Analysis of Zebrafish Optic Tectum Visual Processing Before and After Optic Nerve Crush

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ANALYSIS OF ZEBRAFISH OPTIC TECTUM VISUAL
PROCESSING BEFORE AND AFTER OPTIC NERVE CRUSH

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of the Department of Psychology
Western Kentucky University
Bowling Green, Kentucky

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Master of Arts

By
Angela Lynn McDowell
August 2002
ANALYSIS OF ZEBRAFISH OPTIC TECTUM VISUAL PROCESSING BEFORE AND AFTER OPTIC NERVE CRUSH

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Director of Thesis

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# Table of Contents

<table>
<thead>
<tr>
<th>Acknowledgments</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure Titles</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract</td>
<td>vii</td>
</tr>
</tbody>
</table>

Chapter 1: Introduction and Purpose ....................................................1

Models of Neural Regeneration .........................................................2

The Visual System and Neural Regeneration .......................................3

Zebrafish as an Animal Model ..........................................................5

The Zebrafish Visual System Anatomy ..............................................7

Zebrafish Visual System Physiology ...............................................10

Zebrafish Neural Regeneration .........................................................12

Summary, Purpose, & Hypotheses .........................................................15

Chapter 2: Method .............................................................................16

Participants ......................................................................................16

Apparatus ..........................................................................................16

Procedures .........................................................................................18

Chapter 3: Results .............................................................................22

Overview of Analysis .........................................................................22

Comparing the ERG and TER Responses ...........................................25

Comparing Tectal Responses Before and After Optic Nerve Crush .......27

Chapter 4: Discussion .......................................................................31
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adult Zebrafish Tectal Response</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>Normal TER ON-Response Spectral Sensitivity</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>Normal ON-Response Spectral Sensitivities</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>Normal TER OFF-Response Spectral Sensitivity</td>
<td>51</td>
</tr>
<tr>
<td>5</td>
<td>Normal OFF-Response Spectral Sensitivities</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>Normal TER Spectral Sensitivities</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>Comparing ONC Groups</td>
<td>54</td>
</tr>
<tr>
<td>8</td>
<td>90 dpc ON-response Spectral Sensitivity</td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>TER ON-Response Spectral Sensitivities</td>
<td>56</td>
</tr>
<tr>
<td>10</td>
<td>90 dpc OFF-Response Spectral Sensitivity</td>
<td>57</td>
</tr>
<tr>
<td>11</td>
<td>TER OFF-response Spectral Sensitivities</td>
<td>58</td>
</tr>
</tbody>
</table>
The visual system processes information at various levels. Initial processing takes place in the retina, which then sends information to the optic tectum, the first visual brain center in lower vertebrates, for further processing. There were two main goals of this study. The first goal was to obtain tectal evoked responses (TER) from adult zebrafish and to compare them to previous electroretinogram (ERG) spectral sensitivity data (Bilotta & Harrison, 1999). The second purpose of this study was to examine neural regeneration in the adult zebrafish at various times post-crush and to compare visual processing of these subjects to the normal subjects. The optic nerve tracts of the zebrafish’s right eye were damaged via optic nerve crush and subjects were tested at one of 5 times post-crush: 3, 14, 28, 42, or 90 days post-crush (dpc). Complete TER spectral sensitivity functions were obtained (n=10) and compared to ERG data. The TER ON-response was consistently about one log unit less sensitive than the ERG b-wave (ON-response) across the entire spectrum (320-640 nm). The results show that the cone contributions to TER and ERG responses were different, particularly
at the short and middle wavelengths. TER OFF-response sensitivity and the ERG
d-wave sensitivity were both sensitive to ultraviolet and short wavelengths, but
the TER OFF-response sensitivity dropped considerably to the middle and long
wavelengths. Thus, it appears that the retina and the tectum process visual
information differently. In addition to comparing ERG and TER responses, TER
spectral sensitivity functions were obtained for 90 dpc subjects (n=7) and
compared to the control data. No significant differences were found between the
TER ON-response of the 90 dpc and the control subjects. In fact, their respective
spectral sensitivities appear to have the same cone contributions. The only
difference was that there was more variability in the 90 dpc subjects than in
control subjects. However, the OFF-responses of the control subjects were
considerably more sensitive to the ultraviolet wavelengths than the OFF-responses
of the 90 dpc subjects. The results show that there was a general trend in optic
nerve regeneration over time. This study has provided valuable information about
the differences and similarities in visual processing of different levels of the visual
system. In addition, this study has demonstrated the successful repair of the
functional properties of CNS neurons in this species. All of this information
further enhances the usefulness of the zebrafish as a model for vision science and
neuroscience.
Chapter 1

Introduction and Purpose

In recent decades, neural regeneration has become an important area of research in neuroscience primarily because there is an urgency to understand why higher vertebrates' central nervous system (CNS) tissue does not regenerate, even though lower vertebrates' CNS tissue does regenerate. The underlying reason for this urgency is that if neuroscientists can understand how CNS tissue of lower vertebrates regenerates, then that knowledge could be applied to repairing damaged CNS tissue in higher vertebrates, including humans. One particular goal of this study is to advance our knowledge about whether an injury to a specific CNS tissue in a lower vertebrate will indeed repair itself and to what extent.

Although the visual system will be used as the CNS model in this study, the knowledge gained will likely advance our understanding of the entire CNS system (the brain and spinal cord), because there is a certain degree of similarity between all CNS neurons. Another specific goal of this study is to compare processing at a higher visual processing center, the zebrafish optic tectum, with processing at an earlier level of the visual system (the zebrafish retina). This comparison is an important one to make because it will provide a more complete picture of how the visual system processes the visual world. For example, this study should elucidate which photoreceptors are sending information to the optic tectum, which can be compared to the photoreceptors that are processing information in the retina.
Models of Neural Regeneration

Sensory systems, especially the visual system, are good models for the advancement of the study of neural regeneration for several reasons. One reason is that the visual system allows researchers to have precise control over the stimulus that initiates the visual process. Also, structures of the visual system, such as the retina, optic nerve, and even brain structures in some animal models, are more accessible because they are located more superficially than the spinal cord and many other brain areas. In addition, even though the visual system is simpler in terms of its cell numbers and information processing, the functional properties of its cells are much the same as the more complex areas of the CNS. This similarity is important because the results of studies that use the visual system as a model can be applicable to understanding the more complex systems as well.

The optic nerve is an excellent structure within the visual system model to investigate neural regeneration because it possesses all the advantages listed above, such as accessibility and simplicity. Relatively speaking, the optic nerve is considerably more accessible to the researcher than many other areas of the CNS. This accessibility is crucial because it is important to minimize the use of invasive techniques in order to reduce damage, such as excessive bleeding, during the procedures. The simplicity of the optic nerve arrangement also makes this model very attractive. More specifically, the optic nerve typically processes information unidirectionally from the retina to the brain (see Bilotta & Saszik, 2001). This processing style is especially important when making inferences about cell function via physiological recording. If information were processed
multidirectionally, or in a synaptic web-like entanglement, then understanding and analyzing electrophysiological data would be more complicated.

*The Visual System and Neural Regeneration*

The study of neural regeneration by means of the visual system is quickly becoming rich in history. Roger Sperry (1963) was one of the first researchers to demonstrate neural regeneration through his work with newts. Sperry showed that severed optic nerve tracts in newts would regenerate, and that they would reattach to the same neural connections in the optic tectum, the primary visual processing center in the lower vertebrate brain, that they had prior to injury. Since those initial studies, amphibians and other lower vertebrates have remained popular animal models in this area due to their abilities to regenerate CNS tissue.

*Lower vertebrates.* Neural regeneration of the visual system of lower vertebrates has been rigorously investigated during the last two decades, particularly with the teleost fish, the goldfish. Northmore (1989a, b) quantitatively examined regenerating retinotectal pathways using electrophysiological techniques. In his studies, goldfish optic nerves were crushed, and at varying times post-crush, multiunit physiological recordings of tectal evoked responses (TER) were obtained from the optic tectum. Although weak responses to a high contrast stimulus were seen in some subjects prior to 20 days post-crush (dpc), Northmore (1989a) reported that the goldfish tectum did not typically respond to a vertical rotating black stripe until 20 dpc, nor to flashing light emitting diodes (Northmore, 1989b) until around 40 dpc. By 40 dpc, there was a reappearance of ON- and OFF-responses to the light stimulus, as well as a decline in synaptic
connectivity width (which is indicative of reorganization) (Northmore, 1989a). Furthermore, Northmore (1989b) reported that both the tectal response and the synaptic width of the retinal axons did eventually regain the previous levels of sensitivity and organization that existed prior to optic nerve crush, by about 80-100 dpc. Northmore also reported that there are differences in recovery times between receptive field types and visual stimuli types. The OFF-receptive fields recovered quicker (first seen around 20 dpc) than ON-receptive fields and dual ON/OFF-receptive fields (first seen around 40 dpc) (Northmore, 1989b).

**Higher vertebrates.** The mouse and rat have long played important roles in the study of visual processing, and regeneration is no exception. Recently, Chierzi, Strettoi, Cenni, and Maffei (1999) discovered that mice whose optic nerve neurons over-expressed the protein Bcl-2 had a better chance of survival after sustaining an optic nerve crush than normal wild-type mice did. This study is an important one because mice and other higher vertebrates do not naturally regenerate CNS neurons. However, Chierzi et al. showed that improvement in regeneration capabilities under favorable conditions for cellular regrowth was possible. This study shows that CNS tissue of higher vertebrates can regenerate, thereby illustrating the importance of understanding the natural regenerative process in lower vertebrates. If the natural process of regeneration is fully understood, then therapies to facilitate regeneration can be developed.

**Limitations of present models.** Although there are many advantages to the above models, they have some limitations as well. Higher vertebrates do not naturally regenerate CNS neurons; thus they cannot be used as models for normal
or natural neural regeneration. There are also some issues with the goldfish model. These include the ability to generalize the results to humans. For example, DeMarco and Powers (1991) provided evidence that goldfish ERG responses do not exhibit color opponency which is typically indicative of color vision, even though behaviorally goldfish exhibit that they can discriminate between wavelengths (Neumeyer, 1985). This contradiction between physiological and behavioral data is cause for concern when generalizing the results, because this discrepancy does not exist between the two levels of analysis in higher vertebrates (De Valois & Morgan, 1974). Due to the limitations of these animal models, a more appropriate model is desirable.

Zebrafish as an Animal Model

Just as the visual system is an excellent system to study neural regeneration, it is equally important to have a good animal model. The zebrafish (Danio rerio) is potentially an excellent model for studying neural regeneration. Given the inconsistencies and limitations for research using the goldfish as a model for color vision, the zebrafish has become an increasingly popular animal model to study both neural development and regeneration during the last decade (see Bilotta & Saszik, 2001). There are many reasons for their popularity. There is evidence suggesting that zebrafish have the necessary requirements for color vision; in fact, their preliminary visual processing center, the retina, possesses all of the anatomical features and many of the same functional features of higher vertebrates including mammals (Hughes, Saszik, Bilotta, DeMarco, & Patterson, 1998). The extensive genetic analyses that have been done with this animal model
are another advantage with the zebrafish as a model for neural regeneration. Because zebrafish breed so prolifically, require relatively low maintenance, and are very economical to maintain, geneticists have been able to map out the zebrafish’s genetic makeup (Postlethwait, Johnson, Midson, Talbot, Gates, Ballinger, & et al., 1994). This knowledge allows geneticists and other researchers to manipulate the zebrafish’s genetic information to produce mutations in their visual system (see Bilotta & Saszik, 2001). These mutations are typically characterized by a specific cell-type loss or function loss. For example, the recessive mutation known as no optokinetic response c (nrc) produces blindness in zebrafish and has been tested using behavioral and physiological measures (Allwardt, Lall, Brockerhoff, & Dowling, 2001). The retinotectal mutation known as macho has been used to examine the role of neural activity within the developmental process (Gnuegge, Schmid, & Neuhauss, 2001). The retinal ganglion cells of the mutant macho do not generate action potentials, and as a result of this lack of neural activity, the retinotectal pathway does not develop normally. The macho mutation results in a disorganized and unrefined retinotopical map. These mutations allow scientists to examine zebrafish behavior in the absence of specific neural elements in order to draw conclusions about the functions of those elements. Another advantage to using the zebrafish is that they are highly valuable for studying embryonic development. Because zebrafish reach maturity within three months, the entire developmental process is considerably shorter than in most vertebrates. Finally, the zebrafish also brings with it additional and unique anatomical visual processes because it possesses an
The ultraviolet cone photoreceptor as well as the other three cone types typically possessed by many vertebrates including humans (Branchek & Bremiller, 1984). The ultraviolet photoreceptor can be studied to answer questions concerning species differences as well as to test current theories of color vision.

The Zebrafish Visual System Anatomy

Retinal anatomy. As in most vertebrates, the zebrafish retina has three distinct nuclear layers consisting of five types of cells and two plexiform layers, where synaptic connections between the five cell types exist (Dowling, 1987). The outer nuclear layer consists of rod and cone cell bodies, the inner nuclear layer contains three retinal cell types: horizontal, bipolar, amacrine cells, and the ganglion layer contains the ganglion cell bodies. The outer plexiform layer consists of the synaptic connections between the rod and cone photoreceptors, bipolar cells, and horizontal cells, and the inner plexiform layer consists of the connections among bipolar, amacrine and ganglion cells.

The zebrafish visual system is comprised of two main processing centers; one is the retina, where preliminary processing takes place and the other is the optic tectum, the brain area that receives information from the retina and then further processes it. The retina consists of several cell types, which have been studied developmentally. Branchek and Bremiller (1984) provided initial research on zebrafish retinal development by studying the photoreceptors. They found five types of photoreceptors: rods, long single cones, short single cones, double cones with principal outer segments and accessory outer segments. Each anatomically different photoreceptor possesses a unique photopigment with different peak
sensitivities. For rods, the peak sensitivity of the photopigment is 500 nm (Saszik & Bilotta, 1999); the peak sensitivities of each of the cone photoreceptors are 362 (short-single or U-cones), 415 (long-single or S-cones), 480 (accessory double cone or M-cones), and 570 nm (principal double cone or L-cones) (Robinson, Schmitt, Harosi, Reece, & Dowling, 1993). At two days post fertilization (dpf), photoreceptor cell nuclei (neuroblasts) and their inner segments (ellipsoids) were visible with an electron microscope, but the outer segments were not yet visible. However, by 2.5 dpf the outer segments of the nuclei could also be seen with an electron microscope. It was shown that these initial cells eventually developed into cones, but rod cell nuclei were not seen until eight dpf. The entire photoreceptor mosaic could be identified by 12 dpf. Weak electrophysiological responses were seen by a few days postfertilization, but adult-like responses were first recorded around 14 dpf (Branchek & Bremiller, 1984).

Retinotectal pathway. The anatomy of the retina is initially somewhat confusing, because it appears to be wired “backwards.” The most anterior portion of the retina contains the ganglion cells, the neurons that first encounter light. However, even though the ganglion cell layer is the first layer within the retina to encounter light, it is the last cell layer to process light. When light enters the retina, it first passes through the ganglion cells and then around each connecting cell until it reaches the back of the eye, where the photoreceptors reside. The photoreceptors absorb and process the light. Their electrical signal is then processed by each connecting cell and finally is processed by the ganglion cells. The signal travels down the axons of the ganglion cells, which actually form what
is known as the optic nerve. These fibers are organized in a specific manner as regard each axon’s location within the bundle and the manner in which each axon forms a synaptic connection onto the optic tectum (Burrill & Easter, 1995; Kaethner & Stuermer, 1992). Research has shown that retinotectal synaptic connections are spatially opposite. For example, the dorsal retinal axons synapse onto the ventral tectum and the ventral retinal axons synapse onto the dorsal tectum (see Bilotta & Saszik, 2001). Zebrafish, as well as most other lower vertebrates, have a complete crossover of their optic nerve tracts. This crossover means that all ganglion cell axons exiting from one eye synapse onto the contralateral (opposite side) optic tectum; whereas in humans there is no complete crossover. A portion (approximately half) of the axons from each eye synapse onto the contralateral and the ipsilateral (same side) optic tecta (Sekuler & Blake, 2001). This provides another advantage of studying lower vertebrates’ visual system, since each eye sends information to only one tectal hemisphere. Because all of the retinal information exiting the eye will be received by one tectum the researcher will have full access to all retinal information received by that tectum.

**Optic tectum.** In lower vertebrates, the optic tectum is the brain center that receives input from the optic nerve fibers and further processes that information. In higher vertebrates, the optic tectum, or the superior colliculus as it is called in higher vertebrates, is just one of several secondary visual processing centers. However, in most lower vertebrates, including the zebrafish, the optic tectum is the primary brain structure for visual processing (Rupp, Wullimann, & Reichert, 1996). The response properties of adult zebrafish tectal cells do not seem to be
nearly as simple to examine as the response properties of retinal cells. Research thus far has classified individual tectal cells according to their response characteristics to different stimuli (Sajovic & Levinthal, 1982a, b). Sajovic and Levinthal presented 16 types of stimuli, varying in color, mobility, shape, contrasts, and size, and found four distinct types of tectal receptive fields. For example, the type that was reported to be the most common is type I. Sajovic and Levinthal (1982a) defined type I to be spontaneously inactive in the dark, biphasic (at onset and offset), and to display positive spikes/bursts.

*Zebrafish Visual System Physiology*

Although it is essential to study anatomical processes when attempting to understand a particular behavior, it is equally important to study physiological processes. Examining the physiology of an animal is more likely to provide insight about behavioral function than examining just anatomy. For example, even though goldfish possess an ultraviolet sensitive cone, there is very little physiological evidence for U-cones contributing to the electroretinogram (ERG) and tectal evoked responses (Cassidy & Bilotta, 2000). On the other hand, behavioral studies of goldfish have shown that U-cones contribute to the goldfish behavioral sensitivity (Hawryshyn, 1991; Hawryshyn & Beauchamp, 1985; Neumeyer, 1985).

*Retinal physiology.* The ERG is a measure of the gross electrical potential of retinal neurons to the onset and termination of a light stimulus. The ERG displays several distinct response characteristics, known as the a-wave, b-wave, and d-wave. The a-wave is defined as the initial voltage-negative response.
occurring immediately (first 16 ms) after the onset of the light stimulus. The a-wave is believed to represent the response summation of the photoreceptors, because photoreceptor responses hyperpolarize (become more negative) to light. The b-wave is defined as the large initial voltage-positive response following the a-wave and occurs at the onset of the light stimulus as well. Furthermore, the b-wave is typically defined as the response summation of ON-bipolar cells that depolarize in response to the onset of the light stimulus. The d-wave is defined as the large voltage-positive response immediately following the termination of the light stimulus. The d-wave reflects the response of OFF-bipolar cells that depolarize (become more positive) in response to the termination of the light. The ERG has been used to examine visual sensitivity to the entire spectrum of visible light for many animal models including the zebrafish.

Hughes et al. (1998) studied adult zebrafish cone contributions to the photopic ERG spectral sensitivity function. They reported that zebrafish appear to possess color opponent mechanisms (L-M and M-S) that have been accepted as a basis for potentially having color vision. In addition, they reported that the ERG b-wave component appears to receive input from all four cone types, including the two opponent contributions mentioned above and two nonopponent contributions from the U-cones and S-cones. In addition, the spectral sensitivity of the zebrafish ERG was examined developmentally, and the results showed that age positively correlated with a general increase in sensitivity in the ERG b-wave response (Saszik, Bilotta, & Givin, 1999). They also reported sensitivity differences between the cone types, with the U-cones being the most sensitive, followed by a
decrease in sensitivity across each cone type (S-, M-, and L-cones, respectively). Interestingly, Sazzik, et al. (1999) found that the ERG contribution from the U-cones in developing zebrafish was similar in sensitivity to the adult zebrafish U-cone contribution. Thus, the U-cone contribution appears to be adult-like very early in development while the other cone contributions continue to develop with age.

*Optic tectum.* Due to the complexity of recording from the tectum with a relatively small animal, only one set of studies has been done that examined the physiology of the adult zebrafish optic tectum. Sajovic and Levinthal (1982a, b) successfully recorded from single units of the adult zebrafish optic tectum. This study is very important because it provides evidence that recording from the adult zebrafish tectum can be done successfully. However, their research was limited to examining and quantifying visual properties of receptive fields within the tectum, and did not evaluate spectral sensitivity or the cone contributions to the tectal cells.

*Zebrafish Neural Regeneration*

Very little research on neural regeneration has been done in the zebrafish. Initially research suggested that the zebrafish might not be able to regenerate damaged CNS neurons, especially optic tectum neurons (Schmatolla & Erdman, 1973; Rahmann, 1968; cited in Marcus, Delaney, & Easter, 1998). However, recently Marcus et al. (1998) provided indirect evidence suggesting that the zebrafish optic tectum may be capable of regenerating. After injecting the thymidine analog, BrdU, and a BrdU tracing element into developing zebrafish,
neurons were specifically labeled with the BrdU agent. They hypothesized that if neurogenesis, or the creation of new cells, occurred then there should be more labeled cells within the optic tectum at the later intervals, indicating that the labeled cells were mitotic. After mounting and counterstaining the anatomical sections onto slides, the neurons were examined. Marcus et al. were then able to count the number of labeled cells at various times postfertilization. They did indeed find a significant increase in the number of BrdU labeled cells at the later time intervals and demonstrated an exponential increase in the number of labeled cells as the amount of time postfertilization increased. Because cell division is indicative of regeneration capabilities, they concluded that there is evidence for neurogenesis within the zebrafish. Therefore it is likely that zebrafish, like the goldfish, can regenerate neural cells.

Unfortunately, their study provided only indirect anatomical evidence; they did not provide direct anatomical, physiological, or behavioral evidence of neural regeneration. Because the optic nerve was not damaged, there is no direct evidence supporting the zebrafish’s ability to regenerate CNS neurons and one cannot assume the functional ability of any structure to be "normal" until it is empirically tested. Interestingly, Maeyama and Nakayasu (2000) reported finding evidence for differences in neural development in the zebrafish brain. Similar to Marcus’ study, Maeyama and Nakayasu found evidence that the zebrafish optic tectum continued to undergo cell division into adulthood. However, they reported that replicating cells became increasingly restricted to certain areas near the margins of the optic tectum as the animal continued to develop, and they found no
evidence of continued neurogenesis in zebrafish brain structures other than the optic tectum and cerebellum. Maeyama and Nakayasu suggested that there was a dual system for neural regeneration, with some systems capable of further development and others not capable of regeneration.

Vihtelic and Hyde (2000) provided evidence that the adult albino zebrafish retina does regenerate after light induced retinal apoptosis; retinal cells were exposed to light levels so intense that the cells were destroyed. They found that even though the retinal cells did regenerate, the fish did not regain the well organized cone distribution across the retina (i.e., the cone mosaic) that they had prior to the retinal damage. Cameron and Carney (2000) provided similar evidence, in that retinal regeneration occurs following surgical destruction of the retina, but the establishment of the well organized cone mosaic did not reach previous levels. These two studies are important because they provide evidence of the potential for regeneration capabilities in the zebrafish retina. However, there are limitations with these two studies. Primarily, neither study addresses the issue of the functional capability of the zebrafish retina after regeneration. Again, one cannot assume that possessing the anatomical structures would be enough to imply normal function. Visual function can only be tested by physiological or behavioral means to gain a complete and accurate picture of the regeneration process. Secondly, there has not been any research examining regeneration at the higher level of visual processing to date. It is equally important to study the process of zebrafish optic nerve regeneration to gain insights into understanding and facilitating regeneration at this higher level of visual processing.
Summary, Purpose, & Hypotheses

The proposed study will use electrophysiological techniques to further examine the visual processing of the optic tectum, with two specific goals in mind. The first goal will be to compare the spectral sensitivity based on the massed electrical responses of the optic tectum (tectal evoked responses or TER) to previously obtained ERG spectral sensitivity functions (Bilotta & Harrison, 1999). The spectral sensitivities and cone contributions between the two levels of the visual system will be examined and compared. The second goal of this study is to functionally test neural regeneration by means of an optic nerve crush. This testing will be accomplished by comparing the spectral sensitivity functions of the TER before and after optic nerve damage. Based on research showing that the process of regeneration is an unorganized event, it is hypothesized that the optic nerve will indeed regenerate, but not to full visual function.
Chapter 2

Method

Participants

The participants were adult male and female zebrafish (*Danio rerio*) approximately 3 to 4 cm in length. They were bred in-house or bought from a local pet store and then kept in several tanks with a water temperature of about 28.5° C. The participants were kept on a 14 hr light on/10 hr light off cycle, and the animals were fed Tetramin basic flakes tropical fish food daily at a regularly scheduled time. They were housed in the lab for at least two weeks prior to use. All housing and experimental procedures were approved by Western Kentucky University’s Institutional Animal Care and Use Committee on January 3, 2000.

Apparatus

*Optical system.* The light stimulus was administered by a two channel optical system. The light from the test channel was generated by a 150-W xenon arc lamp (Spectral Energy, Westwood, NJ, Model LH/150). The projected light from the arc lamp was collimated by passing through a quartz lens, and then it entered a water bath, which cooled the light temperature by absorbing the infrared energy. After the light was cooled, it was refocused by a quartz lens onto an optical shutter (Uniblitz, Rochester, NY, Model LS62M2), which was controlled by the laboratory computer. After the light passed through the shutter opening, it was re-collimated and then passed through a monochromatic interference filter with a bandwidth of 10 nm (Oriel, Stratford, CT, Model 54161 & Andover, Salem, NH, Model FS10-50). The
wavelengths presented varied from 320 nm to 640 nm in multiples of 20 nm. The light then passed through a series of neutral density filters (Reynard Corporation, San Clemente, CA, Model 398), which controlled the stimulus irradiance. Finally, the light passed through a polka dot beam splitter (Oriel, Stratford, CT, Model 38106) where half of the light was focused onto one end of a liquid light guide (Oriel, Stratford, CT, Model 77556). The other end of the guide was positioned in front of the fish’s right eye.

In addition to the monochromatic stimuli presentation, a white light was focused onto the fish’s eye as the background stimulus. A 250-W quartz halogen light bulb (Oriel, Stratford, CT, Model 6334) generated the white light, and it passed through a glass filter to cool the light temperature by removing the infrared energy. The light then passed through a quartz lens and was focused onto an optical shutter. The light was re-collimated and passed through a neutral density filter to control the stimulus irradiance. The light was then reflected off a mirror and focused onto the backside of the polka dot beam splitter, where half of the white light was mixed with half of the monochromatic light, and directed towards a quartz lens that focused it onto the liquid light guide. The neutral density filters were chosen so that the subjects were exposed to a background irradiance of 5 μW/cm².

*Electrophysiological apparatus.* During the electrophysiological procedure, the subject was positioned in a stereotaxic holding device. A small tube connected to the holding chamber was inserted into the fish’s mouth for the purposes of respirating and maintaining proper anesthetization. A water-anesthesia solution was passed through this tube continuously throughout the experiment. Recording electrodes
consisted of an ERG electrode, which was a 36-gauge chlorided silver wire (WPI, Sarasota, FL, Model AGW0530) encased in a glass pipette filled with a teleost saline solution; the tip size of the electrode was approximately 20 im. A reference electrode, which was a 36-gauge chlorided silver electrode, was used. The electrical signals were sent to an a/c differential amplifier (Grass Instrument Co., W. Warrick, RI, Model P55) with a bandpass of 0.1 to 100 Hz. Signals were sent to a 60-MHz dual-channel oscilloscope (Tektronix, Beaverton, OR, Model 2215A) and to the laboratory computer for storage. The data acquisition rate was 1000 Hz.

**Procedures**

*Optic nerve crush procedures.* Fish were anesthetized with a 0.04% tricaine methanesulfonate (MS-222) solution until respiration ceased. The subject’s dorso-ventral eye muscles were cut, and the eye was slightly pulled out to expose the optic nerve. Titanium surgical tweezers #5 (WPI, Sarasota, FL, Model 14400), with a tip-size of 0.05 mm, were used to pinch the nerve for 2-3 seconds. The eye was appropriately repositioned, and the subject was placed back into its home tank. In addition to these subjects, several fish served as a sham group. This group was treated exactly the same as the optic nerve subjects, except the optic nerve was not crushed. The surgical procedures were the same as for the optic nerve crush subjects, the muscles were cut, and tweezers were placed around the exposed optic nerve for 2-3 seconds. However, the tweezers did not actually touch or damage the optic nerve. This procedure was necessary to show that the surgical procedure itself was not responsible for any differences between the control group and the treatment groups. An antibiotic, Maracyn (Mardel Laboratories, Glendale Heights, IL, Model M-2119),
was administered to the home tank of all experimental and sham subjects to prevent infection. The dosage was 200 mg for every 10 gal tank, administered for five consecutive days.

**Electrophysiological procedures.** The zebrafish were anesthetized with a 0.04% MS-222 solution until respiration slowed down. Then a local anesthetic, a 5% lidocaine ointment (E. Fouger & Co., Melville, NY), was spread over the area of the skull that was directly above the optic tectum. The subject was positioned under a dissecting microscope (WPI, Sarasota, FL, Model 13301). A small drill (Fine Science, Foster City, CA, Model 18000-17) with a drill bit size of 0.7 mm was used to make small skull punctures. The punctures aided in the removal of the skull in order to record from the tectum. Other surgical instruments were also used to expose the tectum. Vannas iridectomy scissors (WPI, Sarasota, FL, Model 14364) were used to make incisions connecting the small drill holes. A stainless steel Tyrell surgical hook (WPI, Sarasota, FL, Model 14136) or tweezers (WPI, Sarasota, FL, Model 14400) were used to gently remove the skull. After the surgical procedure was finished the subject was given a 20 µg intramuscular injection of the paralytic gallamine triethiodide (Sigma, St. Louis, MO, Model G8134) and placed into the stereotaxic device described earlier.

Once in the recording chamber, the electrodes were placed into position. The TER electrode was placed in the superficial layers of the left tectal hemisphere and the reference electrode was positioned in the left nostril. The fish was given 3-5 minutes to adapt to the background light prior to the presentation of the monochromatic stimuli. Stimuli were presented in sets of ten and each presentation
set consisted of one wavelength at one stimulus irradiance. The stimulus irradiance started at a maximum or minimum level of irradiance and was then appropriately decreased/increased in log units of 0.5 until all desired irradiances were administered. Once all irradiances were administered, the next wavelength was presented. To obtain spectral sensitivity data, seventeen wavelengths were presented (from 320 to 640 nm in 20 nm steps). Presentation order was counterbalanced across subjects. Responses were recorded 50 ms prior to the stimulus (baseline), during the 500 ms stimulus, and then 500 ms post-stimulus.

*Testing schedule procedure.* Three different groups were tested. The first group, the control group, consisted of ten subjects. These subjects did not receive an optic nerve crush and were not on a specific time constraint for testing. However, the data for the control group were collected entirely before the testing of the other groups. A complete TER spectral sensitivity series was obtained for these subjects.

The second group, the sham group, consisted of three subjects and was tested at the same time the initial optic nerve crush subjects were tested (at 3 dpc). At this point, it was not necessary to obtain complete spectral sensitivity data. The purpose of this testing was to determine the effect of the surgical procedures on the tectal response. Therefore, testing was limited to presenting four wavelengths near each cone’s peak sensitivity (360, 420, 480, and 560 nm) at one or several suprathreshold irradiances (determined by the control group data). The responses to these four wavelengths were used to ensure that there was no selective damage to any of the cone contributions to the response. The sham group was necessary to show that the
subjects’ responses were affected only by the actual optic nerve crush and not by any of the additional surgical procedures used en route to crush the optic nerve.

The last group was the optic nerve crush group. Subjects in this group were tested at different times post-crush. The times chosen post-crush were 3, 14, 28, 42, and 83-91 dpc. Initially, the optic nerves of ten subjects were crushed, and at 3 dpc three of these subjects were tested by presenting each of the four wavelengths at one suprathreshold irradiance. This testing was necessary to test whether the optic nerve crush had been successful.

The remainder of the optic nerve crush groups were tested on a restricted schedule with a 14 day interlude between each level tested. Responses were recorded from five subjects at 14, 28, and 42 dpc; four wavelengths at a suprathreshold irradiance were used to test these subjects. Seven subjects for the 90 dpc (83-91 dpc) were tested, and a full spectral sensitivity series was gathered on these subjects in order to compare these results to the control subjects’ tectal spectral sensitivity.
Chapter 3

Results

Overview of Analysis

The main goals of this study were to compare two visual levels of the zebrafish (the retina and the tectum) and to examine the functional recovery of optic nerve regeneration. Thus, the results section of this project is divided into two major headings: comparing the ERG and TER responses and comparing tectal responses before and after optic nerve crush. To achieve these goals, several procedures were required. The following section describes the analytical procedures, which include obtaining tectal evoked responses, deriving tectal spectral sensitivities, modeling the tectal spectral sensitivity data, determining significant differences in the data by using several 2 (group or condition) X 17 (wavelength) mixed design analyses of variance (ANOVAs). Group or condition is the between-subjects variable and wavelength is the within-subjects variable. Significant condition by wavelength interactions were examined using the Tukey-Kramer post hoc test.

Deriving tectal evoked responses. The TER is a gross electrical recording of neural activity from the surface of the optic tectum. Figure 1 shows a sample TER response of a normal zebrafish to a 340 nm stimulus at several irradiances. The ordinate shows the TER response averaged over 10 stimulus presentations and the abscissa represents time (ms). The stimulus duration (500 ms) is indicated by the raised horizontal bar along the abscissa. Notice that there are positive ON-
and OFF-responses and that the amplitude of the responses is dependent upon the
stimulus irradiance; that is, as the stimulus irradiance is increased the response
amplitude is increased. Since the TER response possesses both ON- and OFF-
responses, each response component was analyzed separately.

Deriving spectral sensitivity. To examine the cone contributions to the
various response components, a spectral sensitivity function for each type of
response must be obtained. To derive spectral sensitivity the peak amplitudes of
the TER responses were determined for every stimulus irradiance at each
wavelength. Log response amplitudes versus log stimulus irradiance functions
were calculated for each wavelength. Linear regression analyses of the functions
were made, and the sensitivity at each stimulus wavelength was obtained. The
sensitivity was calculated by choosing an arbitrary response criterion and
determining the amount of light needed to obtain that response criterion. The
reciprocal of this irradiance value represented the sensitivity of the subject to that
wavelength. Each subjects’ spectral sensitivity data was averaged across the
spectrum (320 to 640 nm) for the control subjects and the 90 dpc subjects for both
the ON- and OFF-response components.

In addition to obtaining spectral sensitivity functions from the TER of
normal and 90 dpc subjects, data were gathered at four wavelengths (360, 420,
480, and 560 nm) for five subjects at various times post-crush prior to 90 dpc.
Gathering data at these four wavelengths was done to determine the
developmental sequence of the return of the TER response following optic nerve
crush.
**Cone modeling.** A multiple mechanism model was used to examine the cone contributions to each response component for the control and 90 dpc spectral sensitivity functions. This model has been previously used by the Bilotta laboratory (Hughes et al., 1998) and the equation for each mechanism had the form:

\[
S_\lambda = (k_1 x A_{1\lambda}) + (k_2 x A_{2\lambda})
\]

where \(S_\lambda\) was the sensitivity at wavelength \(\lambda\), \(A_x\) was the absorptance of a cone type \(x\) at wavelength \(\lambda\), and \(k_1\) and \(k_2\) were the weights assigned to the cone inputs. When \(k_2\) was positive, the mechanism consisted of two additive components. When \(k_2\) was negative, the mechanism consisted of two opponent components, and when \(k_2\) was zero, there was only one cone contribution to the response. A nonlinear regression analysis was used to find the best-fit cone weights to the spectral sensitivity data.

The specific wavelength range for each mechanism was based on the modeling procedures from Hughes et al. (1998). The advantage of this multiple model was that it allowed the weights of a specific cone type to vary across the spectrum. For example, the M-cones can provide an excitatory contribution to one mechanism (e.g., M-S) and an inhibitory contribution to another mechanism (e.g., L-M). See Hughes et al. (1998) for a discussion.

**Inferential statistics.** In order to statistically compare the spectral sensitivity function of the various TER responses, several 2 (control vs. 90 dpc) X 17 (wavelength) mixed design ANOVAs were used to test the optic nerve
regeneration. In addition, following a significant interaction, the Tukey-Kramer post hoc tests were used to determine which wavelengths were significantly different between the groups.

Comparing the ERG and TER Response

The spectral sensitivities based on the TER ON- and OFF-responses were compared to the ERG b- and d-wave spectral sensitivities. The ERG b- and d-wave spectral sensitivity data were obtained from Bilotta and Harrison (1999). They derived the spectral sensitivity functions from the b- and d-wave components based on responses to a 500 ms stimulus, the same stimulus duration used in this study. Bilotta and Harrison (1999) found that the ERG spectral sensitivity function based on the b-wave component was best-fit by a multiple mechanism model that possessed both color opponent (M-S and L-M) and nonopponent mechanisms (U-only and S-only). They also found that the ERG d-wave component receives U-S, S-U, and M+L cone mechanisms.

The average spectral sensitivity function of the TER ON-response component is shown in Figure 2. The symbols depict the data, the line represents the best-fit model to the data, and the letters indicate cone contributions to the model. The present study found that the ON-response of the TER was also best fit by a multiple mechanism model that possessed color opponent (L-M) and nonopponent mechanisms (U+S and M-only).

The spectral sensitivity functions of the TER ON-response and the ERG b-wave are shown in Figure 3. The filled squares depict the ERG data and the open squares represent the TER data. The lines represent the best-fit models and the
letters indicate the cone contributions to each model; the ERG model is indicated by asterisks. There appear to be a consistent difference between the ERG and TER of about one log unit of absolute sensitivity across the spectrum. In addition, there appear to be both differences and similarities in cone contributions to the ERG and TER. For example, while both the ERG and the TER responses contain L-M mechanisms, only the ERG contains an opponent M-S mechanism and only the TER contains an U+S mechanism.

The average spectral sensitivity function of the TER OFF-response component is shown in Figure 4. The symbols depict the data, the line represents the best fit model to the data, and the letters indicate the cone contributions to the model. The TER OFF-response mechanisms are U-S, S-U, M-only, and L-only.

The spectral sensitivity functions of the OFF-responses of the ERG (d-wave) and the TER are shown in Figure 5. The filled circles depict the ERG data and the open circles represent the TER data. The lines represent the best-fit models and the letters indicate the cone contributions to each model; the ERG model is indicated by asterisks. There appear to be differences between the absolute sensitivities. The TER appears to be more sensitive than the ERG at the ultraviolet wavelengths, but is less sensitive than the ERG at the short, middle and long wavelengths. In addition to these differences, there are some notable similarities between the TER and the ERG. Namely, both the TER and the ERG have U-S and S-U mechanisms.

The TER ON-response and the TER OFF-response were examined for differences in sensitivity and cone contributions to their spectral sensitivities in
Figure 6. The open squares depict the ON-response and the open circles represent the TER OFF-response. The lines represent the best-fit models and the letters indicate the cone contributions to each model; the ON-response model is indicated by asterisks. There appears to be a similar trend across the spectrum with the U-S and U+S contributions being the most sensitive, sharp declines in sensitivity of the S-cones and slight declines of the M- and L-cones. The U-cones appear to be a little more sensitive in the OFF-response than in the ON-response, but the L-cones appear to be slightly more sensitive in the ON-response than in the OFF-response.

Comparing Tectal Responses Before and After Optic Nerve Crush

In order to examine the timing of recovery following optic nerve crush, the presence of responses were examined at several times post-crush. In addition, a group of ‘sham’ fish were examined in order to determine whether the surgical procedures produced any additional damage. The percentage of responses seen for each optic nerve crush group and the sham group is shown in Figure 7. A “response” was defined as any TER response at any of the four wavelengths. Five subjects were tested for each group, except for the 90 dpc subjects where 10 subjects were tested.

The results show a general increase in the presence of a response, with an increase in the number of days post-crush. However, there are two minor exceptions. The first difference is that the 28 dpc had a 0% response rate, but the 14 dpc had a 20% response rate. Secondly, although the shams were more successful than most of the optic nerve crush groups, the 90 dpc had the same response rate. It is important to note that the results show that the optic nerve
crush procedure was successful, because the 3 dpc group showed no responses. However, it should also be mentioned that the ‘sham’ subjects were not normal. Interestingly, sensitivity calculations could not be made on the majority of the data for subjects equal to or less than 42 dpc, because the responses were so small and/or abnormal.

The average spectral sensitivity function of the TER ON-response component for subjects at 90 dpc is shown in Figure 8. The symbols depict the data, the line represents the best-fit model to the data, and the letters indicate the cone contributions to the model. The cone contributions to the spectral sensitivity of the TER ON-response for the 90 dpc subjects appear to be U+S, M-only, and L-M.

The spectral sensitivity functions of the TER ON-response for 90 dpc subjects with control subjects are shown in Figure 9. The filled circles depict the 90 dpc subjects (model contributions indicated by asterisks) and the open circles represent the control subjects. ON-responses for the 90 dpc subjects were compared to the ON-responses of the control subjects by using a 2 (control vs. 90 dpc) X 17 (wavelength) mixed design analysis of variance (ANOVA). The within-subjects variable was wavelength (320-640 nm) and the between-subjects variable was group type (control vs. 90 dpc). These analyses determined whether tectal ON-responses reappeared and whether there were significant differences between the spectral sensitivity functions before (control) and after the optic nerve crush (90 dpc).
Results show that 90 dpc subjects (n=7) do indeed regenerate their optic nerve neurons. Spectral sensitivity data of the 90 dpc subjects were compared to those of the control subjects (n=10). No significant main effect was found for the between subjects variable (group), $F(1, 15) = .15, p > .05$; nor was a significant interaction found for wavelength by group, $F(16, 240) = .43, p > .05$. Interestingly, the TER control and 90 dpc subjects appear to possess exactly the same cone mechanisms; furthermore when graphically comparing the data points at each wavelength, most of the means fall on top of each other. The only obvious difference between the TER control subjects and the TER 90 dpc subjects is in the amount of variability at each wavelength. The 90 dpc subjects have more variability than the control subjects.

The average spectral sensitivity function of the TER OFF-response component of the 90 dpc subjects is shown in Figure 10. The symbols depict the data, the line represents the best-fit model to the data, and the letters indicate the cone contributions to the model. The multiple mechanism model was used to determine the cone contributions to the spectral sensitivity of the TER OFF-response for the 90 dpc subjects. The model revealed cone mechanisms of U-only, S-only, and M+L. It should be noted that the model excluded the data at 320 nm.

The spectral sensitivity functions of the TER OFF-response for the 90 dpc subjects with the TER OFF-response of the control subjects are shown in Figure 11. The most striking difference appears to be that there is no opponency in the 90 dpc group, whereas opponency exists at the U-S and S-U mechanisms for the control subjects. In addition, there appears to be more variability in the 90 dpc
subjects' spectral sensitivity data than the control subjects' spectral sensitivity data. The filled squares depict the 90 dpc subjects (model contributions indicated by asterisks), and the open squares represent the normal subjects. OFF-responses for the 90 dpc subjects were compared to the OFF-responses of the control subjects by using a 2 (control vs 90 dpc) X 17 (wavelength) mixed design ANOVA. The within-subjects variable was wavelength (320-640 nm) and the between-subjects variable was group type (control vs. 90 dpc). These analyses determined whether tectal OFF-responses reappeared and whether there were significant differences between the spectral sensitivity functions before (control) and after the optic nerve crush (90 dpc).

Results show that no significant main effect was found for group, F(1, 15) = .08, p > .05, but a significant interaction was found for wavelength by group, F(16, 240) = 4.77, p < .01. The Tukey-Kramer post-hoc test was done on all 17 wavelengths to determine where the significant differences were, and significant differences were found at 340, 360, and 640 nm.
Chapter 4

Discussion

There were two main goals for this project. The first goal was to compare retinal spectral sensitivity with tectal spectral sensitivity, and the second goal was to functionally test neural regeneration by means of an optic nerve crush. In addition, there were some broader purposes for conducting this study. The zebrafish has become an important model throughout science, but especially in the visual sciences and developmental neurosciences. This study provides further evidence of the viability of this animal model because it demonstrates that the visual system is an excellent model to study neural systems, including the study of neural regeneration (Bilotta & Saszik, 2001).

Obtaining zebrafish tectal data is important to further enhance the utility of this animal model. Spectral sensitivity data had not been examined in many fish models, and it can provide valuable information about photoreceptor activity before and after optic nerve crush. However, previous research in the area of neural regeneration in fish has primarily utilized the goldfish, but as mentioned previously, the goldfish has limitations that the zebrafish does not have, such as the disparity between the behavioral and physiological data.

Comparing the ERG to the TER

One purpose of this study was to examine the similarities and differences between retinal and tectal physiology. These properties were compared at both stimulus onset and stimulus termination. There are several notable similarities at
stimulus onset. The ERG and the TER spectral sensitivities indicate that the ERG and TER responses both receive contributions from all four cone types, demonstrate opponency, and demonstrate a high level of sensitivity to ultraviolet wavelengths. In addition, the opponency is the same for both levels of the visual system, consisting of an L-M mechanism. This opponent L-M or red-green mechanism appears across a variety of species with color vision, including primates (Sperling & Harwerth, 1971). When comparing the ON-responses of the TER and the ERG, differences can also be seen. Namely, there are cone contribution differences to the model at the ultraviolet, short, and middle wavelengths. These findings indicate that there are differences in visual information processing between the retina and the tectum. Another difference between the ON-responses of the ERG and the TER is that there is more variability in the TER spectral sensitivity data. This increase in variability is likely due to the added complexity of zebrafish tectal neurons compared to their retinal neurons (Sajovic & Levinthal, 1982a, b). Sajovic and Levinthal (1982a, b) have shown that there are four different types of tectal cells that respond differently to various stimuli.

The OFF-responses of the ERG and TER also contain some similarities and differences. Again, both models have four cone types contributing to the model, both have opponent and nonopponent contributions, and both have a high degree of sensitivity to ultraviolet wavelengths. Furthermore, the opponent contributions are more similar in the OFF-response than in the ON-response; both the spectral sensitivities of the ERG and TER OFF-response contain U-S and S-U
components. However, the TER OFF-response is more sensitive at the ultraviolet wavelengths than is the ERG OFF-response, but decreases sharply across the spectrum. The ERG OFF-response is somewhat less sensitive at the ultraviolet wavelengths, but remains highly sensitive across the remaining wavelengths. Consistent with the ON-responses, the TER OFF-response spectral sensitivity also has more variability across the spectrum. The variability is most likely due to the added complexity of zebrafish tectal neurons (Sajovic & Levinthal, 1982a, b).

This study also compared the TER ON- and OFF-response spectral sensitivities. Again, there are similarities and differences between the two spectral sensitivities. Both have a high degree of sensitivity to the ultraviolet wavelengths, both have opponent and nonopponent contributions, and both receive contributions from all four cone types. In addition, both models display a similar trend in sensitivity, with the responses to ultraviolet wavelengths being the most sensitive, followed by sharp declines in sensitivity across the visible spectrum. However, the opponent contributions are not the same for the ON- and OFF-response spectral sensitivities. The ON-response spectral sensitivity appears to have more opponency at the long and middle wavelengths (L-M), whereas the OFF-response spectral sensitivity appears to have more opponency at the ultraviolet and short wavelengths (U-S and S-U).

The ON- and OFF-responses of goldfish optic nerve spectral sensitivities have also been examined. DeMarco and Powers (1991) found three cone type contributions to the goldfish ON-response (short, middle, and long). In addition, they found an opponent mechanism at the middle wavelengths. The spectral
sensitivity of the goldfish optic nerve (ON-response) has some general similarities to the zebrafish. Both have overlapping cone type contributions (S, M, and L-cones) and possess similar opponent contributions (inhibitory M). However, there are some noticeable differences as well. The goldfish optic nerve spectral sensitivity does not have a U-cone contribution nor are the opponent and nonopponent contributions identical to the zebrafish’s tectal spectral sensitivity (ON-response). The spectral sensitivity of the OFF-response for the goldfish optic nerve and the zebrafish tectum also contain similarities and differences. Again, DeMarco and Powers (1991) found three cone types contributing to the goldfish OFF-response spectral sensitivity (S, M, and L-cones). An opponent contribution was also found in the OFF-response (inhibitory S). However, the goldfish OFF-response does not contain a U-cone contribution, but the zebrafish tectal OFF-response does. In addition the opponent and nonopponent contributions are not the same as in the cone mechanisms of the zebrafish.

Comparing Tectal Responses Before and After Optic Nerve Crush

The primary goal of this study was to examine optic nerve regeneration. As cited in Marcus et al. (1998), previous studies by Schmatolla and Erdman (1973) and Rahmann (1968) had suggested that the zebrafish tectum was not capable of regeneration. Contrary to those initial studies, Marcus et al. (1998) suggested that regeneration was possible, but because no testing was done to functionally demonstrate regeneration, conclusive results could not be made. The present study supports the conclusions made by Marcus et al. (1998). This study demonstrates the establishment of tectal spectral sensitivity (control subjects), the
complete elimination of tectal responses via optic nerve crush (3 dpc), and then the complete regeneration of tectal responses (90 dpc) to their pre-crush status.

The results of the control and 90 dpc TER ON-response spectral sensitivities are remarkably identical. Not only do both spectral sensitivities contain contributions from four cone types but also the cone mechanisms are exactly the same; they both possess the opponent (L-M and M-S) and nonopponent (U-only and S-only) mechanisms. In addition, they are virtually identical in absolute sensitivity. The only minor difference between the ON-responses of the control and 90 dpc groups is the increase in variability for the 90 dpc group. However, this result is not entirely surprising, because goldfish studies have reported similar findings (Northmore, 1989a, b; Cassidy & Bilotta, 2000). This increase in variability is possibly caused by several factors. Incomplete synaptic reorganization could cause more variability, the amount of damage during the crush procedure is also a potential factor, and the 90 dpc group had fewer subjects (n=7) than the control group (n=10).

Another interesting point to elaborate on is the differences found between the control and 90 dpc OFF-response spectral sensitivities. Unlike the ON-responses, the OFF-responses do differ considerably. The only similarity is that both spectral sensitivities contain contributions from four cone types. However, no opponency was found in the 90 dpc group, even though it was found in the control group. There were differences in sensitivity between the control and 90 dpc groups across the spectrum. The control group had a higher sensitivity at the ultraviolet wavelengths, but was less sensitive than the 90 dpc group at the middle
and long wavelengths. Furthermore, post-hoc tests revealed significant differences at 340, 360, and 640 nm. Thus, the OFF-response following optic nerve damage is less sensitive to ultraviolet wavelengths than is the case for control subjects. Therefore, the connection between the U+S cones in the TER OFF-response may not be complete by 90 dpc. However, because the same opponent mechanisms are evident in the 90 dpc ON-response that were found in the control subjects’ ON-response, it appears there is a difference in regeneration activity related to the ON- and OFF-responses. It is possible that there is a time difference or that they represent two types of mechanisms. Interestingly, Maeyama and Nakayasu (2000) found evidence supporting a dual system of regeneration in the zebrafish tectum. Maeyama and Nakayasu (2000) described marginal zones containing stem cells along the edges of the zebrafish optic tectum. However, they noticed that the stem cells were very specific in their migratory pathway. Maeyama and Nakayasu (2000) reported that some zones were “active zones” and some zones were “largely inactive.” It is possible that a dual system could account for the differences in regeneration between the ON- and OFF-responses.

There appear to be differences between zebrafish optic nerve regeneration and goldfish optic nerve regeneration. Northmore (1989b) reported that goldfish OFF-responses were first detected in optic nerve crush fish. Although the present study was not able to pinpoint which response type reappeared first in zebrafish (ON, OFF, or ON/OFF), the present study did demonstrate that the ON-response regenerated to its pre-crush state by 90 dpc, whereas the OFF-response failed to do so completely by 90 dpc. However, comparisons between our data and goldfish
data are limited because different methods were used to test regeneration. Northmore (1989a, b) tested zebrafish neural regeneration with different stimuli than the present study did. This study presented monochromatic stimuli at various irradiances to fish, but Northmore’s study (1989a, b) used a flashing red LED and a black and white stimulus to test fish.

Although this project was successful in addressing the two main goals, there are some changes that future studies should address. The 14, 28, and 42 dpc times do not seem to represent optimal times of cellular regrowth. However, because this is the first study to functionally test regeneration of zebrafish optic nerve neurons, specific optimal time ranges were not available. The times chosen were based on goldfish data, but after seeing many non-responses and the amount of variability in the responses that were found at these times, perhaps extended ages, such as 35, 55, and 75 dpc would be more appropriate test ages. Likewise, it would be interesting if the 90 dpc time was further extended to see whether the variability differences would cease to exist and whether the OFF-response spectral sensitivity would return to normal.

Another important aspect that should be addressed is the noticeable degree of difference between performing the optic nerve crush procedure on goldfish and the zebrafish. Prior to testing, zebrafish appeared to have more eye swelling and attrition than goldfish undergoing the same procedures. The zebrafish is much smaller than the goldfish, and it is our collective observation that the crush procedure is likely more stress-inducing to zebrafish because of this size.
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Figure Captions

**Figure 1.** TER responses to various stimulus irradiances under a broadband background irradiance of 5 μW/cm². Each waveform represents the average response to 10 stimulus presentations. For all averaged TERs, the baseline responses were set to zero microvolts. The stimulus wavelength is 340 nm and was presented for 500 ms. The horizontal line depicts stimulus onset and termination. The negative values associated with each response depict the log stimulus attenuation where a log irradiance of -3.0 represents 12.0 log quanta s⁻¹ cm⁻².

**Figure 2.** Spectral sensitivity of normal TER ON-response. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. Each square represents the mean sensitivity averaged across 10 subjects. The line represents the best-fit model and the error bars indicate ± 1 SEM. The cone contributions that best-fit this model are U+S, M-only, and L-M.

**Figure 3.** ERG and TER normal ON-response spectral sensitivities. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. The open squares represent the mean sensitivity for TER ON-response (n=10); the line represents the best-fit model. The cone contributions that best-fit this model are U+S, M-only, and L-M. The filled squares represent the mean sensitivity for ERG b-wave (n=10), the line represents the best-fit model. Asterisks indicate the model for the ERG b-wave. The cone contributions that best-fit this model are U-only, S-only, M-S, and L-M. Error bars represent ± 1 SEM.
**Figure 4.** Spectral sensitivity of normal TER OFF-response. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. Each circle represents the mean sensitivity averaged across 10 subjects. The line represents the best-fit model and the error bars indicate ± 1 SEM. The cone contributions that best-fit this model are U-S, S-U, M-only, and L-only.

**Figure 5.** ERG and TER normal OFF-response spectral sensitivities. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. The open circles represent the mean sensitivity for TER OFF-response (n=10); the line represents the best-fit model, and the error bars represent ± 1 SEM. The cone contributions that best-fit this model are U-S, S-U, M-only, and L-only. The darkened circles represent the mean sensitivity for the ERG d-wave (n=10); the line represents the best-fit model. Asterisks indicate the model for the ERG d-wave. The cone contributions that best-fit this model are U-S, S-U, M-only, and L-only. The error bars represent ± 1 SEM.

**Figure 6.** Spectral sensitivity of the TER ON- and OFF-responses. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. The open circles represent the mean sensitivity for TER OFF-response (n=10); the line represents the best-fit model. The cone contributions that best-fit this model are U-S, S-U, and M+L. The open squares represent the mean sensitivity for the TER ON-response (n=10). Asterisks indicate the model for the TER ON-response. The cone contributions that best-fit this model are U+S, M-only and L-M. The error bars represent ± 1 SEM.
Figure 7. Percentage of tectal responses seen for each group tested at various times post-crush. Fish were tested at 3, 14, 28, 42, and 90 dpc; in addition, sham subjects were also tested. The ordinate represents the response percent and the abscissa represents the various groups tested at different days post-crush. Five subjects were tested for all groups, except for the 90 dpc group, where ten subjects were tested.

Figure 8. TER ON-response spectral sensitivity for 90 dpc subjects. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. Each circle represents the mean sensitivity averaged across 7 subjects. The line represents the best-fit model and the error bars indicate ± 1 SEM. The cone contributions that best-fit this model are U+S, M-only, and L-M.

Figure 9. TER OFF-response spectral sensitivity for 90 dpc subjects. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. Each square represents the mean sensitivity averaged across 7 subjects. The line represents the best-fit model and the error bars indicate ± 1 SEM. The cone contributions that best-fit this model are U-only, S-only, and M+L. It should be noted that the sensitivity value at 320 nm was excluded from the model.

Figure 10. TER ON-response spectral sensitivities for normal and 90 dpc subjects. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. The open circles represent the mean sensitivity for the ON-response (n=10) of the normal subjects; the line represents the best-fit model. The cone contributions that best-fit this model are U+S, M-only, and L-M. The filled
circles represent the mean sensitivity for the ON-response (n=7) of the 90 dpc subjects; the line represents the best-fit. The cone contributions that best-fit this model are U+S, M-only, and L-M. Asterisks indicate the model for the TER ON-response of the 90 dpc subjects. The error bars represent ± 1 SEM.

**Figure 11.** TER OFF-response spectral sensitivities for normal and 90 dpc subjects. The ordinate represents log absolute sensitivity and the abscissa represents the stimulus wavelength. The open squares represent the mean sensitivity for the OFF-response of the normal subjects (n=10); the line represents the best-fit model. The cone contributions that best-fit this model are U-S, S-U, M-only, and L-only. The filled squares represent the mean sensitivity for the OFF-response of the 90 dpc subjects (n=7); the line represents the best-fit model. The cone contributions that best-fit this model are U-only, S-only, and M+L. Asterisks indicate the model for the TER ON-response of the 90 dpc subjects. The error bars represent ± 1 SEM.
Adult Zebrafish Tectal Response

Stimulus wavelength: 340 nm
Normal ON-Response Spectral Sensitivities

Log Absolute Sensitivity (q/s/cm²)

Wavelength (nm)

U*  S*  M - S*  L - M*

ERG b-wave*

Tectal ON-response
Normal OFF-Response Spectral Sensitivities

Log Absolute Sensitivity (q/s/cm²)

- U - S
- S - U*
- M + L*
- U - S*
- S - U
- M
- L

- ERG d-wave*
- Tectal OFF-response

Wavelength (nm)
Tectal Responses After ONC

Response Percent

Days Post-Crush

3 14 28 42 90 shams
TER ON-Response Spectral Sensitivities

Log Absolute Sensitivity (q/s/cm²)

- U + S
- U + S*
- M
- M*
- L - M
- L - M*

90 dpc*

Normal

Wavelength (nm)
90 dpc OFF-Response Spectral Sensitivity

Log Absolute Sensitivity (q/s/cm²)

Wavelength (nm)
TER OFF-Response Spectral Sensitivities

Log Absolute Sensitivity (q/s/cm²)

Wavelength (nm)

90 dpc
Normal

U - S
S - U
U*
S*
M + L*
M
L