression of mRNA AChE-R more efficiently than it did for mRNA AChE-S (data not shown), supporting previous observations of particular vulnerability of nascent mRNA transcripts to the antisense effect.27,35,56 These results further suggest that the N-AChE-R protein (and/or other nascent AChE variants induced under photic damage, such as N-AChE-S) play a detrimental role, exacerbating photoreceptor injury after exposure to bright, damaging light. This role can be explained in at least two alternative ways. First and foremost, the damaging role of the expressed AChE may be due to its catalytic activity. ACh has been shown to suppress the production of proinflammatory cytokines20 and to act via nicotinic receptors as a neuroprotector against excitotoxicity.41,42 In fact, in a mammalian model of glutamate-induced excitotoxicity, ACh was found to protect GCS from death.13 Thus, overexpression of AChE by exposure to bright light may act to reduce the neuroprotection offered by endogenous ACh. In addition, recent reports show a nonenzymatic enhancement of apoptosis by AChE in cultured cells.19,20 Because retinal cell death after light exposure is by apoptosis,44,45 involving light-induced expression of AP1,1,39 our findings may reflect a tissue-specific causal role of N-AChE-R in the apoptosis of light-stressed photoreceptors. That suppression of nascent, but not the preexisting AChE, sufficed to reduce light-induced retinal damage supports the notion that distinct AChE variants play different roles in the retina. The similarity of their catalytic properties further supports the notion that nonenzymatic properties of AChE were involved.

Exposure to damaging light also induced expression of N-AChE-R in bipolar cells and ganglion cells, raising the possibility that light-induced retinal damage is not limited to the photoreceptors, but reflects apoptotic processes in a variety of retinal neurons. Future experiments are needed to find out whether N-AChE-R accumulates in retinal (and other) neurons after other stressful episodes such as increased pressure (glaucoma), metabolic stress (diabetes), and ischemia, where it may play detrimental role(s) promoting neuronal and specifically retinal damage.

Antisense treatment has been suggested as a potential therapeutic approach in cases in which specific silencing of gene expression is desired.47 However, this treatment is considered to be of limited value in the treatment of neurologic and retinal diseases because of its restricted transport across the blood–brain barrier38 and the blood–retinal barrier (BRB). Our current findings present the photic stress-disrupted BRB8 as an exception to this rule by demonstrating that intraperitoneally injected Monarsen blocked N-AChE-R expression in the retina. That photic damage injury facilitates the functioning of Monarsen in the retina further raises the possibility of treating other retinal diseases in which the BRB is damaged (e.g., clinical and...
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References


