Article

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Role of ON and OFF Visual Pathways in Rod- and Cone-Driven Flicker Responses

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Purpose: To evaluate the effects of various retinal neurotransmitters on temporal resolution, particularly, on the Critical Flicker Fusion Frequency (CFF), which has been previously applied in ophthalmic pathophysiologic research.
Methods: A binocular physiologic electroretinogram was performed on adult mice. Animals in the control group were injected in the right eye with 1 µL of phosphate-buffered saline (PBS). Animals in the experimental group were injected in the left eye with 1 µL of PBS and in the right eye with 1 µL of PBS to which different molecules were added: 2-amino-4-phosphonobutyric acid (APB), Glutamate, γ-aminobutyric acid (GABA), 6,7-dinitroquinoxaline-2,3-dione (DNQX), Bicuculline, Glycine, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Initially, rod response was recorded and later the cone response.
Results: APB suppressed the rod-driven, but not the cone-driven flicker response. The other agents severely affected the lower flickering frequency response amplitude, in particular, at 3 Hz. The threshold of CFF was lowered from 50 Hz to 40 Hz after applying APB, Glycine, and HEPES. GABA remarkably enhanced rod-driven and cone-driven flicker response at 3 Hz, whereas Glutamate and GABA/Glutamate only did in rod-driven flicker response.
Conclusions: Both ON and OFF visual pathways were implied in cone-driven response, but only the ON visual pathway appears to play a relevant role in rod-driven flicker response. Flicker response seems to be enhanced by horizontal cells both in rod-driven and cone-driven response. In addition, due to the greater sensitivity of the flicker at low frequencies, it is suggested that pathophysiological studies should be carried out at said frequencies.

Keywords: CFF; flicker response; neurotransmitter; retina; mice

Introduction

Temporal resolution is defined as the ability to discern luminance changes over time, which allows the visual system to collect and process light information. Once the visual system cannot distinguish a high-frequency flickering light from a constant or continuous light, it is called Critical Flicker Fusion Frequency (CFF) to the upper cut-off threshold for a given flicker frequency [1]. The CFF is a complex function of the visual system that reflects biological activity in retinal cells. Currently, changes in CFF cannot be ascribed to a specific cellular level. However, this test is a technically simple, economic, fast, and valuable in the clinical routine [2]. Due to its relevant role, the CFF has been widely used in ophthalmological diagnosis, including glaucoma and ocular hypertension [3], optic neuropathy [4,5], media opacity [6], and age-related macular degeneration [7]. Regarding its efficiency in detecting rapid changes, the CFF is further used to assess brain nervous system function, that is described as alertness, attention, and cortical arousal in humans [8], such as diagnosing minimal hepatic encephalopathy [9,10], and the early detection of Alzheimer's disease [11].

The CFF has also been used to compare the temporal resolution capabilities of different animals, such as monkey [12], dogs [13], rats [14], domestic chickens [15], and mice [16,17]. All these studies were electrophysiologically approached, through recordings such as electroretinogram (ERG), and/or behaviorally measured, appearing the potential clinical application of CFF. Although some studies are related to the effect of some drugs on the CFF, almost all of them are studies with psychoactive drugs [18–22]. But, to our best knowledge, it has rarely been assessed the functional role of neurotransmitters in the temporal resolution, despite the fact that various types of neurotransmitters transmit the majority of visual information from photoreceptors to the optic nerves in the retina.

Noticeably, visual circuits rely upon the dark and light adaptation state and the strength of stimuli [23]. In rod-driven response under scotopic condition, visual signaling mainly flows from rod photoreceptors to rod bipolar cells, and subsequently, to ON ganglion cells via amacrine II cells [24]. However, in cone-driven response, visual information is transmitted directly from cone photoreceptors to ON and OFF cone bipolar cells, and then, to ON and OFF ganglion cells, respectively. Meanwhile, visual information is divided into ON and OFF pathways starting from the outer plexiform layer [25]. This is because that in response to a light stimulus, the release of Glutamate from the photoreceptors ceases, depolarizing ON-bipolar cell (rod bipolar cells and ON-cone bipolar cells) and hyperpolarizing OFF-cone bipolar cells, due to their dendrites expressing inhibitor metabotropic glutamate receptor 6 (mGluR6) and excitatory ionotropic glutamate receptors α-amino-3-hidroxi-5-metilo-4-isoxazolepropionic acid (AMPA) and Kainate, respectively [26].

There is a great similarity in retinal structures and cell types between mouse and human [27]. Furthermore, both species share fundamental properties of temporal psychophysics. For example, Weber adaptation in response to low frequency flicker and illumination-dependent increases in critical flicker frequency as predicted by the Ferry-Porter law [28]. To improve our understanding of visual temporal resolution, the ERG was used to pharmacologically assess flicker frequency sensitivity to different neurotransmitters in mice retina. The goal is to provide evidence that cone-driven and rod-driven flickering response involve different visual pathways.

Materials and Methods

Animal Model, Legal Protection, and Maintenance

Adult (12–24 weeks of age), healthy, male and female C57BL/6J wild-type mice (The Jackson Laboratory, USA) were used. No differences in ERG amplitudes between sexes or animal ages have been shown in this ranges. The animals were kept in ventilated racks with cages, under light and dark cycles of 12:12 hours, free access to diet and water *ad libitum*. The rooms were kept at a temperature of 21 °C, with a relative humidity of 55%. The procedures have followed the directive 2012/63/UE and the Spanish RD n° 53/2013, approved by the ethics and government committee of the Community of Madrid (Proex 143/17).

Animal Preparation and Intravitreal Drug Injection

For better observing the flicker response, the experiment was performed into two subgroups. Dark-adapted animals (more than 12 hours) were anesthetized with an intraperitoneal injection of a saline solution (0.9% NaCl) containing ketamine (Ketamidor, Laboratorios Karizoo, S.A. Barcelona, Spain) and xylazine (Xilagesic, Laboratorios Calier, S.A. Barcelona, Spain). The dose of ketamine was 100 mg/kg, while that of xylazine was 5 mg/kg. In the first group or control group, the left eye of the animal was not manipulated, while the right eye was injected with 1 µL of phosphate-buffered saline (PBS) just behind the limbus using a Hamilton microsyringe (Hamilton Company, Reno, NV, USA) with a 34 gauge needle. In the second group or experimental group, the left eye of each animal was injected with 1 µL of PBS as a control, while the right eye was injected with 1 µL of a PBS solution containing one of the following drugs: 2-amino-4-phosphonobutyric acid (APB) (25 mM), Glutamate (100 mM), γ-Aminobutyric acid (GABA) (100 mM), 6,7-dinitroquinoxaline-2,3-dione (DNQX) (30 mM), Bicuculline (10 mM), Glycine (10 mM), Strychnine (25 mM), or a combination of these compounds, which are either agonists or antagonists of retinal neurotransmitters: GABA (100 mM)/Glutamate (100 mM), and DNQX (30 mM)/Bicuculline (10 mM). Meanwhile, the effect of pH buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (25 mM) was also tested. The final concentrations of the pharmacological agents used were based on an adult mouse vitreous volume of 5 µL. All drugs and agents were purchased from Sigma Aldrich (St. Louis, MO, USA) and were prepared fresh in PBS, unless otherwise indicated.

To prepare for recording full-field flicker, the pupils of the animals were dilated with one drop of 1% tropicamide (Colircusí tropicamide, Alcon Cusí, Barcelona, Spain). The animals were then placed at the center of the home-made Ganzfeld dome. During the recording, the body temperature of the animal was maintained at 37 °C using a water-circulation warming pad. To prevent the corneal surface from drying out and to facilitate the transmission of electrical signals, a drop of 2% methylcellulose (Methocel, Omnivision, Puchheim, Germany) was applied to the corneal surface. The anaesthesia and intraocular injections were performed under dim red room illumination. Afterward, the mice were kept in complete darkness for 10 minutes before recording to allow the drugs to work effectively and preserve the animals' full dark adaptation.

Signals Recording and Light Stimulation

To record the flicker response, Burian-Allen corneal electrodes were placed on the visual axis, approximately 2–3 mm from the cornea of each eye. The reference electrode was carefully placed on the mouth to prevent the mouse from swallowing its tongue, while the ground needle electrode was placed at the base of the tail. These electrodes were connected to an AC amplifier (Grass®, Astro-Med Inc., West Warwick, RI, USA). The signal acquisition, analysis, and storage were performed using the Power-Lab-ADI® and Labchart® v8 software (Power-Lab-ADI® and Labchart® v8 software, Oxford, UK). The electrophysiological response that was recorded underwent amplification ($\times$1000), filtering, which included a high pass filter of 0.1 Hz, and a low pass filter of 1000 Hz, and digitized at a rate

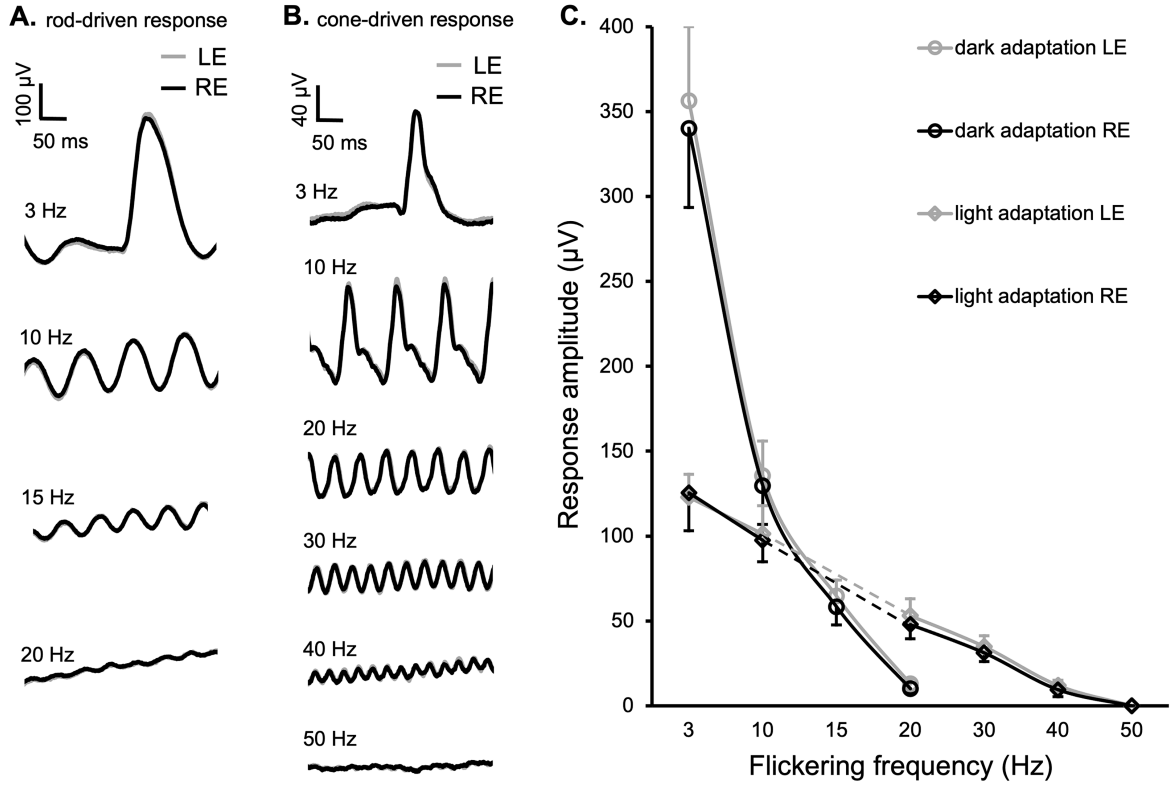

Fig. 1. Flicker response to various flickering frequencies under dark and light adaptation. (A) Representative recording waves in the rod-driven flickering response from 3 Hz to 20 Hz under dark adaptation. (B) Representative recording in the cone-driven flickering response from 3 Hz to 50 Hz under light adaptation. (C) Flicker response amplitude to a series of flickering frequencies under dark-adapted and light-adapted condition. Each data point on the graph represented the mean of the right and left eye of six animals. Error bars were standard deviations. A phosphate-buffered saline (PBS) solution of 1 µL was injected into the right eye, while the left eye was not manipulated in any way. LE, left eye; RE, right eye.

of 2K. The whole recording time is 1000 milliseconds, with 100 milliseconds of prestimulation. The flicker response was measured in a silent room without distracting noises and across the central visual field.

The white light stimuli used in the experiment were produced by an LED-based Ganzfeld dome. The mice were placed in a platform localized in the center point of the Ganzfeld dome and the stimulus was viewed from a distance of 15 cm, giving an angular stimulus size of 20°, while fixation was central. The light intensities were gauged using a calibrated photometer (GOSSEN MAVO MONITOR, Germany). The light intensities utilized in our study were based on the full-field flash ERG [23], which is a standard for measuring the electrical response of the retina to light stimuli, but adapted for mouse recordings. Specifically, the rod-driven response (scotopic condition) and the cone-driven response (photopic condition) were measured, the intensity of the applied light stimulus was 0.01 and 15 $cd{\cdot}s{\cdot}m^{-2}$, respectively. The experiment started recording from rod-driven response at an intertrials interval of 10 seconds to preserve the animals' dark adaptation status. Following the initial measurements, the mice were exposed to 15 minutes of light adaptation under a background light of 32 $cd{\cdot}s{\cdot}m^{-2}$, which saturated the rod-driven responses. Then, the cone-driven response was recorded at an intertrial interval of 1 second. To ensure stable flicker responses and minimize the impact of noise and artifacts, at least 20 light responses were averaged to stimuli of the flickering frequency at values of 3, 10, 15, and 20 Hz under dark adaptation, and at values of 3, 10, 20, 30, 40, and 50 Hz under light adaptation. Once ending the experiment, animals' eyes were checked and corresponding data were discarded if mice presented cataract. Mice were killed with sodium pentobarbital, intraperitoneally administered (Doletal®, Vetoqimol, Madrid, Spain).

Flicker Analysis

In the analysis of flicker response, the first recording to the onset of the flicker, which may resemble a single full-field flash ERG, was excluded [23]. In this study, response amplitudes of three continuous waves were measured from their corresponding peak to their preceding baseline, and then all the measured amplitude were averaged for analysis. The first measured response amplitude always started from 500 milliseconds after light stimulation, except for measuring 3 Hz flicker response. At this frequency, there were

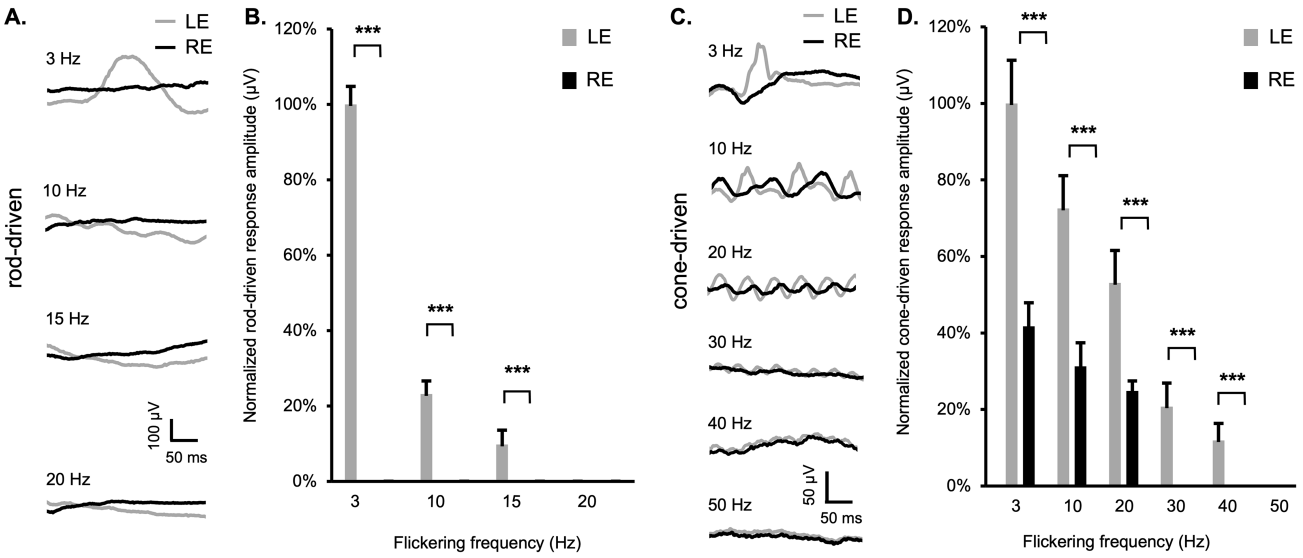

Fig. 2. Flickering responses induced by 2-amino-4-phosphonobutyric acid (APB) under dark and light adaptation. (A) Under dark adaptation, representative recording waves of flicker response from 3 Hz to 20 Hz. (B) Normalized rod-driven flickering response amplitude. (C) Under light adaptation, representative recording waves of flicker response from 3 Hz to 50 Hz. (D) Normalized cone-driven flickering response amplitude. The illustrated waveforms showed the recording signal from 400–700 ms after the onset of light stimuli. Data were normalized to the response amplitude of the control eye in response to the flickering frequency at 3 Hz. Mean ± standard deviation (SD), n = 3. LE, left eye, that is the control eye, indicated in grey; RE, right eye, that is the experimental eye, indicated in black. ***, $p < 0.001$.

only four waves in the whole response; thus, its last three waves of the response amplitude were measured, although one of those waves were before 500 ms. When the flicker frequency was higher than 3 Hz, of note, the wave was discarded if there were two peaks or more, and so, another continued wave was measured.

The flicker responses amplitude was analyzed. The data from different animal groups were analyzed and graphed using Excel software (version 16.53, Microsoft, Redmond, WA, USA). The results were presented as the mean and standard deviation (SD). To determine whether each data set followed a normal distribution, the Shapiro-Wilk test was used beforehand. A statistical comparison of the mean was performed using the Student's *t* test for normal distributions, and the Mann–Whitney U test for non-parametric distributions by IBM SPSS statistical 23.0 package (SPSS Inc., Chicago IL, USA). *p* values of less than 0.05 were considered statistically significant.

Results

Normal Flickering Recording

In the control group, data were obtained after exposure to a series of steady flicker stimuli with increasing frequencies, both under dark and light adaptation (n = 6) (see Fig. 1). Intravitreal injection of 1 µL of PBS was administered to the right eye of each animal, while the left eye was not injected with anything. The amplitude of the flicker response gradually decreased as increased the flickering frequency, both under light and dark adaptation. When stimulated with low flicker frequencies, the amplitude of the response was greater in dark adaptation conditions, particularly at 3 Hz, than in light adaptation.

None of the light adaptation conditions showed any statistically significant differences in the response amplitude between the right and left eyes (in the rod-driven response: $p = 0.471$ (3 Hz), $p = 0.627$ (10 Hz), $p = 0.265$ (15 Hz), and $p = 0.206$ (20 Hz); in the cone-driven response: $p = 0.748$ (3 Hz), $p = 0.568$ (10 Hz), $p = 0.181$ (20 Hz), $p = 0.079$ (30 Hz), $p = 0.231$ (40 Hz), flickering response was fused at 50 Hz). Thus, the flicker response was not affected by the manipulation caused by the intravitreal injection. In addition, it was observed that no animal could discern flickering responses to 50 Hz stimuli, under adaptation to light. However, in dark adaptation, the limit was lower so that only two of six animals could respond to stimuli of 20 Hz. The CFF for the mouse cone-mediated visual pathway in response to 15 cd·s·m^{-2} was 50 Hz, whereas for rod-mediated vision in response to 0.01 cd·s·m^{-2}, it was about 20 Hz.

Effects of Intravitreal Injection on Flickering Recording

Effects of 2-Amino-4-Phosphonobutyric Acid

APB is a Glutamate agonist, and its intravitreal injection at 25 mM (n = 3) produced different effects under dark and light adaptation. Notably, flicker response in rod-driven (Fig. 2A,B) was abolished by APB, whereas in the

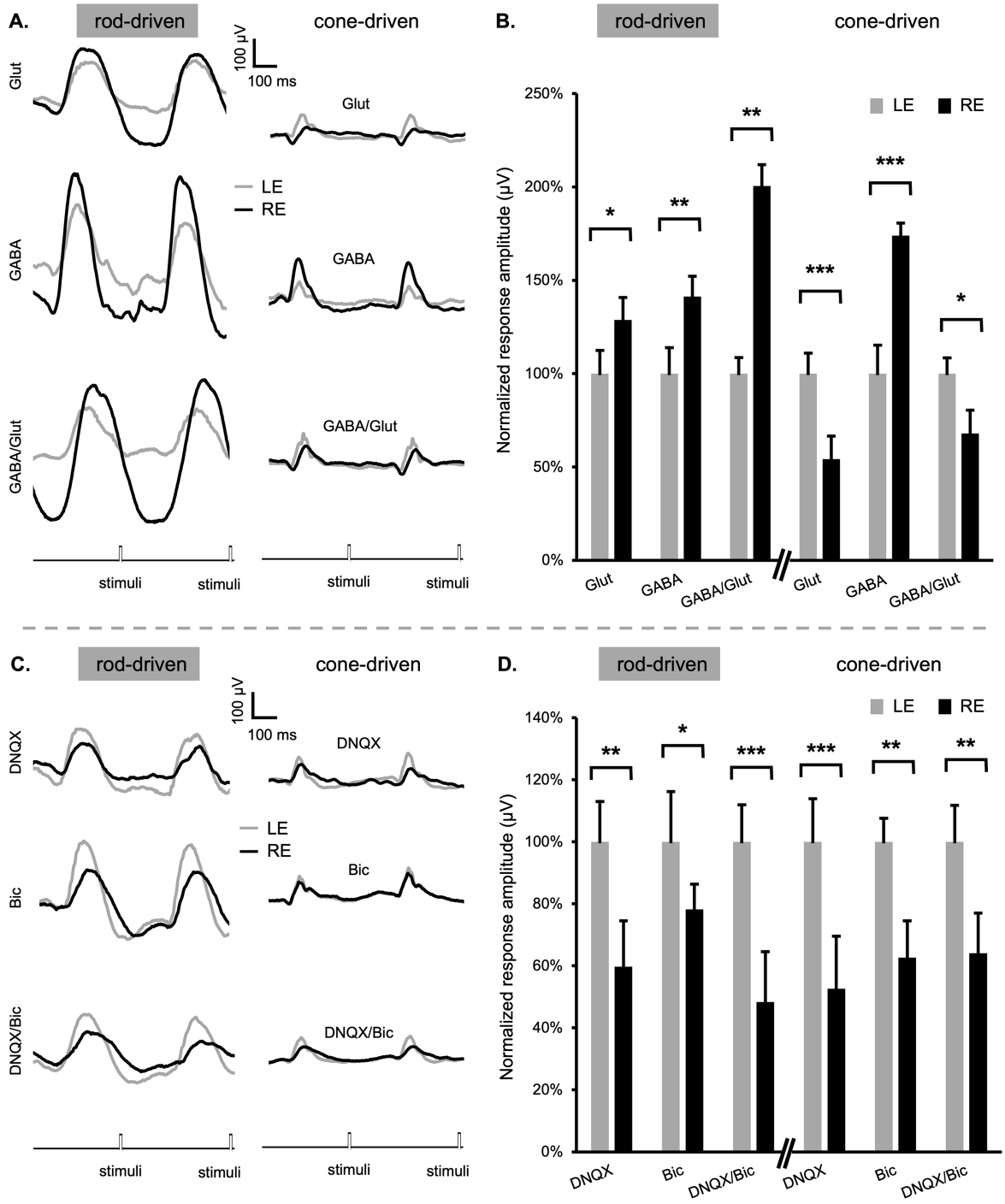

Fig. 3. Scotopic and photopic flickering 3 Hz response induced by Glutamate, γ-aminobutyric acid (GABA), and their antagonists. (A) Representative recording waves after application of 100 mM Glutamate, 100 mM GABA, and (100 mM/100 mM) GABA/Glutamate. (B) Normalized flicker response induced by Glutamate, GABA, and their combination under dark and light adaptation. (C) Representative recording waves after application of 30 mM 6,7-dinitroquinoxaline-2,3-dione (DNQX), 10 mM Bicuculline, and their combination under dark and light adaptation. (D) Normalized flicker response induced by DNQX, Bicuculline, and their combination under dark and light adaptation. The illustrated waveforms showed the recording signal from 400–1000 ms after the onset of light stimuli. Data were normalized to the response amplitude of the control eye in response to the flickering frequency at 3 Hz. Mean ± SD, n = 3–6/group. GABA, γ-aminobutyric acid; Bic, Bicuculline; DNQX, 6,7-dinitroquinoxaline-2,3-dione; Glut, Glutamate; LE, left eye, that is the control eye, indicated in grey; RE, right eye, that is the experimental eye, indicated in black. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

cone-driven response (Fig. 2C,D), it was significantly reduced at 3 Hz ($p < 0.001$), 10 Hz ($p < 0.001$), 20 Hz ($p < 0.001$) and was fused at 30 Hz.

Effect of Glutamate, γ-Aminobutyric Acid, and Their Antagonist

The low flicker response, particularly at 3 Hz, was severely altered when the right eye of animals was intravitreally injected with Glutamate (100 mM, n = 4), GABA (100 mM, n = 3), or GABA/Glutamate (100 mM/100 mM, n = 6) in co-administration, compared to the left eye (Fig. 3A,B). Concretely, the flicker response at 3 Hz was significantly augmented in scotopic conditions by all three of them (Glutamate, $p = 0.013$; GABA, $p = 0.005$; GABA/Glutamate, $p = 0.007$), but suffered a significantly reduction by Glutamate ($p < 0.001$), and GABA/Glutamate ($p = 0.022$) in photopic conditions. Meanwhile, GABA increased the flickering response amplitude at 10 Hz (Right eye vs Left eye, 227.62 ± 10.52 vs 77.01 ± 9.16, mean ± SD, $p < 0.001$, n = 3) in scotopic condition and at 10 Hz (Right eye vs Left eye, 123.45 ± 12.95 vs 70.95 ± 10.79, mean ± SD, $p < 0.001$, n = 3) and 20 Hz (Right eye vs Left eye, 104.22 ± 12.66 vs 60.43 ± 8.25, mean ± SD, $p = 0.026$, n = 3) in photopic condition. No statistical differences were found in the response amplitude between the right and left eyes in any other flickering frequencies.

DNQX and Bicuculline are antagonists of the AMPA/Kainate receptors and the GABAa receptor, respectively, so their application should have the opposite effect. Actually, the 3 Hz flicker response was significantly decreased in both scotopic and photopic responses after intravitreal injection of DNQX (n = 3, $p = 0.001$ in rod-driven response, $p < 0.001$ in cone-driven response) or Bicuculline (n = 3, $p = 0.039$ in rod-driven response, $p = 0.001$ in cone-driven response), or co-administered DNQX/Bicuculline (n = 6, $p < 0.001$ in rod-driven responses, $p = 0.001$ in cone-driven responses) (Fig. 3C,D). Once the flickering frequency was higher than 3 Hz either under dark or light adaptation, the amplitude of flickering responses was not altered ($p > 0.05$) after the application of Bicuculline, whereas was significantly reduced by the application of DNQX/Bicuculline (in rod-driven response: $p = 0.004$ (10 Hz), $p = 0.007$ (15 Hz), flickering response was fused at 20 Hz; in cone-driven response: $p < 0.001$ (10 Hz, 20 Hz, and 30 Hz), $p = 0.021$ (40 Hz), flickering response was fused at 50 Hz).

Effect of Glycine and Its Antagonist-Strychnine

After intravitreal injection of Glycine (n = 3), a considerable reduction of the flickering response at 3 Hz ($p < 0.009$), 10 Hz ($p < 0.039$), 15 Hz ($p < 0.013$) was induced under scotopic conditions (Fig. 4A,C). Under photopic conditions, the flicker response was relevantly weakened by Glycine at 3 Hz ($p < 0.012$), 10 Hz ($p < 0.009$), unchanged once higher 10 Hz ($p > 0.05$), and almost fused at 40 Hz (Fig. 4B,D).

Strychnine is an antagonist of Glycine receptors, its application (n = 3) was supposed to induce opposite effect from Glycine. However, said opposite effect was only observed in cone-driven flicker response at low frequencies (3 and 10 Hz) (Fig. 4F,H), which is the only difference with the intravitreal injection of Glycine. In addition, a reduction of the CFF threshold from 50 to 40 Hz was observed. On the other hand, flicker responses under rod-driven conditions were reduced in a similar way to that induced by Glycine (Fig. 4E,G).

Effects of 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

After intravitreal injection of the pH buffer HEPES (pH 7.4, n = 6) into the right eye of the animals, it was observed that the flicker response was significantly reduced when compared to the flicker response of the left eye of the animals, both under scotopic ($p < 0.001$ (3 Hz, 10 Hz), $p = 0.002$ (15 Hz)) and photopic conditions ($p < 0.001$ (3 Hz, 10 Hz, 20 Hz, 30 Hz)) (Fig. 5). Of note, under photopic conditions, the flickering response was fused at 40 Hz, instead of 50 Hz.

Discussion

The flicker response selectively reflects the activity of the rod and cone visual system, under light and dark adaptation, respectively [23]. The evaluation of the flicker response showed a scotopic CFF of 20 Hz and a photopic CFF of 50 Hz in C58BL/6J mice. The higher CFF under photopic conditions is a consequence of its increase with illumination level [29], allowing the eye to see substantially higher flicker rates under bright lighting than under dim lighting. These differences are also the result of sensitivity regulation, which controls the overall gain of the system [30]. All of this needs to be taken into account when designing behavioral experiments. Other studies have suggested that the ability to detect the flicker response depends on several factors, such as the frequency of the modulation, the intensity of the average illumination, or the area of the retina in which the stimulus is produced [8]. In any case, CFF is a complex and integrative function of the entire visual system, making it difficult to correlate changes in CFF with a specific dysfunction at a certain level of signal transduction [7]. Thereby, in this study, different flickering frequencies under dark and light adaptation state were applied. To better understand how works the injected agents in retina, a diagram of these retinal connections of the ON and OFF pathways is shown (Fig. 6).

In response to a light stimulus, photoreceptors stop releasing glutamate. The response of ON bipolar cells (rod bipolar cells and ON-cone bipolar cells) dendrites is depolarizing, due to the expression of metabotropic glutamate receptor 6 (mGluR6); while the OFF cone bipolar cells dendrites is hyperpolarizing due to the expression of

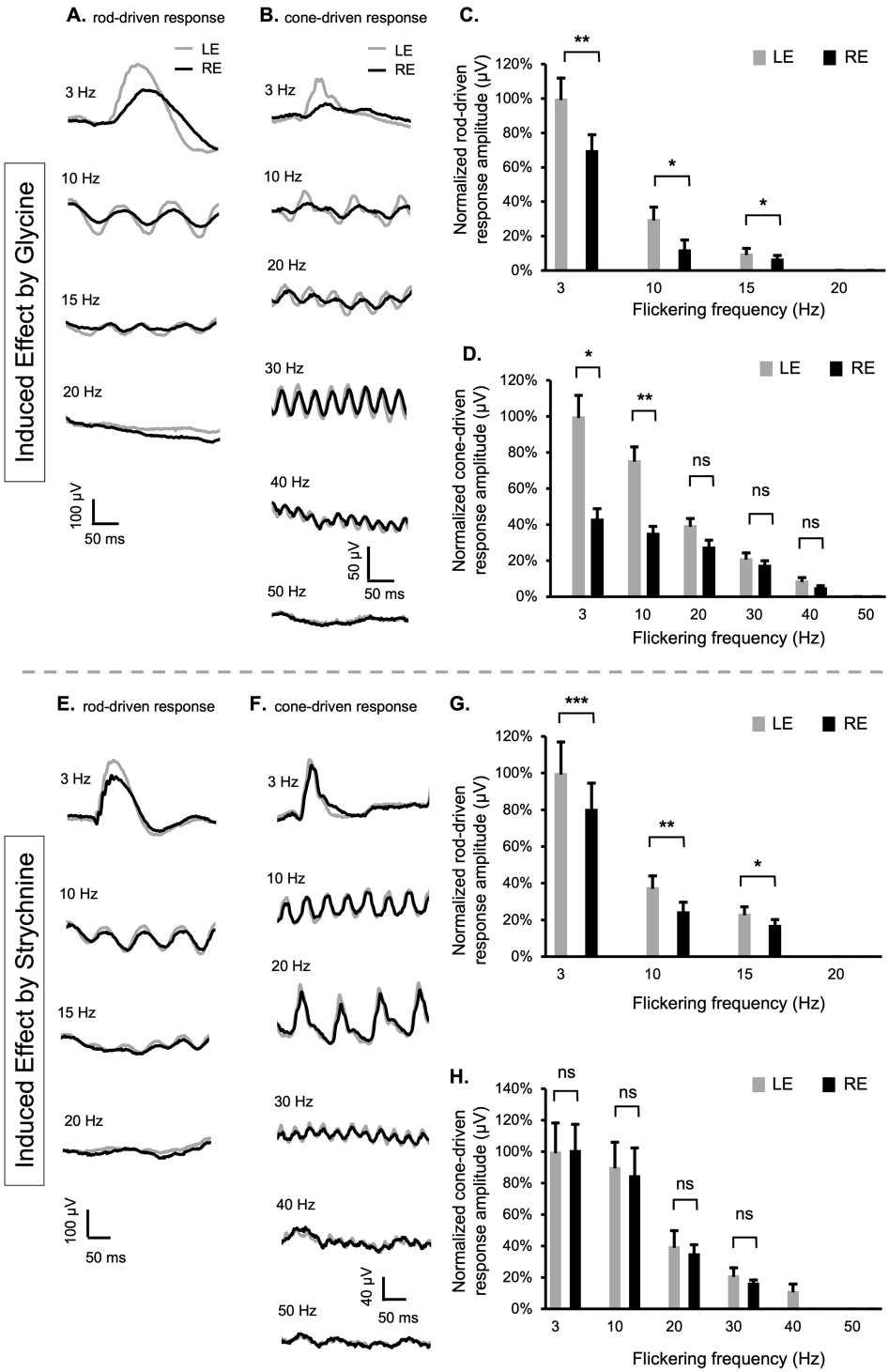

Fig. 4. Scotopic and photopic flickering response induced by Glycine and Strychnine. Representative recording waves of flicker response after intravitreal injection of 10 mM Glycine in the rod-driven response from 3 Hz to 20 Hz (A) and cone-driven-response from 3 Hz to 50 Hz (B). The illustrated waveforms showed the recording signal from 400–700 ms after the onset of light stimuli. Normalized flicker response amplitude in the rod-driven response (C) and cone-driven response (D). Representative recording waves of flicker response after intravitreal injection of 25 mM Strychnine in the rod-driven response from 3 Hz to 20 Hz (E) and cone-driven-response from 3 Hz to 50 Hz (F). The illustrated waveforms showed the recording signal from 300–700 ms after the onset of light stimuli. Normalized flicker response amplitude in the rod-driven response (G) and cone-driven response (H). Data were normalized to the response amplitude of the control eye in response to the flickering frequency at 3 Hz. Mean ± SD, n = 3/group. LE, left eye, that is the control eye, indicated in grey; RE, right eye, that is the experimental eye, indicated in black. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no statistical significance.

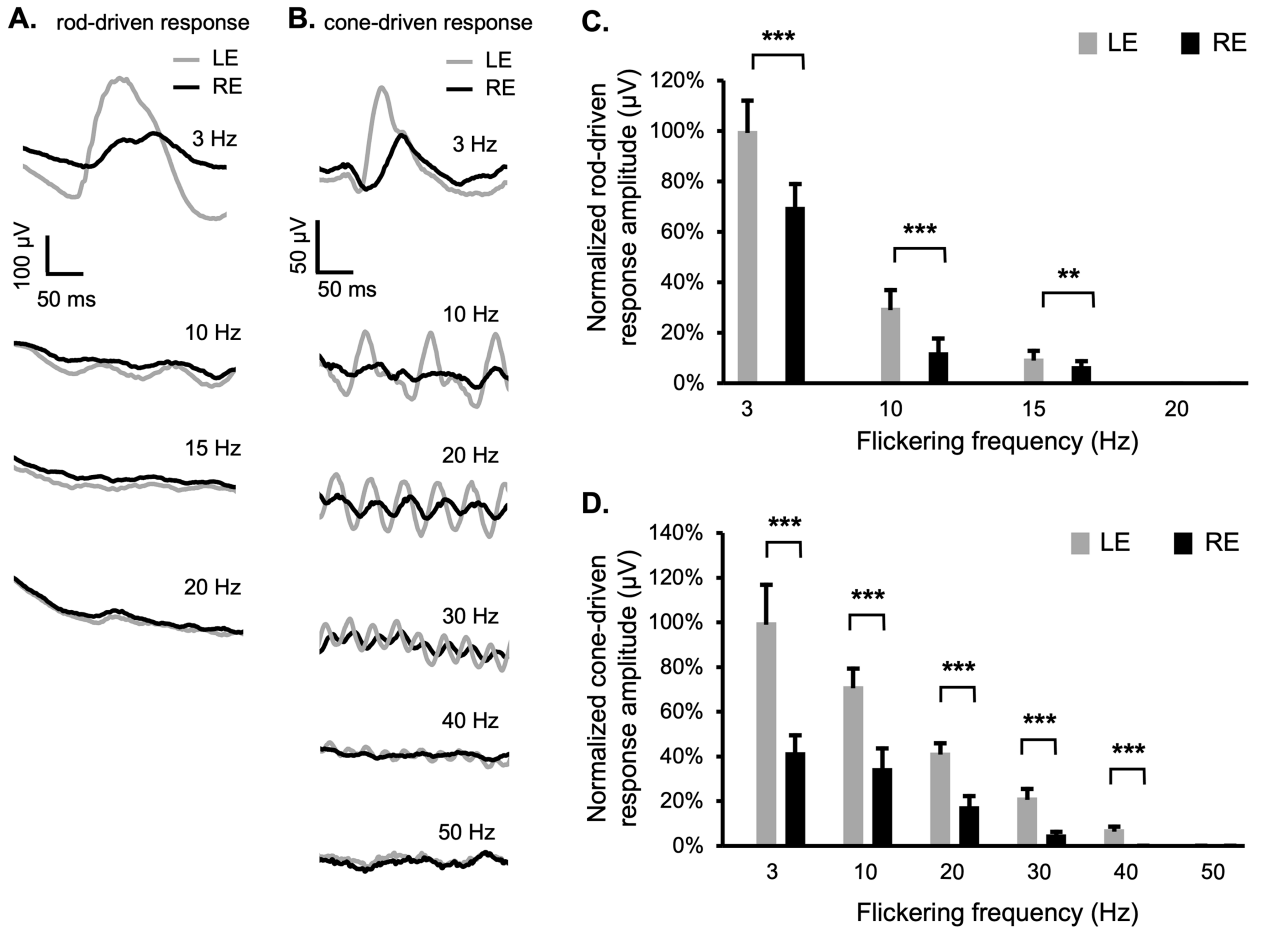

Fig. 5. Scotopic and photopic flickering response induced by 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Representative recording waves of flicker response after intravitreal injection of 25 mM HEPES in the rod-driven response from 3 Hz to 20 Hz (A) and cone-driven-response from 3 Hz to 50 Hz (B). The illustrated waveforms showed the recording signal from 400–700 ms after the onset of light stimuli. Normalized flicker response amplitude in the rod-driven response (C) and cone-driven response (D). Data were normalized to the response amplitude of the control eye in response to the flickering frequency at 3 Hz. Mean ± SD, n = 6. LE, left eye, that is the control eye, indicated in grey; RE, right eye, that is the experimental eye, indicated in black. **, $p < 0.01$; ***, $p < 0.001$.

ionotropic glutamate receptors AMPA/Kainate [26]. Glutamate released by photoreceptors in the dark by acting on mGluR6 receptors inhibits signal transmission, while acting on AMPA/Kainate receptors is excitatory [31].

APB is capable of blocking visual transmission from photoreceptors to ON bipolar cells [31], but not to OFF cone bipolar cells. This is because that APB is an agonist of metabotropic mGluR6 receptors located in dendrites of ON cone bipolar cells and rod bipolar cells. On that account, the injected APB abolished the rod-driven but not all the cone-driven flicker response, due to under photopic conditions, the OFF pathway of cones still being remained. Therefore, in scotopic conditions, light response is abolished, while in photopic conditions is only decreased. The effects of APB show that the ON pathway of cones shares a synaptic mechanism with the rods, while the OFF pathway of cones is different, so that when blocking the former with APB only the OFF pathway of cones remains. These differences would allow rod and cone visual pathways to be assessed independently by measuring CFF. According to that, other studies have not shown flicker response on mGluR6-/- mice below 5 Hz, since at these frequencies flicker responses are dominated by the rod pathways [32]. However, it should not be ignored the OFF rod pathways (Fig. 6A) since that rod visual signaling could flow from rod to cones photoreceptors through gap junctions (the second rod visual pathway), and/or flow from rod photoreceptors to OFF cone bipolar cells trough chemical synapses (the third rod visual pathway) [33]. Hence, two possible reasons to explain why no signals were recorded after blocking the ON visual pathway using APB in the rod-driven flicker response: the OFF visual pathway is not involved in and/or signals of the OFF visual pathway were too extremely small to be recorded.

Glutamate and GABA are two of the major neurotransmitters in the retina. Glutamate, whose receptors exist on all retinal neurons except photoreceptors, is continuously released in the dark by photoreceptors, but its release is inhibited by light stimuli. In this way, the injected Glu-

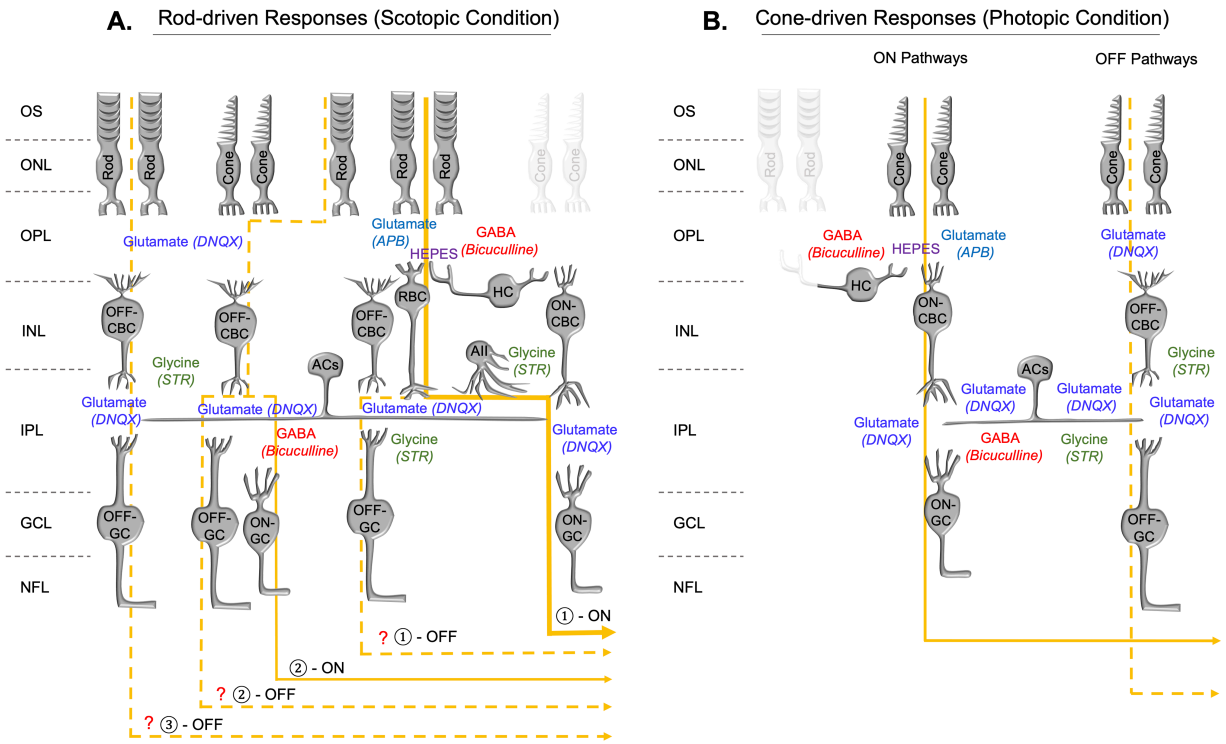

Fig. 6. Schematic of the ON and OFF visual pathways under dark and light adaptation conditions, main neurotransmitters, and membrane receptors expressed on retinal cells. (A) Under dark-adapted condition, flicker responses possibly involves three rod visual pathways. Light stimuli hyperpolarizes rod photoreceptors. Then, rod signals are mainly transmitted to rod bipolar cells, in the first rod-pathway. From here, through AII amacrine cells, inputs go to axonal terminal of ON cone bipolar cells (① - ON) or to OFF cone bipolar cells through gap junctions (① - OFF). In the second rod-pathway, light signal could flow from rod to cones photoreceptors through gap junctions flows to OFF cone bipolar cells through chemical synapses. The third rod-pathway involves gap junctions between rods that make chemical synapses with some OFF cone bipolar cells, which transmit the signal to OFF ganglion cells. (B) Under light-adapted condition (cone pathways), light also hyperpolarize cone photoreceptors. Cone signals are transmitted to ON-cone bipolar cells at the onset of light stimuli and to OFF-cone bipolar cells at the offset of light stimuli. Modulating the transmission of the signal in the outer plexiform layer would be the horizontal cells and in the inner plexiform the amacrine cells. Neurotransmitters and their antagonists are shown (in parentheses) at the various synapses. RBC, rod bipolar cells; ON-CBC, ON cone bipolar cells; OFF-CBC, OFF cone bipolar cells; HC, horizontal cells; ACs, Amacrine cells; ON-GC, ON ganglion cells; OFF-GC, OFF ganglion cells; OS, outer segments of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cells layer; NFL, nerve fiber layer; STR, strychnine; ?, Currently, it is unsure whether flicker response involves these OFF rod visual pathways.

tamate adds to the one released in the dark, causing Flicker waves of greater amplitude, in particular, at 3 Hz. However, the injected Glutamate exerts the opposite effect in light adaptation since Glutamate inhibits the ON pathway and enhances the OFF pathway. Application of DNQX (ionotropic glutamate receptor blocker) produced the opposite effect in scotopic, but not in photopic conditions. All their responses are a significant decrease of their amplitude.

Contrary to what was observed with Glutamate, GABA increased the amplitude of flicker waves, both in scotopic and photopic conditions; while their antagonist Bicuculline decreased the amplitude of flicker waves, both under dark-adapted and light-adapted conditions. Hence, GABA seemly acts as a modulator to increase the Flicker signal.

The flicker response was significantly reduced after intravitreal injection of HEPES, regardless of stimulus frequency, both under scotopic and photopic conditions. HEPES is a pH buffer and therefore protects against pH alteration within a limited range [34–36]. Its application seems to avoid the pH changes that normally occur during visual stimulation under physiological conditions. It is known that one of the mechanisms of neuronal regulation in the retina is the lateral pathway through the horizontal cells. This suggests that the most likely site involved is the cleft of the synaptic triad, which is formed by horizontal cells coincide with the axon terminals of the photoreceptors and the dendrites of ON cone bipolar cells [37]. Horizontal cells release GABA, but also contain GABA receptors and AMPA/Kainate receptors [26,36]. In addition, horizon-

tal cells are permeable to HCO_3^- [38,39]. Because aspects of the triadic synapse, such as the role that the axon terminals of horizontal cells play in flicker rate, remain unknown, further study is needed. However, current data suggest that horizontal cells play a fundamental role in the genesis of the flicker response, concretely, improving or strengthen the flicker response.

Glycine receptors (GlyR) are known to induce inhibitory postsynaptic currents. In this study, the injected Glycine relevantly decreased the flicker response in rod-driven independent of the flickering frequency, whereas such a decrease in cone driven response was only observed at low frequency. These might be due to a modulation occurs in the retinal circuits to transmit visual information when lighting changes, and/or different subtypes of GlyR were expressed by different retinal neurons. Indeed, GlyRs are mainly expressed on amacrine cells and OFF cone bipolar cells in the adult retina [40–42], correspondingly, plays a critical role for the visual transmission in the rod-driven and cone-driven response. However, there are functional differences in glycinergic receptors. On the other hand, OFF cone bipolar cells receive kinetically fast glycinergic inputs due to expressing GlyR α1 y β subunits (Decay time constant, $\tau \sim 5$ ms) [43], which causes high-amplitude currents. In contrast, in rod-driven response, amacrine II cells contain GlyR α3 subunits with medium-fast kinetics ($\tau \sim 11$ ms) and narrow field amacrine cells express $\alpha 2\beta$ and $\alpha 4\beta$ with slow kinetics ($\tau \sim 27$ ms) [44]. For these reasons, a broad range of flickering frequencies of light, both in photopic and scotopic intensities, can be processed in the retina.

Noticeably, the application of Strychnine (antagonist of GlyR) weakened rather than augmented the flicker response in rod-driven response as it was supposed to. This might be due to the rod-driven visual pathways involving amacrine cells containing GlyRs. In addition, approximately 40% of amacrine cells' subpopulation synthesizes and releases Glycine [40,45]. Glycinergic transmission is mediated by receptors permeable to the Cl^- ion and thereby is primarily inhibitory; however, depending on whether the Cl^- ion gradient is positive or negative concerning the resting membrane potential, glycinergic transmission can also be excitatory [46]. In any case, its exciting action is quite unknown at the moment.

Studies have shown that the low-frequency flicker response is more vulnerable to intravitreal injection agents, particularly at 3 Hz, both under dark and light adaptation state. There are two possible reasons. First, the flicker response reflects the entire temporal characteristic to low-frequency light stimulation, without loss of information. However, as the flicker frequency increases, the time to process visual information is severely shortened, reducing the integration time of the system, and subsequently, causing an overlapping of recording waves. Thereby, it is only capable of reflecting the processing main temporal area of visual transmission without reflecting some information corresponding to retinal feedback, such as the kinetic sum of inner retinal neurons, amacrine cells and/or Müller cells. A second reason necessarily involves the retinal processes to determine flicker sensitivity that is common in the retina of mice and humans. One acts as a temporary low-pass filter, involving signal processing within photoreceptors as its main element. The other is a high-pass filter, which consists of an inhibitory feedback network formed primarily by horizontal and amacrine cell connections in the inner and outer plexiform layer [43,44]. The function of the low-pass filter will be lost if the drugs applied affect the transmission of visual information from the photoreceptors to the bipolar cells, as suggested by studies; the high-pass filter will be altered, if the drugs applied affect the visual transmission through the horizontal or amacrine cells.

Conclusions

Each type of retinal neurontransmitter is critical for preserving the normal flicker response. In this way, GABA released by horizontal cells may enhance the flicker response in a lateral retinal pathway. This study suggests that both ON and OFF visual pathways were implied in cone-driven response. However, only the ON visual pathway appear to be relevant in rod-driven flicker response. In addition, due to the greater sensitivity of the flicker at low frequencies, it is suggested that pathophysiological studies should be carried out at said frequencies.

Availability of Data and Materials

All Data and Materials are available.

Author Contributions

PdlV and FL designed the research study. FL, HL and AGO performed the research. FL, HL, AGO and FG analyzed the data. FL wrote the first draft of the manuscript. PdlV and FG performed writing – review & editing the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors assume responsibility for all aspects of the work and guarantee the integrity of the work.

Ethics Approval and Consent to Participate

The animal study protocol was approved by the Committee of the Community of Madrid for the use of laboratory animals (Proex 143/17). Consent to Participate: not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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