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Visual function in regenerating teleost retina following surgical lesioning

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Abstract
Regeneration of the teleost retina following surgical extirpation of 25% to 100% of the neural retina was investigated in goldfish (Carrasius auratus) and sunfish (Lepomis cyanellus). The retina will regenerate following removal of up to 95% of the neural retina, however complete extirpation prevented regeneration. Visual sensitivity was assessed by examining components of the electroretinogram (ERG) and the dorsal light reflex (DLR) during regeneration. B-wave amplitudes in the experimental eyes increased throughout the study and central connections were reestablished as indicated by the progressive improvement in the dorsal light reflex. The recovery of visual function was closely correlated with retinal regeneration. Visual recovery progressed more slowly than following complete cytotoxic destruction of the mature retina (Mensinger & Powers, 1999) because the surgery removed a large number of the pluripotent cell population and restricted the number and distribution of regenerating foci.

Keywords: Regeneration, Retina, Vision, Electroretinogram, Photoreceptors

Introduction
The teleost retina is a dynamic tissue in which neurogenesis occurs at the ciliary marginal zone (CMZ) and throughout the central retina during the life of the fish. Multipotent retinal stem cells in the marginal zone will generate all classes of retinal neurons; however, the stem cells that persist throughout the central retina normally will develop only into rod photoreceptors (Johns & Easter, 1977; Johns & Fernald, 1981; Johns, 1982; Raymond & Hitchcock, 2000; Hitchcock & Raymond, 2004; Raymond et al., 2006). However, following cytotoxic destruction (Maier & Wolburg, 1978; Kurz-Isler & Wolburg, 1982; Raymond et al., 1988) or surgical excision (Hitchcock & Raymond, 1992; Hitchcock et al., 1992; Cameron & Easter, 1995; Raymond & Hitchcock, 1997) of the central retina, the stem cells become pluripotent and differentiate into various classes of retinal neurons in the central retina of mature fish (Raymond et al., 1988; Hitchcock et al., 1992, Cameron & Easter, 1995; Hitchcock & Raymond, 2004). The replacement of retinal neurons in adult goldfish results in complete anatomical regeneration of the retina (Raymond et al., 1988), establishment of normal synaptic connections (Hitchcock & Cirzenza, 1994), and restoration of axonal contacts with the brain (Stuermer et al., 1985).

The plasticity of the teleost nervous system provides an excellent model for observing developmental processes in an adult animal. The teleost optic nerve has been the focus of numerous studies investigating regeneration and synaptogenesis. Sperry (1949) was the first to document the restoration of function following optic nerve transection. More recent studies have demonstrated the capacity of severed optic nerve fibers to reform highly topographical projections and restore visual function (Meyer, 1977, 1980; Schmidt & Edwards, 1983; Northmore & Masino, 1984; Meyer et al., 1985; Callahan & Mensinger, 2007). Retinal regeneration provides a more complex problem, because it involves the genesis of entire classes of retinal neurons, including new central projections to the tectum. Although, the regenerative potential of the teleost retina has been well documented (Raymond et al., 1988; Hitchcock et al., 1992; Cameron & Easter, 1995; Raymond & Hitchcock, 1997; Hitchcock & Raymond, 2004), little work has been done to assess the visual capability of the regenerated tissue. It is imperative to assess the physiology of regenerating systems in addition to the morphology, to ascertain the success of the regeneration.

A previous study (Mensinger & Powers, 1999) demonstrated that up to 50% of the b-wave amplitude of the ERG was restored in the teleost retina following complete cytotoxic lesioning of the mature teleost retina. Unfortunately, optical side effects caused by the cytotoxin, such as shrinkage of pupil diameter and cataract formation, precluded more accurate visual assessment of the regenerated tissue. Retinal regeneration has been documented following surgical removal of small patches of retina without

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compromising the system’s optics (Hitchcock et al., 1992), and therefore offered an opportunity of more accurate assessment of visual function in the regenerating retina.

Materials and methods

Animal husbandry

Goldfish (Carassius auratus) were obtained from Ozark fisheries (Richland, MO) and sunfish (Lepomis cyanellus) were seized from ponds on the McCoy Farm (Dickson, TN). Fish ranged from 4 to 6 cm standard length. Fish were housed in 40–120 L aquaria equipped with mechanical, chemical and biological filtration, and maintained at 20°C on a 12:12 LD cycle. Fish were fed a diet of commercial fish food (TetraMin) supplemented with frozen Artemia sp. All animal care and surgical procedures conformed to institutional, American Physiological Society and Society for Neuroscience animal care guidelines.

Surgical extirpation

Fish were dark-adapted overnight and transferred to opaque containers under dim red light before light onset. Fish were anesthetized in a 0.01% solution of tricaine methane-sulfonate (MS-222, Sigma, St. Louis, MO), removed from their containers, and placed between moist sponges under a dissecting microscope. Retinas were removed within 15 min of light exposure by one of two methods: (1) patch extirpation—the retina was accessed transscerally, and a section of the dorsal retina extirpated; (2) aspiration— the retina was accessed through the front of the eye, and varying amounts of retinal tissue aspirated.

Patch extirpation

The conjunctiva was removed from the eye, and the eye pivoted ventrally in its socket, revealing the dorsal sclera. A small incision was made with a microblade, parallel and posterior to the limbus at approximately the 12 o’clock position. Trans-scleral incisions were made with an iris scissors parallel to the limbus approximately 45° from vertical, followed by a short medial incision. The resulting tissue flap was retracted from the sclera, and inverted to reveal the neural retina and pigment epithelium. The neural retina and the CMZ were teased away from the underlying pigment epithelium, and a minimum of 15% to 25% of the total retinal area was removed. The scleral flap was repositioned and the wound closed with cyanoacrylate glue (Pacer, Rancho Cucamonga, CA). Patch extirpation was performed on goldfish (N = 12) and sunfish (N = 5).

Aspiration

A small incision was made near the limbus at the 12 o’clock position. Lateral cuts were made with an iris scissors along the limbus, approximately 90° in each direction. The resulting eye flap was pulled anterior and downward, displacing the lens anterior, and exposing the interior of the eye. The eye was visually divided into quadrants using the cross hairs of an ocular micrometer, with the optic nerve head serving as an approximate center. Varying amounts (50% to 100%) of the retina were aspirated using a suction pipette (tip diameter 1 mm), attached to a vacuum aspirator. The CMZ was removed concurrently with the neural retina at the margins of the aspirated areas. Because some tissue was also removed from the border of adjacent quadrants, the tissue extirpation estimates should be considered conservative. Presurgical dark adaptation rendered the neural retina amenable to aspiration, limiting (range 0% to 5% of total aspirated area) the amount of PE that was damaged or dislodged. Following tissue removal, the eye was filled with sterile saline (0.9%), and the flap reattached to the limbus with cyanoacrylate. Seventy goldfish and 10 sunfish contributed to this portion of the experiment.

The contralateral eye of each experimental fish was left intact, and served as an intrafish control. Several fish underwent either patch (N = 4 goldfish; N = 2 sunfish) or aspiration (N = 4 goldfish; N = 2 sunfish) sham surgeries, which mimicked the surgical procedures except for retina removal. Following all surgeries, fish were revived by fresh water circulation over the gills, returned to home aquaria, and isolated from tankmates until normal equilibrium was restored. In later experiments, post-operative sunfish were housed separately.

Electroretinogram

Methodology was identical to a previous investigation (Mensinger & Powers, 1999). Briefly, fish were anesthetized in a 0.001% solution of tricaine methane-sulfonate (MS-222, Sigma), placed between sponges in a small Plexiglas aquarium, and the anesthetic solution continuously recirculated over the gills via an intraoral tube. An AgCl2 recording electrode was inserted into an incision at the limbus of the control or experimental eye, and a reference electrode placed into the ipsilateral nare. Light from a regulated tungsten-halogen source (Ealing model 227-1403) was focused on a shutter (Uniblitz model 325b), collimated, and refocused on a 3/8” fiber optic light pipe. The fiber optic was placed 5 cm from the eye, which remained just above water level. The eyes were covered with moist pieces of filter paper. The light beam was adjusted to be perpendicular to the eye and at this distance; the light from the fiber optic covered the entire surface of the eye. The irradiance of the light was maintained at 20 µW cm−2 s−1 as measured by a UDT 40× radiometer. Fish were dark adapted for 15 min, and presented with five consecutive 200 ms flashes of broad band light at 30 s intervals to either the experimental or control eye. The resulting waveforms were averaged by computer. The entire procedure was repeated for the other eye (trial sequence alternated between control and experimental eye), and then the fish was revived with fresh water, and returned to its home aquarium.

Monochromatic stimuli for the spectral sensitivity experiment were provided by placing interference filters (Melles-Griot, half width at half height 8 nm) in the collimated portion of the beam of the apparatus. Intensity response functions were generated via an ascending method of limits, starting with a subthreshold stimulus and increasing intensity until the criterion response (15 µV) was obtained.

Dorsal light reflex

Methodology was similar to the dorsal light reflex used to assess visual function following cytotoxic lesioning (Mensinger and Powers, 1999). Fish were placed individually in a 40-L aquarium equipped with a 15 W overhead fluorescent light. The top of the aquarium was covered with several sheets of white paper to diffuse the light. An opaque barrier with a small port for the videocamera surrounded the aquarium. Fish were dark adapted for one hour, and then videotaped for five minutes following light onset. The fish was filmed from the front of the arena and a 1-cm × 1-cm grid was placed on the back of the aquarium for reference. The DLR was
determined by analyzing single frames of the videotape and calculating the degree of tilt from the fish’s normal, vertical upright position. Five measurements were made at one minute intervals, and the results averaged to calculate the DLR.

Histology

Fish were sacrificed with an overdose of MS-222. Both eyes were removed and fixed overnight in Bouins solution [picric acid; formaldehyde (37% v/v); glacial acetic acid; 15:5:1], the lens removed, and eyecup cut into dorsal/ventral or nasal/temporal halves. This tissue then was dehydrated in an alcohol series, embedded in paraffin, serially sectioned at 8 µm, and stained with Lee stain (0.025% Methylene Blue and 0.025% Basic fuchsin).

Ocular and retinal morphology was quantified with Neurolucida (Microbrightfield, Colchester VT). The retinas (patch N = 7; aspiration N = 10) of the experimental and control eyes were reconstructed, and divided into laminar and non-laminar categories, based on morphological examination. The laminar tissue consisted of retinal regions with mature photoreceptors and normal retinal stratification and included: (1) normal or original tissue; (2) advanced regenerated tissue; and (3) mature CMZ generated tissue. The non-laminar categories consisted of retinal regions that appeared either abnormal or immature and were divided into three categories: (1) immature—containing some retinal stratification but lacking mature photoreceptors; (2) abnormal—multiple and/or inverted retinal layers; and (3) missing—devoid of retinal tissue (Figs. 1, 2).

A minimum of five representative radial sections, spaced at least 100 µm apart, from each half of each eyecup were reconstructed. Radial sections from the contralateral control eye were reconstructed from approximately the same position as the experimental eye. The retinal outline was traced and the length (µm) that each morphological category occupied on the radial section was calculated with the Neurolucida program MORPH (Microbrightfield). The CMZ contribution for the experimental eye was calculated by measuring the retinal length, distal to the last transretinal fusion (defined in results; Fig. 1a). Because control eyes lacked a distinct morphological marker such as a transretinal fusion, the margin was calculated by subtracting the average retinal lengths of control eyes at the beginning of the study, from the average lengths during the course of the study. Light micrographs were made with a Kodak DSC-200 digital camera (Rochester, NY), imported into Photoshop (Adobe, San Jose, CA) and printed with a Kodak XLS-8300 printer without alteration.

Results

Surgery

Both surgical methods proved successful at avoiding the optical side effects previously experienced with cytotoxic lesioning, and pupil diameter and lens clarity were unaffected. Only a few fish (3%) manifested post-operative complications such as infection or fungal growth around the incision and were excluded from the study. Some experimental eyes did experience post-operative shrinkage, primarily the eyes with greater percentages of retina aspirated (≥75%). However, average eye diameter remained within 10% of the control eye.

Surgery did not adversely affect goldfish health, because average survival time of the experimental fish paralleled both unoperated and sham control groups, with several control and experimental
goldfish surviving until the conclusion of the study (580 days). However, surgery negatively impacted the sunfish because postoperative fish exhibited increased aggressive behavior. This resulted in a high number of post-operative deaths, and necessitated that surviving fish be maintained separately for the duration of the study. Even following complete healing of the incisions, sunfish with diminished visual function that had previously cohabited, could not be placed in the same aquarium without fatal implications. This contrasted with sham operated or control groups that could be routinely maintained in schools of five. These post-operative complications resulted in lower numbers of experimental sunfish than goldfish.

Morphology

Regenerated tissue was morphologically distinct from control tissue by slight “imperfections” in the retinal anatomy. The most noticeable difference was small, laminar fusions (Raymond et al., 1988; Hitchcock et al., 1992) that transversed the inner nuclear layer and ganglion cell layers. These areas may denote the union of disparate tissue such as regenerating tissues joining from two foci, merging with original tissue, or with the CMZ. Tissue that was similar in appearance to controls, free of laminar fusions, situated near the retinal border, and bounded medially by a terminal fusion marker, was considered margin generated. These CMZ areas could be only accurately distinguished in the experimental eye when contiguous with regenerating areas (Figs. 1, 2). Table 1 summarizes the morphometrics of the reconstructed retinas.

Patch extirpation

Regenerated retina following patch extirpation (>120 days) showed normal retinal stratification interspersed with laminar fusions at the junction of the surgical incisions. Marginal growth was only observed in areas contiguous with original or regenerated tissue indicating the CMZ was removed with the neural retina. Retinal regeneration into the extirpated zones was completed by day 240, and the regenerated areas appeared free of abnormalities. Total retinal area was within 2% of controls with the regenerated area consisting of 13.2 ± 2.0% of the area of the experimental retina (Table 1). Fig. 1 shows a light micrograph montage of a radial cross-section through a regenerated area 270 days following patch removal. The section was taken from the dorsal portion of the nasal hemisection, close to the nasal-temporal border. Margin generated tissue is visible to the left of the terminal laminar fusion (arrow a). The regenerated area exhibited normal laminar organization, and contained only a single laminar fusion (arrow b) within its borders. The ventral fusion denoted the merger of the regenerating retina with the original retinal tissue, which is visible to the right of the arrow (arrow c).

Aspiration

In contrast to the orderly appearance of the regenerated retina following patch extirpation, the morphology of the advanced stage aspirated retina displayed greater variation, and was characterized by retinal tissue in several stages of development (Fig. 2). In reconstructed aspirated retinas (N = 10, average time 272 days post-surgery), approximately 2.0% of the retina was devoid of neural tissue, and an additional 16.9% of the retina was incomplete or abnormal. The incomplete areas were characterized by inner layers lacking mature photoreceptors, whereas the abnormal retinas included circular clusters of photoreceptors termed “rosettes” (Fig. 2C; del Cerro et al., 1992), significantly thickened portions of neural retina, or detached areas. Thus, an average of 18.9% of the experimental retina was lacking mature photoreceptors or normal stratification and was considered unlikely to contribute to the b-wave of the ERG.

Areas characterized by normal retinal stratification with mature photoreceptors were observed contiguous and expanding outward from the non-extirpated tissue, with 42.3 ± 14.7% of the experimental eye composed of regenerated tissue. CMZ growth was limited to areas contiguous with original or regenerated tissue indicating the CMZ was removed with the neural retina. The average amount of functional retina contributed by the CMZ was 7.8 ± 2.4%. Total functional area (original, regenerated, and margin tissue) comprised 81.1% of the area of the experimental eye, and total retinal (laminar and non-laminar) area of the experimental eye averaged 85.1 ± 6.7% of controls (Table 1).

Electrophysiology

Patch extirpation

ERGs were detectable in excised retinas (N = 8) three weeks following surgery (earlier recordings were not attempted to allow the incision to heal) (Fig. 3). The initial goldfish ERGs were less than expected based on tissue extirpation estimates (15 to 25%), because b-wave amplitudes were approximately 64.2 ± 6.1% of controls three weeks after surgery. The b-wave amplitude showed a moderate increase through day 108, before increasing to 91.0 ± 2.8% of control amplitude by day 225. Continual improvement was noted through day 570 with the experimental eyes in surviving fish (N = 2) indistinguishable from controls (98.0 ± 2.0). Patch extirpation also was used to remove approximately 20% of the sunfish retina (N = 5). Sunfish b-wave amplitude was reduced to 59.8 ± 7.9% of controls, 21 days following surgery, before increasing to 84.3 ± 6.0% by 35 days. At the 175-day mark, the
The b-wave amplitude of the experimental eye had recovered to 93.7 ± 3.1% of the control eye, which was comparable to goldfish recovery (91%) at the same stage (Fig. 3).

**Retinal aspiration**

Fig. 4 plots the percent recovery of b-wave amplitude of the experimental eye compared to its intrafish control eye, versus time (days) after aspiration of goldfish (50% to 100%) and sunfish (50%) retina. Regeneration was not observed in goldfish (N = 15) following complete removal of the retina during the study. Goldfish (N = 8) with 95% of the neural retina removed, displayed very small b-waves at day 52, with amplitudes increasing to 11.1 ± 4.4% of controls by day 210 and averaging 26.0 ± 1.9% of controls by day 270. Removal of 75% of the goldfish retina (N = 12) initially reduced b-wave amplitude to 13.0 ± 6.9% of controls, however b-wave amplitude increased to 40.2 ± 7.9% by day 102, and 64.0 ± 12.7% of controls by nine months. Extirpation of 50% of the goldfish retina (N = 10) initially reduced b-wave amplitude to approximately a third of controls. These retinas displayed steady and continued improvement throughout the study, attaining 70.1 ± 3.0% of control amplitudes at 270 days. Removal of half the sunfish retina (N = 5) reduced b-wave amplitude to 40.4 ± 10.0% of the control eye, before recovering to 74.7 ± 2.8% of controls by 210 days (Fig. 4).

Fig. 5 plots the b-wave amplitude (µV) of the control and experimental eye versus time (days) following 75% retinal removal. The mean amplitude of the control b-waves increased approximately 20% during the experiment. Retinal aspiration initially reduced b-wave amplitude to 9.9 ± 2.1 µV or approximately 15% of controls. The mean b-wave amplitude of the experimental eyes increased to 68.0 ± 6.9 µV by day 240, but remained below pre-surgical level.

The percentage of laminar (original, regenerated, and CMZ tissue) and nonlaminar (partial, abnormal, and missing tissue) retina in the experimental eye was calculated, and compared to total retinal area of controls. These values were then plotted against the percent recovery of the experimental b-wave amplitude. There was a linear correlation ($r^2 = 0.79$) between the percentage of laminar retinal area and b-wave amplitude (Fig. 6).

**Spectral sensitivity**

The dark-adapted spectral sensitivity of several (N = 4) fish with 75% retinal aspiration following recovery of least 60% (range 60 to 72%) of b-wave amplitude (at 550 nm) was tested. The irradiance (photons s$^{-1}$ cm$^{-2}$) required to induce a 15 µV criterion response for each wavelength was plotted versus the wavelength (nm) (Fig. 7). The experimental eyes were approximately 40% less sensitive at 550 nm than controls which correlates with the percentage of b-wave recovery. The composition of the regenerated retina was similar to controls.

**Dorsal light reflex**

Post-operative fish swam with the control eye positioned away from downwelling light. The deviation from the fish’s normal upright position was correlated with the b-wave recovery in the...
experimental eye. Fig. 8A plots the DLR in degrees of vertical deviation versus percent recovery of the b-wave amplitude. Goldfish exhibited a pronounced 18° to 30° deviation following complete aspiration of the retina, and this behavior persisted throughout the study. The degree of recovery of the b-wave amplitude was correlated to the degree of inclination of the DLR. Following aspiration of 75% of the retina, recovery of 20% to 30% of b-wave amplitude reduced the inclination to 10.7° ± 1.2°. B-wave recovery of 40% to 60% produced an average tilt of 4.3° ± 0.8°, whereas 60% to 80% recovery was correlated with a vertical deviation of 3.3° ± 1.2°. The patch excised goldfish initially showed only a minimum deviation (≤5°, 28 days), which was eliminated after b-wave recovery of 95% of controls. Retinal extirpation had a much more profound effect on sunfish equilibrium. Aspiration of 50% of the neural retina caused a DLR of up to 38°. This exaggerated behavior persisted until b-wave function reached approximately 40% of controls when the inclination dropped below 10°. Recovery of 70% to 80% of b-wave amplitude virtually eliminated the tilt (Fig. 8B).

Discussion

The goal of this study was to determine the functional recovery of regenerated retinal tissue following surgical lesioning, and circumvent the cytotoxic effects of eye shrinkage and cataract formation. Because cytotoxically lesioned retina displayed comprehensive morphological regeneration, but regained only 40% to 50% of b-wave amplitude, it was hypothesized that lens occlusion and tissue shrinkage were partly responsible for reduced sensitivity of the regenerated retina (Mensinger & Powers, 1999). Greater visual sensitivity (b-wave amplitude) was achieved than following cytotoxic lesioning. However, surgical extirpation fell unexpectedly short of achieving parity with control eyes during the 280 day window established for regeneration following complete cytotoxic lesioning (Raymond et al., 1988). The following analysis explains the mechanisms behind the unexpected slower recovery rates and associated reduced visual sensitivities following surgical lesioning.

ERG analysis

The ERG was chosen to assess visual function, because it allowed repeated sampling over time, and analysis of the establishment of photoreceptor synapses with the inner retina (b-wave) (Mensinger & Powers, 1999). It had been predicted that regeneration would transpire between the 140 days required for small patch regeneration (Hitchcock et al., 1992), and the 280 days necessary for...
regeneration following complete cytotoxic lesioning (Raymond et al., 1988; Mensinger & Powers, 1999). However, retinal area following patch extirpation did not become statistically indistinguishable (t-test; \( P > 0.20 \)) from controls, until approximately 270 days after surgery. Regeneration lagged further behind in eyes subjected to 75\% aspiration, because these retinas continued to contain incomplete areas, and attained only 85\% of control retinal area by 270 days.

The slower regeneration rates were caused by the different retinal responses after injury. Previous studies (Hitchcock et al., 1992) demonstrated that after surgical removal, neuroepithelial-like dividing cells cluster at the wound edge, and form a “blastema.” It was postulated that the rod precursor or stem cells on the edge of the wound are stimulated to become pluripotent, and generate all classes of retinal neurons (Raymond & Hitchcock, 2000; Otteson et al., et al., 2001; Wu et al., et al., 2001). The blastema at the wound edge contains this “embryonic zone,” and it slowly migrates into the wound from the edges, leaving behind a trail of regenerated tissue (Cameron & Easter, et al., 1995). In the present study, histological examination confirmed that following surgery, only the wound edge was actively regenerating. The absence of regenerating foci in surgically excised or aspirated areas noncontiguous with the migrating blastema indicated that the pluripotent cell population had been removed along with the neural retina. Additionally, both surgical techniques either removed or damaged the CMZ, because retinal tissue was not observed in areas noncontiguous with other regenerating tissue. Therefore, regeneration was limited to the rate that the blastema would migrate into the excised retina. This contrasted with the multiple foci of the cytotoxically lesioned retina, which because of their greater number and wide spread distribution, allowed regeneration to proceed more rapidly.

The current model for fish retinal regeneration is that stem cells in the inner nuclear layer (INL), initiate regeneration (Julian et al., et al., 1998; Otteson et al., et al., 2001; Wu et al., et al., 2001; reviewed in Hitchcock et al., et al., 2004). However, the cellular origins of the regeneration still remain to be confirmed. Recent studies have suggested that Müller glial cells may serve as stem or precursor cells for retina regeneration in the fish (Yurco & Cameron, 2005) and the chicken (Fischer & Reh, 2001). Whatever the mechanism, surgical removal of the neural retina limits regeneration to the edge of the wound margin. Although transdifferentiation of retinal pigmented epithelium (RPE) can result in retinal regeneration in amphibians and recent studies have detailed the process in the newt (Mitsuda et al., et al., 2005) and frog (Yoshii et al., et al., 2007), there has been no evidence of transdifferentiation in fish. The absence of regenerating loci that was not in contact with original or regenerating tissue indicates that the RPE was not the source of retinal regeneration in the fish.

**Origin of b-wave amplitude increase**

Following 75\% aspiration, regenerated and original tissue accounted for approximately 80\% of total retinal area of the experimental eye at day 270. The b-wave amplitude in control eyes increased 20 \( \mu \)V (20\%) during the study. This was attributed to natural margin addition and central neurogenesis of rod photoreceptors. However, experimental b-wave amplitudes displayed a six-fold gain to approximately 60 \( \mu \)V during this period. Thus, margin addition and central neurogenesis in the intact retina was insufficient to account for the gains in the experimental eye and the regenerated retina was the main source for the increase in b-wave amplitude.

However, despite the steady increase in absolute sensitivity, experimental b-waves were only 55.4\% of control b-waves at 270 days, despite 81\% of the experimental eye containing normal, stratified retina. Although there may be several explanations, such as the morphological differences in the regenerated tissue, we believe that the reduced sensitivity may be attributed to our methodology. The intrafish b-wave comparison proved to be an effective method in the cytotoxic lesioning study (Mensinger & Powers, 1999), because the marginal growth zone continued to expand equally in the two eyes, canceling its contribution to the ERG, and allowing a direct comparison of original and regenerated tissue. In the present study, margin addition was restricted in the experimental eye to areas contiguous to original or regenerated tissue. Thus removal of the marginal growth zones in the experimental eye, contributed to lower than expected experimental/control ratios. To more accurately assess the b-wave sensitivity of the regenerated retina, the relative retina area (experimental/control) was multiplied by the laminar, and presumably functional area of the experimental eye. The predicted maximum b-wave amplitude of the experiment eye was 58\%, which is approximately the b-wave amplitude (54\%) determined in the experimental eye (Table 1). Therefore it appears that the sensitivity of the regenerated tissue is close to normal and the regenerated retina is functional.

**Other mechanisms**

Other factors may have also contributed to the reduced sensitivity of the experimental eye. Although presurgical dark adaptation facilitated separation of the retina from the underlying pigment epithelium, small areas of the pigment epithelium may have been removed or damaged, impeding regeneration into these areas. This is consistent with previous studies, which showed that intact PE was necessary for teleost retinal regeneration (Schmidt et al., et al., 1978; Knight & Raymond, 1994). Additionally, abnormalities such as areas of detached retina, abnormally enlarged layers and circles or “rosettes” of photoreceptors (del Cerro et al., et al., 1992) were noted in the aspirated eyes. Although several of these anomalies had well defined photoreceptors, it was unlikely they possessed the proper neural connections to contribute to the b-wave. Our methodology also did not quantify other morphometric features that could have also accounted for reduction in b-wave amplitude of the experimental eyes such as: (1) lower bipolar cell density; (2) lower number of synapses; (3) delay in synapse formation.

**Spectral sensitivity**

Previous studies have shown that the regenerated retinas can contain an abnormal photoreceptor mosaic (Cameron & Easter, 1995) and that cell-type-specific tangential arrays are not restored especially for cone photoreceptors (Vithellic & Hyde, 2000; Cameron & Carney, 2004; Stenkamp et al., et al., 2001; Stenkamp & Cameron 2002). Although the current study did not address the spatial re-organization of the photoreceptors, the spectral sensitivity of the regenerated retina was determined. The regenerated retina was reduced in sensitivity because of lower percentages of functional tissue; however its spectral sensitivity was similar to controls indicating that the relative ratio of photoreceptors and visual pigments was restored. It has been hypothesized that visual pigment distribution after regeneration enables restoration of normal spectral sensitivity (Cameron et al., et al., 1997).
Behavior

The ERGs indicated that the regenerated retina was functional, however the question remained whether central connections were established, and if the new tissue could mediate normal visual function. The dorsal light reflex was used to assess behavioral function; it provided a simple, natural noninvasive test for the establishment of central connections (Silver, 1974; Powers, 1978; Lin & Yazulla, 1994). The DLR has evolved as a camouflage mechanism for pelagic fish acting as a form of counter illumination (Walls, 1967). Fish will tilt their vertical axis in reference to solar position throughout the day, to align the lighter underside with downwelling light to minimize their silhouette to ventrally situated predators. When the visual input to one eye is interrupted or constricted, the fish will adjust its vertical position accordingly, in an attempt to balance the light input to both eyes. The DLR proved an accurate measure, as morphological differences in retinal area as small as 10% were detectable. As regeneration progressed, the postural imbalance was reduced indicating that central connections were being reestablished. This reduction was a product of synaptogenesis and not habituation, because the tilt persisted in animals with permanent retinal damage.

In summary, the retina will regenerate following surgical removal of up to 95% of the neural retina. Regeneration commences at the edge of the wound, and slowly continues over the extirpated area. Visual function was restored, and the spectral sensitivity of the retina matched the control eye. The fish could mediate at least rudimentary visual behavior with the regenerated tissue.

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