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Margaret G. Au
Western Kentucky University

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Margaret G. Au
Department of Psychology
Western Kentucky University

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Dr. Joseph Bilotta
Thesis Advisor
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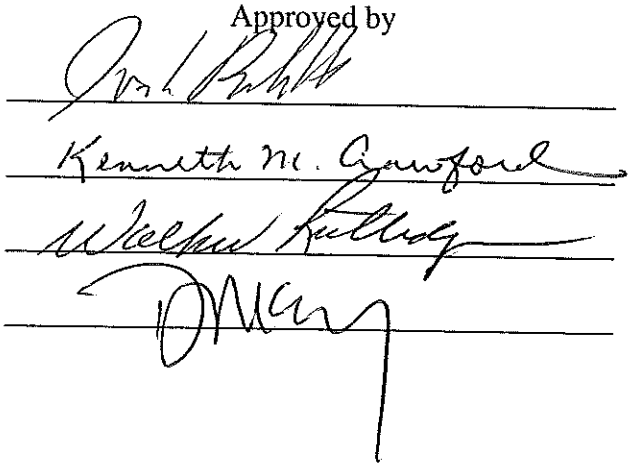

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Abstract

The effect of the glutamate antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) on Off-bipolar cells in the zebrafish (*Danio rerio*) was examined. CNQX has suppressed the *d*-wave component of electroretinogram (ERG) in other species. Here, adult zebrafish received an injection of CNQX or saline solution. They were then presented 200 ms stimuli at 17 wavelengths and several irradiances. Spectral sensitivity was calculated from the *b*-wave components of the ERG responses. Retinal injection of CNQX impacted the *a*-, *b*-, and *d*-waves of the ERG, especially at longer wavelengths. Significant differences in the spectral sensitivity functions of the CNQX and saline groups were found at 460 nm and 540 nm—the location of M – S and L – M color opponent mechanisms; thus, CNQX suppresses the color-opponent mechanisms. The fact that CNQX selectively affects sensitivity at longer wavelengths supports the presence of multiple Off-bipolar cell types. This study sought to further clarify the specificity of Off-bipolar cells and the general visual pathway of the adult zebrafish.

Introduction

The zebrafish (*Danio rerio*) has become an animal of great significance in research. The natural sciences, in particular, have benefited from using the zebrafish as a vertebrate model for genetics and developmental neuroscience. Because large numbers of offspring may be produced quickly and maintained inexpensively, these fish have become a popular with genetic researchers. Additionally, sexual maturity is attained rather quickly, usually by three months postfertilization. This allows extensive genetic screening over several generations in a relatively short time period (Driever, Stemple, Schier & Solinca-Krezel, 1994).

The process of identifying mutant phenotypes and mapping genes has become a priority for developmental and vertebrate geneticists. Neuhauss, Biehlmaier, Seeliger, Das, Kohler, Harris, and Baier (1999) have discovered 400 loci for vision disorders alone through optokinetic and optomotor response assays. Physical assays of genetic mutants as well as normal development also may be monitored unobtrusively via the zebrafish transparent chorion and embryos (Barinaga, 1990).

Vision science and neuroscience have taken advantage of the zebrafish as a means to understand vertebrate visual processing and development (Bilotta, Saszik, & Given, 1999). Anatomical and physiological studies of the visual system have examined the

relationship between cell type and structure. One means that can be used to assess retinal physiology is the electroretinogram (ERG). The ERG measures the gross electrical potentials of retinal neurons in response to visual stimuli. An ERG consists of several waves or components, each correlated with the response of specific types of retinal neurons.

Moving from the outside (i.e., back of the eye) to the inside of the retina, the first cells encountered are photoreceptors. Three types of zebrafish cones (short single, long single, and double) and four cone photopigments have been identified; the double cones possess two outer segments. Photoreceptors (rods and cones) are contained within the outer nuclear layer—the first of three nuclear layers (Dowling, 1987). Horizontal, amacrine, and bipolar cells populate the inner nuclear layer. The ganglion cell layer is the last of the nuclear layers. Signals travel through the direct pathway via the cones, then bipolar cells, and then ganglion cells. The remaining retinal cells types (amacrine and horizontal) provide lateral connections within the retina. Between the nuclear layers, outer and inner plexiform layers connect all of the described retinal cell types (Bilotta & Saszik, 2001). The inner plexiform layer (IPL) is of particular interest because its stratification patterns determine the differentiation between On- and Off- pathways (Connaughton & Nelson, 2000).

On-type cells, such as On-bipolar cells, respond to light with a depolarizing (excitatory) response, while Off-type cells, such as Off-bipolar cells, respond to light with a hyperpolarizing (inhibitory) response. It is important to note that not only do On-bipolar cells respond to light onset with a depolarizing response, but these cells also

respond to light termination with a hyperpolarizing response. Off-bipolar cells respond in an opposite fashion to light onset and termination.

One may eliminate the ERG responses of specific retinal neurons by using neurotransmitter antagonists and agonists. Presumed cellular contributions to the ERG have been isolated pharmacologically in zebrafish (Saszik, Alexander, Lawrence, & Bilotta, 2002) as well as primates (Sieving, Murayama, & Naarendorp, 1994), cats (Gargini, Cemontis, Cervetto, & Bisti, 1999), and other organisms.

A typical ERG response consists of three main components: the *a*-wave, the *b*-wave, and the *d*-wave (Dowling, 1987). A voltage-negative *a*-wave is thought to represent photoreceptor response and hyperpolarizes at stimulus onset. The depolarization of On-bipolar cells contributes to the voltage-positive *b*-wave. At stimulus termination, the voltage-positive *d*-wave occurs from the depolarization of Off-bipolar cells and possibly photoreceptors (Bush & Sieving, 1996).

In adult zebrafish, 13 types of bipolar cells have been identified, which are divided into On- and Off- subtypes, based on the sublamina of the inner plexiform layer (IPL) in which the axon terminates (Connaughton & Nelson, 2000). Two sublaminae (A and B) compose the IPL where synaptic reactions occur. A and B sublamina are defined as zones within the IPL, separated by a glycine-positive band that is free of axon terminals. Variations in the axon terminal sublamina allow for the differentiation of retinal neuron responses. Similar anatomical categories have been used to classify goldfish bipolar cells (Stell, Ishida, & Lightfoot, 1977) and On- and Off- ganglion and amacrine cells in carp (Famiglietti, Kaneko, & Tachibana, 1977). Both On- and Off-

bipolar cells respond to glutamate, with OFF-bipolar cells being depolarized and On-bipolar cells being hyperpolarized.

Photoreceptors release glutamate to activate bipolar cells. Bipolar cell receptor specificity, seen by differences in reactions to glutamate agonists and antagonists, also explains the changes in the membrane potential of bipolar cells. On-bipolar cells depolarize through ion channels controlled through a secondary messenger in the signal transduction pathway. Adult zebrafish On-bipolar cells possess at least one APB (DL-2-amino-4-phosphonobutyric acid) sensitive receptor and at least one glutamate-gated chloride current mechanism, which is insensitive to APB (Saszik et al., 2002). OFF-bipolar cells respond to glutamate via a CNQX-sensitive conductance mechanism (Connaughton & Nelson, 2000).

By pharmacologically "knocking out" cell types, one can assess the components of zebrafish retinal processing, then compare those results to data obtained without the use of neurotransmitter agonists or antagonists. Bipolar cells are worthy of such study. As mentioned above, On-bipolar cells depolarize, while Off-bipolar cells hyperpolarize at stimulus onset. At stimulus offset, On-bipolar cells hyperpolarize and Off-bipolar cells depolarize. Thus, the ERG response consists of the summed contribution of those and other cell types. Responses of one or both types of bipolar cells nullified chemically should lead to the elimination of the *b*- and *d*-waves for On- and Off-bipolar cells, respectively.

The glutamate antagonist CNQX has proven to be effective in eliminating retinal responses in other species. When injected into the eyes of primates, CNQX mitigated the contributions of hyperpolarizing Off-bipolar cells. For ERG data, this elevated the *b*-

wave and created a plateau between the *b*- and *d*-waves but eliminated the actual *d*-wave peak (Sieving et al., 1994).

The purpose of this study was to examine the retinal contributions of Off-bipolar cells to the zebrafish ERG. This was accomplished by pharmacologically isolating components of the zebrafish ERG—specifically, the extent of Off-bipolar cell function. Since zebrafish ERGs possess a *d*-wave, believed to be the result of contributions of the Off-bipolar cell responses and anatomical data acknowledge the presence of these cells, it is hypothesized that the addition of CNQX should block Off-bipolar cells and eliminate or reduce the *d*-wave of the ERG.

Methods

Participants

Adult zebrafish (*Danio rerio*) purchased at a local pet store were examined. Fish were sustained in 14h light/10h dark cyclic illumination. Water temperature was maintained at approximately 28.5°C. Fish were fed daily with frozen or live brine shrimp (San Francisco Bay Brand, Inc., Newark, CA).

Apparatus

Light stimuli were presented using a two-channel optical system. The test channel light source was a 150-W xenon arc lamp (Spectral Energy, Westwood, NJ, Model LH 150). This light was collimated and focused onto an optical shutter. To control stimulus wavelength, inference filters (Oriel, Stratford, CT, Model 54161 & Andover, Salen, NH, Model FS10-50; half-bandwidth of 10 nm) were placed in the light path. Neutral density filters were placed into the collimated light path (Reynard, San Clemente, CA, Model 398) to control stimulus irradiance. The light was focused onto the end of 5-mm-diameter liquid light guide (Oriel, Stratford, CT, Model 77556). The other end of the guide was positioned in front of the right eye of the subject.

A 250-W tungsten-halogen bulb (Oriel, Stratford, CT, Model 6334) with a 24 v/12a DC power supply (Condor, Oxnard, CA, Model F24-12-A+) produced a background light with an irradiance of 5 $\mu W/cm^2$. This suppresses rod contributions in adult zebrafish (Hughes, Saszik, Bilotta, DeMarco, & Patterson, 1998). The test channel was combined with the background light and filled the liquid light guide, directing diffuse white light illumination to the fish's eye.

Procedures

A solution of 0.04% tricaine methanesulfonate was used to anesthetize each subject. Once anesthetized, the subject was intramuscularly injected with 20 μg gallamine triethiodide. A 26-gauge needle was used to puncture the limbus of the right eye in preparation for CNQX injection and ERG electrode placement. The fish was positioned in a small Plexiglass container with a section of plastic tubing then placed inside a Faraday cage.

After placement in the Faraday cage, 0.1μl of a 250 μM solution of CNQX mixed with teleost saline solution (Sigma Chemical, St. Louis, MO) was injected into the right eye of the fish via a pneumatic microinjection pump (WPI, Sarasota, FL, Model PV800). An identical amount of teleost saline (minus CNQX) was injected into the right eyes of fish in the "normal" group. The molarity used allowed for a 22 μM concentration of CNQX in the vitreal chamber, if one assumes that the vitreal fluid is mixed with CNQX. The same final concentration was used by Sieving, et al (1994).

Recording and reference electrodes composed of 36-guage chlorided silver wires were placed in the subject's vitreal chamber and in the subject's left nostril, respectively.

Electrode signals were differentially amplified via an AC amplifier (A-M Systems, Inc., Carlsborg, WA, Model 3000) with a bandpass of 0.1 to 100 Hz. At the same time, the signal was displayed on a digital oscilloscope (Tektronix, Beaverton, OR, Model 340A) and recorded on a computer with a data acquisition board at a rate of 1000 Hz. The liquid light guide was placed in front of the subject's right eye, and the door of the Faraday cage was closed.

Fifteen minutes passed before trials began, allowing for adaptation to the broadband background and allowing the CNQX to take effect. Trials began at each wavelength with stimulus irradiance below threshold and increased by 0.5 or 0.4 log units until the response criterion had been exceeded—an ascending method-of-limits procedure. Three stimulus presentations, each 200 ms long with 800 ms between presentations, constituted each trial. To avoid chromatic adaptation and order effects, the 17 wavelengths between and including 320 and 640 nm were first presented in 40 nm steps. Wavelengths skipped in the first presentation were filled in so that the final data set included 20 nm increments between wavelengths.

Results

There were two major components of data analysis: ERG waveform analysis and analysis of spectral sensitivity functions. ERG waveforms from normal (saline injection only) and CNQX groups were examined and compared. Analysis of the effect of CNQX on spectral sensitivity functions (the *b*-wave and cone contributions) was then completed.

Waveform analysis

Three stimulus presentation waveforms were averaged for each stimulus wavelength and irradiance. Waveforms at 400-nm and 560-nm from subjects injected with only saline (Figs. 1a and 1c) and those injected with 0.1 μL CNQX (Figs. 1b and 1d) are presented in Figure 1. Time in milliseconds is noted on the abscissa and ERG response in microvolts on the ordinate. The 200 ms stimulus onset and termination is noted by a raised bar along each figure. As shown in figures 1a and 1c, normal ERG waveforms at all wavelengths are characterized by a voltage-positive *b*-wave shortly after stimulus onset. At stimulus termination (200 ms), the voltage-positive peak of the *d*-wave occurs. In normal zebrafish, it is unusual to obtain a significant voltage-negative *a*-wave before the *b*-wave at stimulus onset (Hughes, Saszik, Bilotta, DeMarco, & Patterson, 1998).

The injection of CNQX notably changed the ERG waveforms at both 400-nm and 560-nm (Fig. 1b and 1d). Unlike normal zebrafish, subjects injected with CNQX displayed a very large (in Figures 1b and 1d, approximately 40 μV) *a*-wave at 400-nm and 560-nm. In comparison to saline, both wavelengths from CNQX subjects also exhibited much larger *b*-wave peaks. CNQX appeared to have some impact on the *d*-wave as well. The *d*-waves did not completely disappear, however, as indicated by Fig. 1b and 1d. However, ERG responses to stimuli of longer wavelengths (Fig. 1d) showed smaller *d*-wave amplitudes than responses to short wavelength stimuli (Fig. 1b). There was a considerable difference between normal and CNQX ERG *b*-wave to *d*-wave amplitude ratios. In other words, the difference between the microvolt responses of the CNQX group's *b*- and *d*-waves is much greater than the difference between the *b*- and *d*-waves of the control group.

Spectral sensitivity analysis

Spectral sensitivity functions were obtained from the *b*-wave responses. To calculate spectral sensitivity, *b*-wave peak amplitudes for each stimulus wavelength were plotted as a function of stimulus irradiance. Sensitivity was defined as the log stimulus irradiance at each wavelength that produced a criterion response of 20 μV. The actual spectral sensitivity function was graphed by noting the reciprocal log stimulus irradiance (quanta/s/cm^2) at the criterion response for each wavelength.

Figure 2 illustrates the relationship between the criterion log stimulus irradiance (ordinate) and wavelength (abscissa) for normal and CNQX groups. Circles represent the average normal group sensitivity at each wavelength, and squares symbolize the average

sensitivity of the CNQX group. Ten subjects were used for the normal group spectral sensitivity, with eleven subjects comprising the CNQX group. Error bars stemming from these points represent ± 1 S.E.M.

As expected, the normal data possessed characteristic sensitivity peaks near stimulus wavelengths of 362, 415, 480, and 570 nm; these represent the peak sensitivities of the U, S, M, and L cones, respectively (Robinson, Schmitt, Harosi, Reece, & Dowling, 1993). In addition, drops in sensitivity at 460 and 560 nm were found in the normal spectral sensitivity function; these were due to M-S and L-M opponent mechanisms (Saszik et al., 2002). The CNQX group demonstrated greater sensitivity than the normal group, particularly at longer wavelengths—especially at 460 nm and 540 nm.

In order to determine whether there were any significant differences between the groups, a 2 (condition: saline or CNQX injection) by 17 (wavelength) analysis of variance (ANOVA) was performed. The results showed a significant condition by wavelength interaction, $F(16, 304) = 5.307$, $p < 0.005$. Independent t-tests were conducted at each wavelength to determine the specific wavelengths at which there was a significant difference between the normal and CNQX groups. Because multiple t-values were calculated, the value of α had to be readjusted to account for experiment-wise inflation. (The α value is inflated when numerous t-tests are conducted because the possibility that results are due to chance also rises with the number of tests.) A Bonferroni correction for 17 tests was applied, readjusting α to 0.05.

After the Bonferroni correction, only three wavelengths showed a significant difference between the two conditions. Notable wavelengths included 460, 540, and 640

nm. Statistical significance is confirmed visually in Figure 2, as the normal and CNQX points for these wavelengths are particularly distanced from each other.

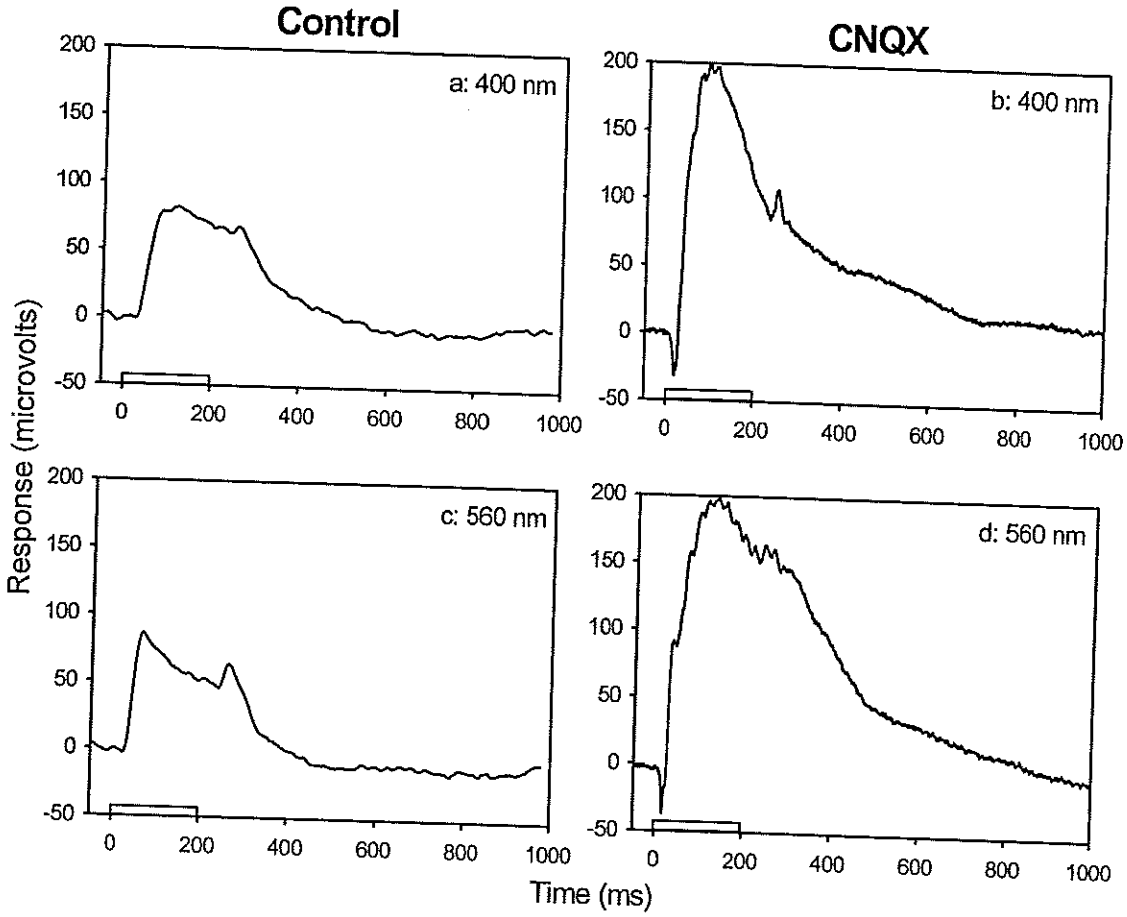

Figure 1: The effect of CNQX (Figs. 1b & 1d) compared to saline/control (Figs. 1a & 1c) on zebrafish ERG waveforms at 400 and 560 nm. Stimuli began at 0 ms and terminated at 200 ms with a background of 5-$\mu W/cm^2$ broadband light. Each waveform represents the average of three stimulus presentations. In all cases, a voltage-positive *b*-wave was present at stimulus onset. Subjects injected with 0.1 µl CNQX possessed a larger voltage-negative *a*-wave prior to the *b*-wave and smaller voltage-positive *d*-wave at stimulus termination (Figs. 1b & 1d) than subjects injected with the same amount of teleost saline (Figs. 1a & 1c) at both wavelengths.

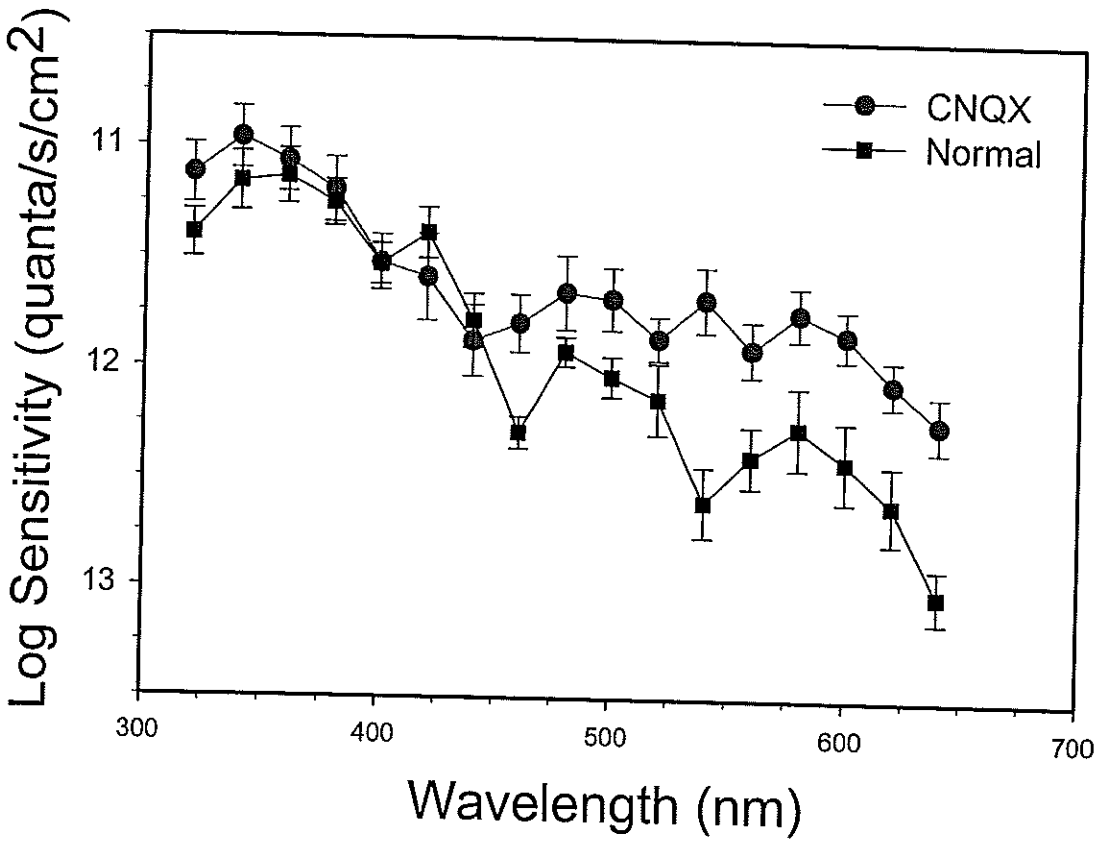

Figure 2: Spectral sensitivity functions for the *b*-waves of zebrafish injected with CNQX (circles, $n = 11$) and zebrafish injected with teleost saline (squares, $n = 10$). Sensitivity represents the reciprocal log stimulus irradiance (quanta/s/cm^2) producing a 20 μV criterion response at each wavelength. A ± 1 S.E.M. is displayed by the error bars. Statistically significant differences between the CNQX and control groups may be observed at 460 nm and 540 nm. These wavelengths normally represent M – S and L – M color-opponent mechanisms in zebrafish.

Discussion

Off-bipolar cell contributions to the zebrafish ERG and spectral sensitivity were examined by comparing those components in fish injected with saline and fish injected with CNQX. The addition of CNQX to the zebrafish retina produced changes in the ERG waveforms and spectral sensitivity of the zebrafish. As initially hypothesized, CNQX did reduce the ERG *d*-wave when injected into retinas of zebrafish. In addition, the amplitudes of the *b*-waves for the group injected with CNQX were substantially larger than those of the saline/normal group. Under normal circumstances, Off-bipolar cells hyperpolarize and On-bipolar cells depolarize in response to light-stimulus onset. On-bipolar cell depolarization is initiated by glutamate from the photoreceptors, which leads to an influx of potassium into the cell, producing a decrease in conductance and the *b*-wave potential (Sieving et al., 1994). Glutamate produces an increase in conductance for Off-bipolar cells, resulting in their hyperpolarization (Connaughton & Nelson, 2000). CNQX compromises the glutamate receptors on Off-bipolar cells, so the unusually large *b*-waves of the CNQX group are likely a result of less Off-bipolar cell hyperpolarization. Normal Off-bipolar cell hyperpolarization may serve to inhibit On-bipolar cell depolarization and shape the *b*-wave. Sieving et al. (1994) suggest a similar "push-pull"

model for primate hyperpolarizing and depolarizing neurons. This mechanism may serve to clamp retinal responses and prevent them from overresponding.

The voltage-negative *a*-waves of the CNQX group were also comparably larger than the normal counterparts. For the CNQX waveforms, the initial rapid drop of this component is not fully understood. Off-bipolar cell hyperpolarization has a "pulling"/inhibitory effect on On-bipolar cell depolarization, resulting in a smaller, more controlled *b*-wave (Sieving et al., 1994). The rapid climb of *b*-waves for fish injected with CNQX could be part of the uninhibited On-bipolar cell response, though this is uncertain. Further studies of the *a*-wave with aspartate (which eliminates all retinal responses except photoreceptors) are required to obtain a better understanding of this ERG component.

Also changing in size was the *d*-wave. As expected, zebrafish injected with CNQX produced ERG waveforms with a reduced *d*-wave contribution. This supports the notion that cells with CNQX-sensitive receptors—the Off-bipolar cells—are the primary producers of the voltage-positive d-wave. This differs from the contributions of cat Off-bipolar cells, which are thought to be less significant (Gargini et al., 1999). Still, remnants of a voltage-positive wave remained at stimulus termination. It is also important to note that the *b*-wave to *d*-wave amplitude ratio for the CNQX group was approximately half the ratio of the normal group. Incomplete suppression of Off-bipolar cells with CNQX has been noted before. The *d*-waves of primate ERGs were not totally eliminated by CNQX, either (Sieving et al., 1994). Though no drug is a panacea, the most likely explanation for the remnant *d*-wave involves multiple receptor types on Off-bipolar cells. DeVries (2000) found two types of kainite receptors and one AMPA

receptor on the Off-bipolar cells of ground squirrels. Perhaps CNQX is an effective glutamate antagonist for only one type of receptor, leaving the others free to respond to light termination. Variability in zebrafish bipolar cell receptor sensitivity has been demonstrated with On-bipolar cells and the glutamate agonist APB (Saszik et al., 2002) and with Off-bipolar cells (Connaughton & Nelson, 2000).

CNQX impacted the spectral sensitivity of the zebrafish ERG response as well. At most wavelengths (especially the longer wavelengths) an increase in sensitivity was displayed for the CNQX condition. A lack of Off-bipolar cell inhibitory hyperpolarization on On-bipolar cell response at stimulus onset likely allows for a larger depolarization, and thus, higher sensitivity. Similarities in sensitivities between the groups at shorter wavelengths suggest that the Off-bipolar cell receptor type is not sensitive to CNQX at these wavelengths. Cells impacted by longer wavelengths, however, seem to be dramatically affected. Similar wavelength-dependent differences have been noted in zebrafish injected with APB, which only significantly reduces the *b*-wave at UV and short wavelengths (Saszik et al., 2002)

Significant differences between the sensitivity of the two groups at 460 and 540 nm indicates a CNQX-induced change in the color-opponent mechanisms of M-S and L-M cones, respectively. Hughes et al. (1998) demonstrated that normal zebrafish spectral sensitivity data produces "notches" or drops in sensitivity at 460 and 540 nm. Notches are indicative of chromatic inhibition—or one cone type inhibiting the contribution of another. When exposed to CNQX, these notches appear to disappear since this is where significant changes in sensitivity take place. CNQX-treated zebrafish are significantly more sensitive at these wavelengths and, therefore, have less inhibition and opponency.

The drug has an effect on the nonopponent M, and L cone type contributions as well as the opponent S and M cone contributors. Those cone contributions impacted by CNQX are likely connected to the Off-bipolar cells with CNQX-sensitive receptors, leaving the other cone types without inhibition. A similar model was proposed for APB (Saszik et al., 2002). Since both M-S and L-M notches are changed with the addition of CNQX, it is likely that either a group of S- and L- cones or only M- cones are attached to the Off- cells with sensitive receptors, while the other group is allowed to respond.

The use of CNQX appears to generate less inhibition in the *b*-wave of the zebrafish ERG. So, like primates (Sieving et al., 1994), the zebrafish has at least one "push-pull" mechanism for hyperpolarizing and depolarizing neurons. Additional investigation determining possible On-bipolar cell hyperpolarizing inhibition of the *d*-wave and Off- cell depolarization at stimulus termination could provide insight into the conditions in which the mechanism is used.

Elucidating the actual group of cones impacted by the loss of most Off-bipolar cell function would also be helpful. Because the wavelengths at which the U- and S-cones contribute to spectral sensitivity were not significantly different between the normal and CNQX conditions, it is likely that the divergence in color opponency at 460 nm and 540 nm (Fig. 2) was due to reduced inhibition from the M- cones. M- cones may be connected to the Off-bipolar cells with receptors sensitive to CNQX, while U-, S-, and L- cones connected to the cells are unaffected by the drug. Further physiological and ERG investigations, in addition to spectral sensitivity analysis of the *a*- and *b*-waves, should be conducted to address this question. Future studies must also examine the impact of dose on cone contributions to spectral sensitivity and magnitude of the ERG *b*-

wave. Sieving et al. (1994) demonstrated an increased reduction of the *d*-wave as CNQX dose increased, but no other analysis was conducted.

The results found here support the existence of multiple Off-bipolar cells, varying by the receptor (DeVries, 2000) and its sensitivity to CNQX. Connaughton and Nelson (2000) also confirm the presence of two populations of Off-bipolar cells in the zebrafish retina—one responding to either kainate or glutamate and one only sensitive to kainate. Another similar study is necessary to characterize these groups by their sensitivity to CNQX.

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