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Metabolic activity in optic tectum during regeneration of retina in adult goldfish

PETER MELZER AND MAUREEN K. POWERS

Department of Psychology and Vision Research Center, Vanderbilt University, Nashville

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Abstract

Retinal and visual function returns following retinal destruction by ouabain in adult goldfish (*Carassius auratus*). Although the precise cellular mechanisms are unclear, the ability to regenerate CNS neurons and connections that subsequently sustain visual behavior is remarkable, especially for an adult vertebrate. In this paper, we ask whether visual stimulation *via* new retinal cells can activate existing cells in the optic tectum, which normally receives the largest retinal projection in this species. The right eyes of adult goldfish were injected with ouabain. After 1–18 weeks the conscious, freely moving fish were exposed to spatially and temporally varying visual stimuli and the resulting tectal metabolic activity was determined with the autoradiographic deoxyglucose method. In normal controls without lesions, visual stimulation produced equally strong metabolic activity in both tectal hemispheres, peaking in the layer where most retinotectal projections terminate ($N = 6$). One week after ouabain injection, metabolic activity in the contralateral, deprived tectum was dramatically reduced ($N = 5$), closely resembling the effect of unilateral ocular enucleation ($N = 5$). However, 9–18 weeks after ouabain injection, metabolic activity in the deprived tectum recovered to a level that was statistically indistinguishable from normal controls ($N = 6$). These findings suggest that, after a comprehensive cytotoxic lesion of the retina, regenerated ganglion cells not only establish new connections with the preexisting optic tectum, but also effectively transmit visual information they receive from newly generated photoreceptors to the "old" tectum.

Keywords: Regeneration, Ouabain, Retina, Goldfish, Tectum, Deoxyglucose

Introduction

The teleost fish retina continues to add new functional neurons throughout life (Johns & Easter, 1977; Johns & Fernald, 1981; Falzett et al., 1988; Powers et al., 1988). New neurons are also produced in response to retinal damage (Lombardo, 1968). For example, exposure to the cytotoxin ouabain leads to severe neural degeneration, yet the retina is capable of completely reconstituting itself from surviving stem cells (Maier & Wolburg, 1978; Raymond & Hitchcock, 1997). Remarkably, the newly regenerated ganglion cells reconnect with the already existing adult optic tectum (Stuermer et al., 1985), the photoreceptors' visual pigments return (Cameron & Powers, 2000), the electroretinogram is restored (Mensinger & Powers, 1999), and several visual behaviors reappear (Lindsey et al., 1995; Mensinger & Powers, 1999).

To support visual behavior, regenerated ganglion cell axons must develop functional connections with more proximal neurons in the visual pathway. Here we ask specifically whether a newly reconstituted goldfish retina establishes functional synapses with its primary projection site, the optic tectum. If not, then connections to other, smaller visual areas must sustain the recovery of visual behavior that is known to occur.

To address this question, we used the autoradiographic deoxyglucose method (Sokoloff et al., 1977) to measure tectal metabolic activity induced by visual stimulation in goldfish with both normal and regenerating retinal projections to separate hemispheres. Preliminary findings have appeared elsewhere (Powers & Melzer, 1995; Powers et al., 1996).

Methods

Goldfish (*Carassius auratus* 4–6 cm sbl) were maintained under standard laboratory conditions (Mensinger & Powers, 1999), anesthetized in 0.04% tricaine methanesulfonate solution (MS 222; Sigma Chemical Co., St. Louis, MO), and revived by flowing aerated fresh water over the gills after all surgical survival procedures. Eleven fish had 1–2 μl 0.4 mM ouabain (Sigma) injected into the vitreal chamber of the right eye through an incision in the ventral limbus (intraocular concentration 30 μM; see Mensinger & Powers, 1999). In five additional fish, the right eye was surgically removed and the eye socket was rinsed with Ringer, filled with Gelfoam (Upjohn, Kalamazoo MI), and covered with a thin layer of cyanoacrylate cement.

Address correspondence and reprint requests to: Peter Melzer, Vanderbilt Vision Research Center, Department of Psychology, 525 Wilson Hall, Vanderbilt University, Nashville TN 37203, USA. E-mail: melzerp@ctrvax.vanderbilt.edu

One week after surgery, the enucleated fish and five of the ouabain-injected fish and were injected intraperitoneally with 2-deoxy-D-[1-^{14}C]glucose (DG; DuPont-NEN, Wilmington, DE; specific activity 50–55 mCi/mmol, dose 120–150 μCi/kg) in 50 μl Ringer solution. Immediately following DG injection, each fish was placed for 1 h into a water-filled 20-cm-diameter glass cylinder surrounded by a moving white cardboard cylinder 27 cm in height, covered with black and white square-wave gratings of varying spatial frequency and orientation under standard laboratory illumination. The stimuli were within the visual range of normal goldfish for such stimuli (DeMarco et al., 1989). Following visual stimulation the fish was deeply anesthetized and pithed, and the eyes and brain were removed and immersed in Bouin's fixative (eyes) or frozen in isopentane at -55°C (brain). Paraffinized retinal sections (7 μm) were stained according to Lee's method (Johns, 1982). Two alternate series of transverse sections (20 μm thick) were cut from each brain in a cryostat at -22°C, mounted on gelatinized slides, dried on a hotplate at 60°C, and autoradiographed along with calibrated [^{14}C]methyl-methacrylate standards on x-ray film (Ektascan EMC-1, Eastman Kodak Co., Rochester, NY) at 5°C. One series was stained for cytochrome oxidase activity (Wong-Riley, 1979); the other was stained with thionin for Nissl substance. The remaining six ouabain-injected fish underwent the same procedure either 9.5, 10, 11, 14, or 18 weeks after injection. Six additional fish without lesions served as normal controls.

Images of DG autoradiograms were digitized with a video camera and examined with NIH-IMAGE (W. Rasband, NIMH, USPHS, Bethesda, MD). Optical density (OD) profiles were recorded across all layers in both tectal hemispheres. Metabolic activity was determined by measuring the average OD across the entire tectal thickness in six sections that were equally spaced along the rostrocaudal extent of the tectum. Pixel-weighted averages of tracer concentration were computed with calibration curves derived from the co-exposed standards (Sokoloff et al., 1977). The percent tracer concentration in the deprived hemisphere compared with the nondeprived hemisphere was used as a measure of functional impairment and recovery, and differences among experimental groups were assessed by means of a nonparametric analysis of variance, followed by a Dunnett's test (SAS, SAS Institute, Cary, NC).

Results

Compared with untreated retinae (Fig. 1A), ouabain-treated retinae 1 week after injection typically had poor stratification, often combined with fused inner and outer nuclear layers (Fig. 1B). The retina in Fig. 1B was swollen, with a paucity of neurons in general and only a few ganglion cells remaining (arrowheads); the photoreceptor outer segments had completely degenerated.

Among the six long-term survivors treated with ouabain, two fish had retinae that did not regenerate. One had no neurons in about half the retina after 18 weeks (Fig. 1C), and the second was completely devoid of neural tissue after 9.5 weeks (data not shown). The retinae of the other four fish appeared remarkably normal (e.g. Fig. 1D, 14 weeks post-ouabain treatment). Although the inner layers were thin, all layers were present and the photoreceptors appeared to be structurally complete.

In normal controls, visual stimulation produced nearly identical bands of activation spanning the medio-lateral extent of both tectal hemispheres (Fig. 2, CTR). Metabolic activity in these bands peaked ~200 μm below the pia (Fig. 3, CTR). The peak corresponds to the *stratum fibrosum et griseum superficiale*, which is the layer where most retinotectal afferents terminate (Northcutt, 1983; Skeen & Northmore, 1984). Lower metabolic activity spread into the adjacent upper and lower layers, that is, *stratum opticum* and *stratum griseum centrale*, respectively. In addition, a thin separate band of lower activity appeared near the white matter. This band may correspond to the *stratum album centrale*. Activation profiles across strata from both sides were indistinguishable in normal controls (Fig. 3, CTR) and side-to-side differences in DG uptake were negligible (Fig. 4, CTR).

One week after ouabain injection (1^{w} p.o.) or enucleation (1^{w} p.e.) metabolic activity in the contralateral tectum appeared dramatically reduced (Fig. 2, bottom). Activation profiles across the deprived strata were flat (red traces in Fig. 3, bottom). In contrast to the findings of Kageyama and Meyer (1988), no shrinkage of the *stratum fibrosum et griseum superficiale* or decrease in its cytochrome oxidase activity was detected. However, DG uptake in the deprived hemisphere was merely half that of the nondeprived hemisphere (Fig. 4) and this diminution was statistically significant.*

Fourteen weeks after ouabain injection, tectal activation *via* the treated eye, with a regenerating retina, had increased (Fig. 2, 14^{w} p.o.). The OD profiles in both hemispheres were similar, with the visual activation *via* the regenerated retinal eye approaching that through the native connection (Fig. 3, 14^{w} p.o.). In the six fish examined 9 weeks or more after ouabain injection, activation in the hemisphere receiving input from regenerated retina averaged 78% of the activation measured in the hemisphere receiving input from the normal projection (Fig. 4), and was not statistically different from normal controls.*

The level of activation in the hemisphere receiving input from the treated eye appeared to vary with the quality of retinal regeneration. For example, the fish whose retina appears in Fig. 1C had poor retinal structure and low tectal activation (a large side-to-side difference; Fig. 4A), while the fish whose retina appears in Fig. 1D had good retinal structure and high tectal activation (a small side-to-side difference; Fig. 4A).

Discussion

Numerous deoxyglucose studies on a variety of species have demonstrated a loss in tectal metabolic activity after enucleation (Altenau & Agranoff, 1979; Skeen & Northmore, 1984; Gorlick et al., 1984; Finkenstädt et al., 1985; McCulloch et al., 1980; Toga & Collins, 1981; Isseroff & Madar, 1983; Toga, 1987). When enucleation is unilateral, any side-to-side differences in resting activity are probably the direct result of the disruption of retinal input, especially a few days after the lesion (Cooper & Thurlow, 1985). But the side-to-side difference can be greatly enhanced by visual stimulation (McCulloch et al., 1980; Batipps et al., 1981; Gorlick et al., 1984) which evokes discharges of intact visual inputs to the nondeprived side and none to the deprived side (Toga & Collins, 1981; Sokoloff, 1981; Thurlow & Cooper, 1989).

In the goldfish, intraocular ouabain injections had a similar effect as enucleation, surely due to the massive reduction of input

*Analysis-of-variance on percent diminution showed greater variability among treatments than within treatment groups ($F = 9.05$; $P = 0.0007$). Enucleated fish and those examined 1 week after ouabain were significantly different from controls (Dunnett's $T = 2.57$; $P \leq 0.05$). Fish tested >9 weeks after ouabain treatment were not statistically different from controls.

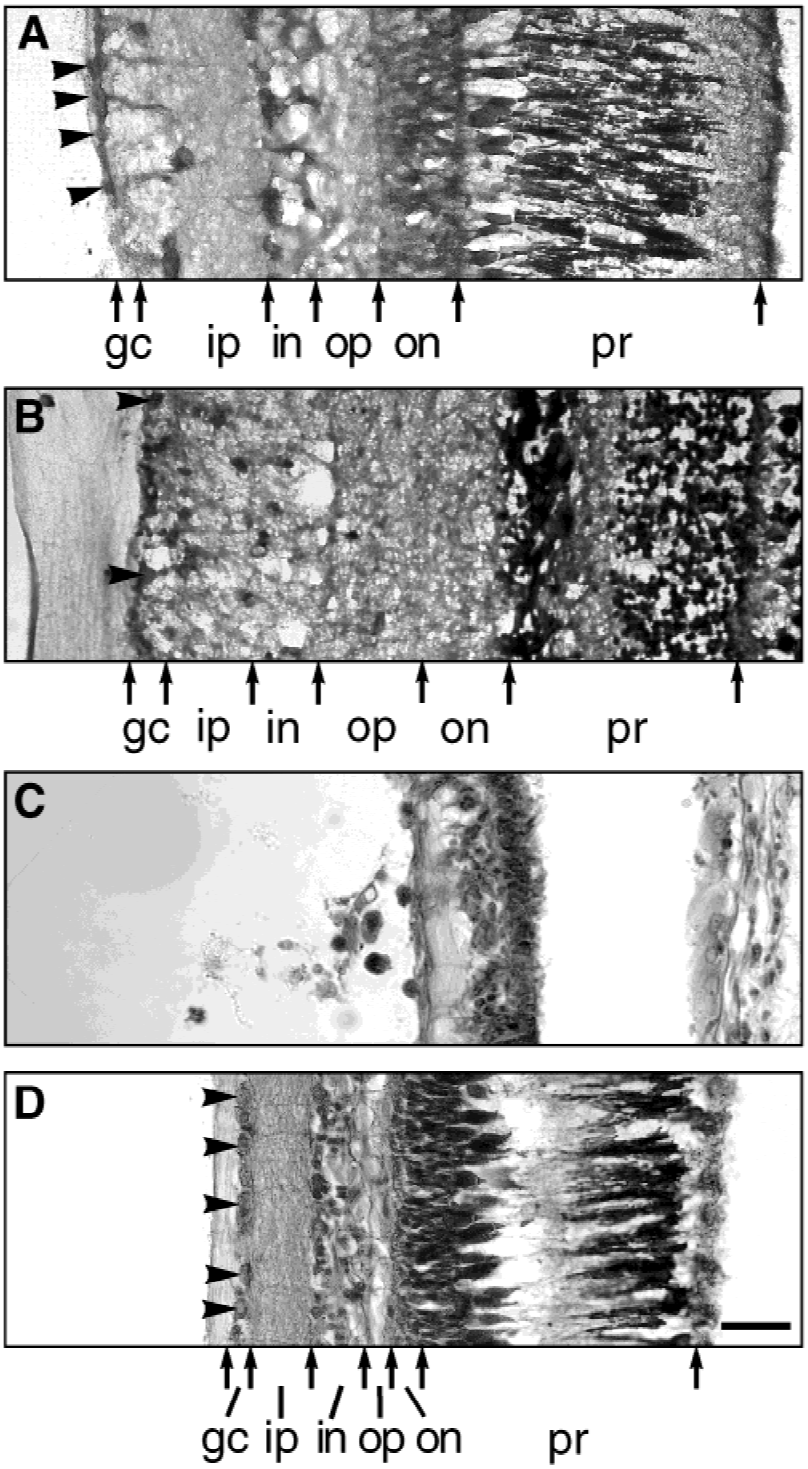

Fig. 1. The retina degenerated and subsequently regenerated following ouabain treatment. Photomicrographs from 7-μm-thick tangential sections cut parallel to the nasal-temporal meridian through the central retina and stained with methylene blue and pararosaniline. Arrowheads point to ganglion cell nuclei. The retina of an untreated eye (A) is compared with retinae one week (B), 18 weeks (C), and 14 weeks (D) after ouabain injection. Note the paucity of ganglion cells during the degenerative phase (B) and the wide extent of retinal reconstitution at long term, ranging from almost none (C) to almost normal (D). Scale bar = 20 μm. gc: ganglion cell layer; ip: inner plexiform layer; in: inner nuclear layer; op: outer plexiform layer; on: outer nuclear layer; and pr: photoreceptor layer.

from the degenerated retina. However, the retina had reconstituted itself in the majority of long-term survivors and tectal metabolic activity recovered nearly 80% of the loss, on average. While it is possible that a minute proportion of the recovered activity may be unrelated to visual input (Zilles et al., 1989), the parallel recovery of tectal metabolic activity and retinal structure strongly suggests that regenerated retinotectal projections do, indeed, develop functional connections with tectal neurons.

Even though functional reconnections are made between retina and tectum, they are apparently incomplete and quite likely abnormal in other ways, at least at the survival times examined in the present study. Lingering abnormalities no doubt contribute to the behavioral deficits that remain after retinal regeneration (Lindsey et al., 1995). Longer survival may improve tectal recovery, and better retinal reconstitution almost certainly will. In accord, a recent study in this laboratory demonstrated a correlation between

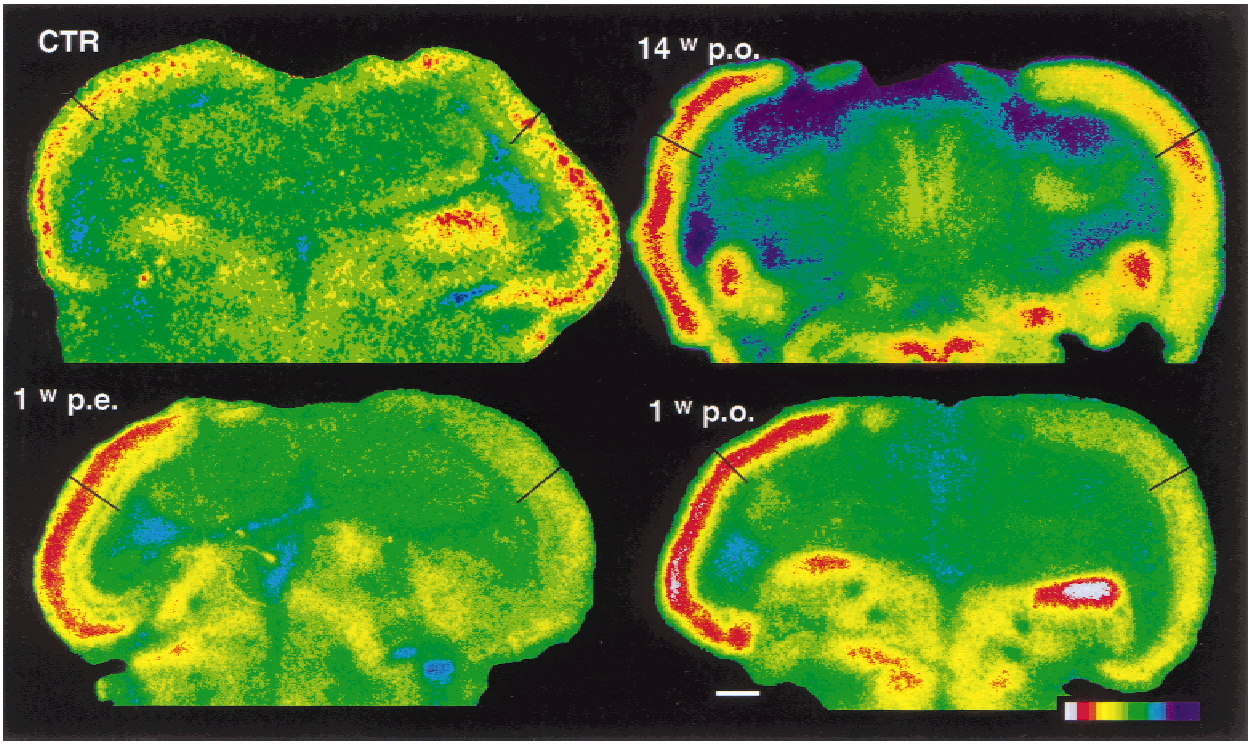

Fig. 2. Visual stimulation initially produced low metabolic activity *via* the damaged retina, but recovery occurred as the retina regenerated. Color-coded images of autoradiograms from 20-μm-thick sections cut transversely through the optic tectum (dorsal is up; the right side of the fish is on the left). After the fish were injected with [^{14}C]deoxyglucose, they were stimulated with black-and-white stripes of varied orientation, direction, and frequency for an hour. Activation patterns are shown for normal controls (CTR), fish examined one week after the right eye was enucleated (1^{w} p.e.), as well as fish examined one week (1^{w} p.o.) and 14 weeks (14^{w} p.o.) after the right eye was injected with ouabain. Blue encodes low and red encodes high metabolic activity (see color bar). The black lines across the tectum indicate the paths along which the optical density profiles of Fig. 3 were taken. Scale bar = 500 μm.

the quality of retinal regeneration and the ability to respond behaviorally to visual stimuli (Powers et al., 1998) suggesting that animals with more highly activated tecta may also perform better behaviorally. Thus, the regeneration of the retina and its connections to the brain need not be perfect for the animal to see, but there does appear to be an association between the overall quality of regeneration and the level of tectal activation.

Anatomical tract-tracing and single- and multi-unit studies will be necessary to determine whether the regenerated projections terminate retinotopically in the tectum, and the extent to which retinal or tectal receptive fields are "normal." Regardless of the precise mechanisms involved however, the return of any level of visual function in a previously blind animal is remarkable and deserves further study.

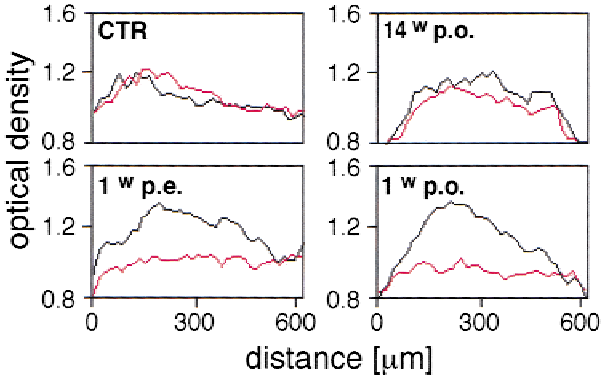

Fig. 3. All tectal layers recovered metabolic activity. Mean optical densities were recorded along five pixel-wide paths from pia to white matter (see Fig. 2 for path locations). The red traces were taken across the left hemisphere, that is, contralateral to the lesion. The black traces were taken across the right hemisphere, which received input from the untreated eye.

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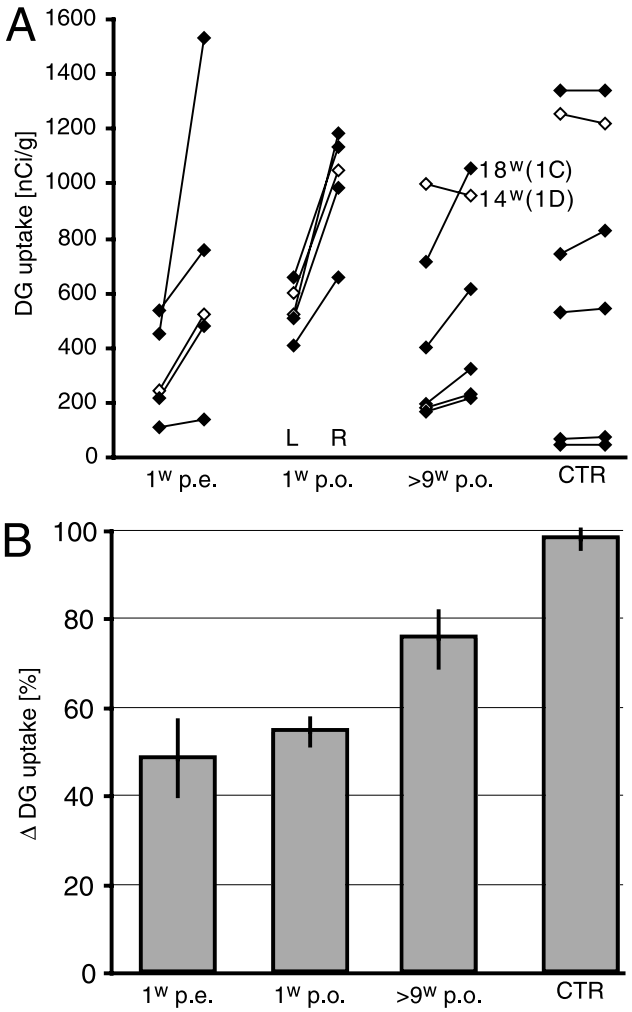

Fig. 4. Metabolic activity in the tectum contralateral to the lesion (L) approached that on the ipsilateral, intact side (R), especially in fish with extensive retinal regeneration. A: Absolute tectal metabolic activity. Each pair of diamonds plots the mean deoxyglucose (DG) concentrations in the left (L) and the right (R) hemisphere for each fish. The unfilled diamonds identify DG uptake in fish whose activation patterns are illustrated in Figs. 2 and 3. Additional labels identify the data from the 18-week fish with poor retinal regeneration (Fig. 1C) and the 14-week fish with good retinal regeneration (Fig. 1D). B: The tectal lobes receiving input from regenerating retinas attained 78% of the activity level on the intact side, on average. Mean DG uptake on the deprived side is plotted as a percentage of DG uptake on the nondeprived side. Scale bars = 1 SEM.

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