Neurogenesis Is Required for Behavioral Recovery After Injury in the Visual System of *Xenopus laevis*

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ABSTRACT

Nonmammalian vertebrates have a remarkable capacity to regenerate brain tissue in response to central nervous system (CNS) injury. Nevertheless, it is not clear whether animals recover lost function after injury or whether injury-induced cell proliferation mediates recovery. We address these questions using the visual system and visually-guided behavior in *Xenopus laevis* tadpoles. We established a reproducible means to produce a unilateral focal injury to optic tectal neurons without damaging retinotectal axons. We then assayed a tectally-mediated visual avoidance behavior to evaluate behavioral impairment and recovery. Focal ablation of part of the optic tectum prevents the visual avoidance response to moving stimuli. Animals recover the behavior over the week following injury. Injury induces a burst of proliferation of tectal progenitor cells based on phospho-histone H3 immunolabeling and experiments showing that Musashi-immunoreactive tectal progenitors incorporate the thymidine analog chlorodeoxyuridine after injury. Pulse chase experiments indicate that the newly-generated cells differentiate into N-β-tubulin-immunoreactive neurons. Furthermore, in vivo time-lapse imaging shows that Sox2-expressing neural progenitors divide in response to injury and generate neurons with elaborate dendritic arbors. These experiments indicate that new neurons are generated in response to injury. To test if neurogenesis is necessary for recovery from injury, we blocked cell proliferation in vivo and found that recovery of the visual avoidance behavior is inhibited by drugs that block cell proliferation. Moreover, behavioral recovery is facilitated by changes in visual experience that increase tectal progenitor cell proliferation. Our data indicate that neurogenesis in the optic tectum is critical for recovery of visually-guided behavior after injury. J. Comp. Neurol. 521:2262–2278, 2013.

INDEXING TERMS: retinotectal system; optic tectum; brain damage; visual avoidance behavior; cell proliferation; stem cell; neural progenitor cell; musashi; PH3; N-β-tubulin; injury response

Pediatric brain injuries can have catastrophic effects depending on the type and location of the injury and the age at which it occurs. Although the developing nervous system displays a greater capacity for recovery after penetrating injury than the adult central nervous system (CNS) (Mackerle and Gal, 2009; Kazim et al., 2011; Plantman et al., 2012), the cellular mechanisms contributing to recovery are not clear. Few experimental models exist to study penetrating brain injury in developing systems (Cernak, 2005; Statler et al., 2011), particularly those that assess a functional behavioral recovery after injury. We were interested in establishing a model to study cellular mechanisms of response to brain injury which would also assess recovery of behavior in intact animals.

The developing nervous system can respond to injury with an increase in endogenous neurogenesis; however, the ability to engage endogenous neurogenesis to regenerate lost brain tissue after damage depends on the type of injury, species, and the age at injury (Magavi et al., 2000; Endo et al., 2007; Ferretti, 2011; Liu and Guthrie, 2011). Studies in developing mammalian systems...
indicate that brain injury can result in increased cell proliferation near the injury site and migration of progenitors and new neurons into the injury site (Sundholm-Peters et al., 2005; Thored et al., 2007; Kim and Szele, 2008); however, survival of newly-generated neurons in the mammalian CNS is extremely low (Thored et al., 2007). Studies on recovery from brain injury have benefited from classical work on nonmammalian vertebrate species, which are capable of axon regeneration, synaptogenesis, and, in some species, the regeneration of brain tissue in response to injury (Endo et al., 2007; Zupanc, 2009). In anurans, such as *Xenopus*, surgical brain lesions trigger proliferation of cells in the ventricular zone (Endo et al., 2007). The proliferative response to injury varies across brain regions and decreases with development from tadpoles to froglets (Filoni et al., 1995). Despite evidence for CNS regenerative capacity in amphibians, it is still not clear whether animals recover lost function after injury or whether injury-induced cell proliferation mediates recovery. We set out to address these questions using the visual system and visually-guided behavior in the *Xenopus laevis* tadpole.

Work in frogs has revealed many mechanisms fundamental to brain development, neuronal plasticity, and learning and memory, which subsequently have been shown to function in mammalian systems, including humans. The frog tadpole offers many experimental advantages for studying mechanisms underlying recovery from developmental brain injury. In particular, tadpoles exhibit optic tectum-dependent visually-guided behavior (Dong et al., 2009; Shen et al., 2011), which we use to assess behavioral recovery from injury. The optic tectum is the primary visual center in nonmammalian vertebrates, integrating multisensory information and governing motor output. Previous work has shown that the optic tectum mediates visual avoidance responses postulated to be required for tadpole survival (Dong et al., 2009; Shen et al., 2011); however, it is not yet clear whether tectal damage in *Xenopus* tadpoles results in a deficit of visual avoidance behavior, whether tadpoles recover the visual avoidance behavior after damage, or whether damage to the tectum induces neurogenesis that is required for recovery of function.

Neurogenesis in the optic tectum occurs in the ventricular proliferative zone throughout larval stages in tadpoles (Straznicky and Gaze, 1972). Newly-generated cells differentiate into neurons and are incorporated into the retinotectal circuit (Gaze et al., 1979). More recent work in our laboratory using incorporation of thymidine analogs such as bromodeoxyuridine (BrdU) (Sharma and Cline, 2010) or in vivo time-lapse analysis of neural cell lineage (Bestman et al., 2012) has shown that cell proliferation and differentiation of progenitor cells in the optic tectum are regulated by visual system input to the tectum. Specifically, we showed that 2 days of visual deprivation causes neural progenitor cells to continue dividing and therefore expands the neural progenitor pool in the optic tectum, whereas visual experience promotes the differentiation of progenitors into neurons (Sharma and Cline, 2010; Bestman et al., 2012). Here we tested whether manipulating neurogenesis through visual experience might affect recovery of visual system function following injury.

### Materials and Methods

#### Animals

*Xenopus laevis* tadpoles of either sex (bred in-house or purchased from either Nasco, Fort Atkinson, WI, or Xenopus Express, Brooksville, FL) were reared in 0.1× Steinberg’s Solution at 22°C with a 12-hour light/dark cycle, unless otherwise noted. All animal protocols were approved by the Institutional Animal Use and Care Committee of the Scripps Research Institute. For visual deprivation experiments, animals were housed in a light-impermeable compartment at 22°C immediately following surgery. After 48 hours, animals were tested for visual avoidance behavior, as described below, then housed in standard 12-hour light/dark conditions until the end of the experiment. All animals were anesthetized in 0.02% MS222 (3-aminobenzoic acid ethyl ester, Sigma, St. Louis, MO) before surgical procedures, and were terminally anesthetized in 0.2% MS222 at the end of the experiment.

#### Visual Avoidance Behavior

We assessed visual avoidance behavior using an assay modified from Dong et al. (2009), as described in Shen et al. (2011). Stage 47 (Nieuwkoop and Faber, 1956) animals were screened for the optomotor response (OMR) to evaluate general health (Roeser and Baier, 2003; Dong et al., 2009; Portugues and Engert, 2009; Shen et al., 2011). Only animals that exhibited a normal OMR were included in visual avoidance assays. Four to five tadpoles were placed in a clear Plexiglas tank fitted with a translucent sheet of 3M (St. Paul, MN) projector screen. Animals were given 1 minute to distribute within the tank and then visual stimuli were presented for 1 minute using a microprojector (3M, MPro110) positioned below the tank. Tadpoles were visualized with infrared (IR) LEDs and videos of the tadpole movements were captured with a Hamamatsu ORCA-ER digital camera. The entire setup was enclosed within a light-tight compartment. Visual stimuli were generated and presented by a custom-written MatLab code (MathWorks and Psychophysics Toolbox extensions, Natick, MA). Behavioral testing was performed at the same time each day. Videos were analyzed...
post-hoc, frame-by-frame, for encounter events and avoidance responses. Data acquisition and analysis were conducted blind to treatment. An avoidance response was scored when a tadpole displayed a sharp turn within 500 ms of an encounter with a dot moving perpendicularly (within a range of 90° ± 15°) toward the eye. Data are presented as an Avoidance Index, or the fraction of the first 10 encounters with moving dots that results in an avoidance response. The Avoidance Index of trials throughout each experiment was normalized to the Avoidance Index determined before treatment on Day 0, to allow comparison of behavioral results across experiments. Every experiment included intact control animals to control for batch-to-batch variation and overall clutch health. Raw data (nonnormalized values) are presented in Table 2. In most cases, 1 minute of recording an animal’s responses to the moving spot stimulus was sufficient to identify 10 encounters; however, a few animals did not have 10 encounters within the 1-minute test period. For cases with more than 5 but fewer than 10 encounters, the Avoidance Index was calculated as the number of avoidance responses divided by the number of encounters within 1 minute. Animals with fewer than five encounters were excluded from the analysis. Out of the 1,458 behavioral trials from the 261 animals analyzed in this study, only 99 trials showed fewer than 10 encounters during the 1-minute recording period (< 7%). Because the stimulus could appear distorted by the edges of the chamber, animals swimming along the edge were not analyzed until they were one body width away from the edge. The Wilcoxon signed-rank test was used to test whether behavior was significantly different from random baseline nonstimulus turning. Each experiment was repeated three times. Data were determined to be normally distributed, so a Student’s t-test (two-sample, two-tailed) was used to test for significant differences between experimental groups. Because the number of surviving animals can change over the course of the experiment, the data are presented as the group average ± standard error of the mean (SEM).

Microscopy and presentation

For live animal imaging and whole-mount immunofluorescence, confocal stacks were collected on an Ultraview VOX spinning-disk system with Volocity 5 software (Perkin-Elmer, Foster City, CA). The system has a Yokagowa CSU-X1 spinning disk confocal attachment mounted on either a Olympus BX61WI microscope equipped with a 20 × 0.95 NA water immersion objective (Figs. 2B–F, 4, 5), or on a Nikon Eclipse FN1 microscope equipped with a 25 × 1.1 NA water immersion objective (Figs. 2G,H, 8). Green and red fluorescent signals were excited with 488 nm and 546 nm laser lines and differentiated with 515(30) and 615(75) filters, respectively. Vibratome sections were imaged with an Olympus Fluoview FV500 laser-scanning confocal microscope equipped with a 20 × 0.8 NA oil-immersion objective (Figs. 6, 7). Green and red fluorescent signals were excited with 488 nm and 546 nm laser lines and distinguished with 505–525 and 550–600 bandpass filters, respectively. Care was taken to minimize pixel saturation during image acquisition. Single optical sections in Figure 2E,F and 3D confocal projections in Figures 4, 5, and 8 were made using Volocity 3D imaging software. The single optical sections in Figures 6 and 7 were made using Metamorph imaging software. Adobe Photoshop (San Jose, CA) was used to adjust contrast uniformly across timepoints within experiments for Figures 2, 4, and 5. In Figure 8 the brightness of Day 7 image was increased compared to the images from previous days to enhance the appearance of fainter processes at that timepoint. Figures were compiled using Adobe Illustrator.

Surgical injury

Stage 47 tadpoles were screened for the OMR as an indicator of general health (Roesser and Baier, 2003; Dong et al., 2009; Portugues and Engert, 2009; Shen et al., 2011) and then screened for visual avoidance behavior. Only animals that exhibited both behaviors were used in experiments. Animals were first tested on Day 0 for a baseline Avoidance Index (Supporting Movie 1). For surgery, tadpoles were anesthetized with 0.02% MS222 and placed on a Kimwipe moistened with MS222 on a stage of a dissecting microscope. A 1 mm diameter glass micropipette pulled into a fine-tipped needle with a 30–40 μm diameter opening was connected to a vacuum with a mild suction and mounted in a micromanipulator. The micropipette tip was positioned perpendicular to the surface of the caudal region of the right tectal lobe and was advanced into the cell body layer, deep to the neuropil to avoid the retinal ganglion cell (RGC) axons which laminate the superficial layers of the tectum. We applied a brief pulse of vacuum aspiration to remove neuronal cells from the tectum, taking care not to disrupt either the ventricular layer of cells or the RGC axons. Injuries were inflicted only to the right tectal lobe. The extent of injury was determined by imaging the dorsoventral extent of both tectal lobes labeled with a lipophilic dye (1 mM FM4-64, Invitrogen, La Jolla, CA) injected into the brain ventricle. Confocal stacks were collected before and after surgery and the tectal volume was determined using Volocity software. We determined the tectal volumes before and after surgery for a subset of animals in each experiment. After a 2-day rest period, animals were rescreened for the OMR to assess general health, visual response, and motor function. Animals that failed to exhibit an OMR
were not used. Injured animals with a normal OMR were then tested with the visual avoidance assay as described above (Supporting Movies 2 and 3). Note that we designed the analysis such that animals are scored for their first 10 encounters with the visual stimulus, thereby making healthy swimming a requirement for animals to be included in the analysis.

Drug treatments
Cell proliferation was blocked with a bath solution containing 20 mM hydroxyurea and 150 μM aphidicolin (Sigma-Aldrich, St. Louis, MO) (Harris and Hartenstein, 1991). Animals were treated for a 24-hour time period from 24–48 hours after surgery. Control animals were treated similarly with drug vehicle, 0.5% dimethyl sulfoxide (DMSO).

Cell proliferation immunolabeling, and imaging
Proliferating cells were labeled with the thymidine analogs iododeoxyuridine (IdU) for whole-mount single-label, or chlorodeoxyuridine (CldU) for double-labeled sections. Cells were labeled by transferring animals to rearing solution containing 3.8 mM IdU or CldU for 2 hours (Sharma and Cline, 2010). For immunolabeling, tadpoles were terminally anesthetized in 0.2% MS222 solution and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) overnight at 4°C.Brains were dissected, rinsed, blocked, incubated with antibodies, described below, and analyzed in whole-mount or 30 μm horizontal vibratome sections.

For both IdU and CldU labeling, brains were treated with 2N HCl for 1 hour at 37°C, rinsed in PBS-Tween 20 (0.3%), and blocked in 5% normal goat serum in PBS-Tween 20 (0.3%) for 1 hour before incubating overnight at 4°C with antibody. For CldU-labeled sections, dissected brains were embedded in gelatin and cut into 30-μm horizontal sections with a vibratome. Sections were blocked in 5% goat serum and 0.3% Tween 20 in PBS for 1 hour before incubating overnight with antibodies. Detection was performed using Alexa-fluor-tagged secondary antibodies (1:200, Molecular Probes/Invitrogen, Eugene, OR). Sections were mounted in ProLong Gold (Molecular Probes/Invitrogen) and imaged with an Olympus FluoView FV500 laser-scanning confocal microscope as described above. Whole-mount brains labeled with IdU were mounted in Vectashield (Vector Labs, Burlingame, CA) and imaged with an Olympus BX61WI fitted with a Perkin-Elmer Ultraview VOX spinning disk confocal attachment as described above. All samples were prepared, photographed, and analyzed in parallel using the same acquisition and analysis settings. Image analysis and fluorescently labeled cell counts for the whole-mount data (Figs. 4, 5) were done in 3D using Volocity (Perkin-Elmer, Waltham, MA) image processing software to mark cells in optical sections, ensuring each cell was counted only once. Image analysis and cell counts for the CldU double-label experiments in vibratome sections (Fig. 6, 7) were performed using Metamorph (Universal Imaging/Molecular Devices, Downingtown, PA). Images were background subtracted and cells were counted in single optical sections the using the “manually count object” feature of Metamorph as described (Sharma and Cline, 2010). For N-beta-tubulin analysis, counts were made in single optical sections from the top and bottom of vibratome sections due to poor antibody penetration specific to this antibody. Two non-neighboring horizontal sections through each optic tectum were used for analysis. Data were determined to be normally distributed. Either the Student’s t-test (two-sample, two-tailed) or the nonparametric Mann-Whitney-Wilcoxon test was used to compare between groups as stated. Data are represented as mean ± SEM.

Antibody characterization
A list of primary antibodies used in this study is provided in Table 1.

<table>
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<th>Antibody</th>
<th>Manufacturer, catalog number</th>
<th>Dilution</th>
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<td>Phospho-Histone H3</td>
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</tr>
<tr>
<td>IdU</td>
<td>BD Biosciences #347580</td>
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</tr>
<tr>
<td>CldU</td>
<td>Accurate/AbSerotec OBT0030G</td>
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<tr>
<td>Musashi1</td>
<td>Abcam ab21628</td>
<td>1:250</td>
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<tr>
<td>N-beta-tubulin I+II</td>
<td>Sigma T-8535</td>
<td>1:200</td>
</tr>
</tbody>
</table>
**Anti-PH3.**

The antibody against phospho-histone H3 (PH3) was obtained from Millipore (Billerica, MA, Cat. No. 06-570; Journal of Comparative Neurology Database and Neuroinformatics Framework antibody registry #310177). This antibody was raised against the synthetic phosphorylated peptide ARK[pS]TGGKAPRKQLC coupled to keyhole limpet hemocyanin, according to the manufacturer’s information. This sequence corresponds to the N-terminus of human histone H3 (aa 7–20) phosphorylated at serine 10. The manufacturer tested the specificity of this antibody by western blot and found that it recognized a single band of 17 kDa in colcemid-arrested HeLa cells as predicted. This antibody has been reported to detect mitotic cells in several species, including the optic tectum of chick, fish, and *Xenopus*, where staining is concentrated in mitotically active nuclei that line the ventricular layer of the tectum, similar to the expression we report (Tibber et al., 2006; Schmidt and Derby, 2011; Wirsching et al., 2012). We found that the antibody labels select nuclei in the *Xenopus* tadpole tectal proliferative zone that display morphological features of nuclei in various phases of mitosis.

**Anti-IdU.**

The mouse monoclonal antibody against IdU was obtained from BD Biosciences (San Jose, CA, Cat. No. 347580; Journal of Comparative Neurology Database and Neuroinformatics Framework antibody registry #400326). This antibody was raised against 5-iodo-2’-deoxyuridine conjugated to ovalbumin and binds to 5-bromo-2’-deoxyuridine as well as 5-iodo-2’-deoxyuridine, according to the manufacturer’s information. This antibody specifically labels nuclei that have incorporated BrdU or IdU into their newly-synthesized DNA during the S-phase of the cell cycle (Schmidt and Derby, 2011). We have previously shown that this antibody labels cells in the proliferative layer of the *Xenopus* optic tectum in animals that have been exposed to BrdU or CldU (Sharma and Cline, 2010). Additionally, we demonstrated anti-CldU specificity by showing that this antibody shows no labeling in *Xenopus* tadpoles exposed to IdU (Sharma and Cline, 2010), consistent with other studies in the literature (Sullivan et al., 2007).

**Anti-musashi1.**

The rabbit antibody against Musashi1 (Msi1) was obtained from Abcam (Cambridge, MA, Cat. No. ab21628). This antibody was generated against a KLH-conjugated synthetic peptide corresponding to a region within the first 25 amino acids of the human Msi1 protein. On immunoblots of mouse embryonic brain tissues, human neuroblastoma cell lines, and mouse neural progenitor cells, the antibody recognizes a 39 kDa band corresponding to the expected size of Msi1, which can be blocked with the immunizing peptide, according to the manufacturer. In tissues, the antibody shows greater labeling in proliferative cells and less labeling as cells differentiate and mature, and has a similar localization pattern to the 14H1 anti-Msi1 antibody used in Kaneko et al. (2000). The *Xenopus* homolog of musashi1 is 84% identical and 92% similar to the human musashi1 within the first 25 amino acids against which the antibody used here was generated. Our previous work demonstrates that musashi1-immunoreactive cells are present in *Xenopus* in the proliferative layer lining the ventricle of the optic tectum (Sharma and Cline, 2010). Moreover, we demonstrated that morpholino-mediated knockdown of the *Xenopus* homolog of Msi1 results in a significant decrease in Msi1-immunoreactivity compared to controls (Sharma and Cline, 2010). Our current data show an expression pattern similar to what we had previously reported.

**Anti-β-tubulin I+II.**

The mouse monoclonal antibody against β-tubulin I+II was obtained from Sigma-Aldrich (Cat. No. T8535), derived from the JDR.3BB hybridoma cell line originally generated by Banerjee et al. (1988). The antibody was generated against a chemically synthesized peptide corresponding to the carboxy-terminal sequence of the human β-tubulin isotype II (EEREEDDEEA) coupled to bovine serum albumin (BSA), which specifically recognizes an epitope located on the isotypes I and II of human β-tubulin and crossreact with bovine, chicken, mouse, pig, rat, and frog, according to the manufacturer. The antibodies have been used to affinity purify β-tubulin isotypes I+II from chick brain extracts.
In vivo time-lapse imaging of optic tectal neurons

Stage 47 animals were anesthetized in 0.02% MS222, and injured in the right tectum, as described above, and then electroporated with the fluorescent protein (FP) expression construct, pSox2bd::gal4UASturboGFP, described in detail (Bestman et al., 2012). For electroporation, plasmids (0.02 μg/μl) were injected into the midbrain ventricle, and voltage pulses were applied with a Grass SD9 stimulator across the midbrain using platinum electrodes (four pulses of 35V in each polarity) to electroporate cells lining the tectal ventricle (Haas et al., 2002; Bestman et al., 2012). Starting the next day, animals were anesthetized and the left and right optic tectal lobes were imaged once a day over the next 7 days with a Perkin Elmer spinning disk confocal as described above (Bestman et al., 2012).

Retinotectal axon imaging

Animals were subjected to unilateral injury in the right tectal lobe and then euthanized and fixed within 1 hour of injury in 4% paraformaldehyde in PBS. After 24 hours of fixation, retinal ganglion cells were labeled with DiI by pressure-injecting DiI solution (Vybrant DiI from Invitrogen/Life Technologies, V-22885, used according to manufacturers recommendations) into the contralateral retinas of stage 47 tadpoles, as described (Witte et al., 1996). Control animals were uninjured. After 3 weeks tecta were imaged as described above.

RESULTS

Xenopus tadpoles exhibit an avoidance response to moving stimuli

*Xenopus laevis* tadpoles display an innate avoidance behavior to moving objects. This behavior is mediated by the optic tectum, a region of the midbrain that receives and processes visual input. We used a behavior assay modified from Aizenman and colleagues (Dong et al., 2009), described in detail in Shen et al. (2011). Briefly, animals are placed in a clear Plexiglas chamber fitted with a piece of projection film; a projector below the chamber displays a stimulus and a camera above the chamber records both the movement of the animals and the movement of the stimulus (Fig. 1A). The behavior assay tests whether swimming animals avoid visual stimuli by determining their swimming trajectory in response to visual stimuli. Animals turn in response to moving spots of certain sizes that approach their eyes at approximately a right angle. Our analysis scores the first 10 encounters that swimming animals have with the visual stimuli, thereby making healthy swimming a requirement to be included in the analysis. Data are presented as an Avoidance Index, or the fraction of times that individual animals respond to the stimulus out of 10 encounters with the moving spot stimuli. Animals do not react to stationary stimuli (Fig. 1B,i–v) and only change their trajectory in response to an encounter with a moving stimulus (Fig. 1B,vi–x; Supporting Movie 1). Both wildtype and albino tadpoles preferentially avoid moving spots corresponding to diameters larger than or equal to 0.4 cm (Fig. 1C). We therefore presented stimuli of 0.4 cm diameter and used albino tadpoles for all subsequent experiments.

Developing an injury paradigm in the Xenopus optic tectum

We set out to establish an injury model in which postsynaptic cells in the *Xenopus* optic tectum were damaged, and in which we could assess recovery of function using the visual avoidance assay. Complete ablation of the optic tectum prevents the visual avoidance behavior (Dong et al., 2009); therefore, we tested whether partial damage to the tectum, inflicted by gentle vacuum aspiration to remove tectal neurons unilaterally (Fig. 2A) might compromise the visual avoidance behavior. This method causes minimal bleeding, inflammation, infection, and death and results in consistent damage. After surgery, animals swim normally and display an optomotor response (Roers and Baier, 2003; Dong et al., 2009; Portugues and Engert, 2009), indicating that they are generally in good health, that the surgery did not damage...
the retinal ganglion cell outputs in the non-tectally-mediated visual system, and that the animals swim normally. The region of damage could be seen if the brain was imaged with DIC optics immediately after surgery (Fig. 2B,C). To assess the damage in the tectal lobe and to determine whether the uninjured lobe showed volume changes in response to damage to the contralateral tectum, we labeled the brain with the fluorescent membrane dye, FM4-64, and collected confocal images of live anesthetized animals before and after surgery. The surgical injury decreased the volume of the damaged tectal lobe (18.5 ± 1.5%) without changing the volume of the undamaged tectal lobe (Fig. 2D–F). Dil injections into the retina contralateral to uninjured or injured tecta show that the retinotectal axons are not damaged by the surgery (Fig. 2G,H).

Xenopus tadpoles recover visual avoidance behavior

We next tested whether partial tectal injury affected the visual avoidance behavior and whether animals could recover from injury. On the first day of the experiment (Day 0), stage 47 tadpoles were surgically injured by aspirating a focal region of optic tectal cells (black) from the caudal right tectal lobe, caudomedial to the region of retinal axon innervation (gray). B,C: DIC images show the tectal lobe before surgery (B) and after surgery (C). Arrows in C indicate wound area. D: Quantification of the tectal volumes before and after surgery. Surgery decreases the volume of the right tectal lobe without affecting the volume of the left tectal lobe. n = 16 animals, P < 0.01. E,F: Single optical sections of fluorescent FM4-64 membrane dye labeling before (E) and after surgery (F). The tectal lobes are outlined in white. The injury site in the right lobe was between the arrows. G,H: Dil labeling of the RGC axons in intact (G) and injured (H) tecta. Injury does not alter gross morphology of the RGC axon arbors. Scale bars = 100 µm in B–F; 30 µm in G,H.

Figure 1. Xenopus laevis tadpoles exhibit an avoidance response to moving stimuli. A: Visual avoidance behavior apparatus. Animals are placed in the chamber and a moving stimulus is presented from below. Video images are captured from above. B: Still frames of a video sequence showing stage 47 tadpoles’ behavioral response to the upward moving stimulus (66.67 ms/frame, every other frame is shown). Tadpoles do not respond to stationary dots (B, i–iv, left panels) and swim in a straight trajectory, as shown in the drawing (B, v). In contrast, tadpoles exhibit an avoidance behavior in response to moving stimuli (B, vi–ix, right panels) and abruptly change their trajectory upon an encounter with a perpendicularly approaching dot, indicated by arrow in the drawing of the swim trajectory (B, x). Time stamps shown in panels B–ix also correspond to Bvi–xi. C: Both pigmented and albino tadpoles similarly display a strong avoidance behavior to a stimulus size of 0.4 cm in diameter (n = 12 animals each).

Figure 2. Unilateral injury to the optic tectum. A: Schematic of the injury paradigm. Stage 47 tadpoles were surgically injured by aspirating a focal region of optic tectal cells (black) from the caudal right tectal lobe, caudomedial to the region of retinal axon innervation (gray). B,C: DIC images show the tectal lobe before surgery (B) and after surgery (C). Arrows in C indicate wound area. D: Quantification of the tectal volumes before and after surgery. Surgery decreases the volume of the right tectal lobe without affecting the volume of the left tectal lobe. n = 16 animals, P < 0.01. E,F: Single optical sections of fluorescent FM4-64 membrane dye labeling before (E) and after surgery (F). The tectal lobes are outlined in white. The injury site in the right lobe was between the arrows. G,H: Dil labeling of the RGC axons in intact (G) and injured (H) tecta. Injury does not alter gross morphology of the RGC axon arbors. Scale bars = 100 µm in B–F; 30 µm in G,H.
TABLE 2.
Avoidance Behavior Raw Data

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<th>Group</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 3</th>
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<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
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<td>3</td>
<td>Intact</td>
<td>74.1 ± 1.5 (53)*</td>
<td>73 ± 1.1 (42)</td>
<td>74 ± 1.7 (40)</td>
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<td>Sham</td>
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<td>29 ± 1.3 (20)</td>
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<td>56 ± 3.2 (14)</td>
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<td></td>
<td>Injured + CDBs</td>
<td>24.6 ± 1.4 (12)</td>
<td>37 ± 2.4 (9)</td>
<td>48.4 ± 3.4 (8)</td>
<td>57 ± 2.1 (10)</td>
<td>n.t.</td>
<td>66.9 ± 3.4 (4)</td>
<td>n.t.</td>
</tr>
<tr>
<td>9B</td>
<td>Intact</td>
<td>74.7 ± 1.6 (47)</td>
<td>77 ± 1.7 (16)</td>
<td>76.4 ± 1.3 (32)</td>
<td>76.9 ± 2.8 (8)</td>
<td>77 ± 2.7 (11)</td>
<td>77.1 ± 3.6 (7)</td>
<td>74.7 ± 1.9 (19)</td>
</tr>
<tr>
<td></td>
<td>Injured</td>
<td>25.6 ± 1.5 (30)</td>
<td>29.9 ± 1.6 (23)</td>
<td>43.3 ± 3.7 (9)</td>
<td>54.5 ± 2.3 (10)</td>
<td>63.7 ± 2.7 (10)</td>
<td>67.8 ± 1.6 (14)</td>
<td>n.t.</td>
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<tr>
<td></td>
<td>Injured + Dark</td>
<td>28 ± 1.7 (31)</td>
<td>42.6 ± 1.8 (38)</td>
<td>59.6 ± 3.6 (9)</td>
<td>63.4 ± 2.2 (9)</td>
<td>66.2 ± 1.7 (17)</td>
<td>71.1 ± 1.8 (14)</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

Avoidance behavior data for each timepoint and each experiment presented. Data are presented as the average percent avoidance ± standard error of the mean with animal number (n).

*Animals were scored and then divided into groups for subsequent treatments.

Avoidance behavior data for each timepoint and each experiment presented. Data are presented as the average percent avoidance ± standard error of the mean with animal number (n).
Cell proliferation increases after injury

The experiments described above show that focal tectal injury significantly impairs visual avoidance behavior and that animals recover the behavioral response over the course of a week. We hypothesized that recovery from injury is based on the generation of new neurons, which then integrate into the damaged circuit and result in recovery of function. To test this hypothesis, we first determined whether cell proliferation increases after damage to the optic tectum. Injured animals were assayed for the cell proliferation marker PH3, which labels actively dividing cells in the mitotic phase of the cell cycle. Surgically injured animals were fixed and their brains were processed for anti-PH3 immunofluorescence and imaged as whole-mounts.

Analysis of individual confocal sections shows that PH3-immunoreactive cells are located in the ventricular layer, as expected. Images were analyzed as individual confocal sections, but are presented as confocal Z-projections of the entire tectum. The apparent widespread distribution of PH3-positive cells in the flattened Z-projection image of the whole-mount tectum is due to the curvature of the ventricular layer (see Bestman et al., 2012). We counted the total number of PH3-positive cells in both the injured right tectum and the intact control left tectum. Consistent with our previous data showing a developmental decrease in cell proliferation (Sharma and Cline, 2010), the number of PH3-positive cells in control animals decreases over the course of the experiment (Fig. 4A–D,K). Within 1 day after injury, PH3-positive cells were already significantly increased in the injured tectal lobes compared to the intact tectal lobes (P < 0.05; Fig. 4A,B,K). Two days after surgery the numbers of PH3-immunoreactive cells in the injured tectum had increased further, and were significantly greater than the number of PH3-positive cells in the intact tectum (P < 0.01) (Fig. 4C,D,K). The significant increase in PH3 labeling in the injured tectum, compared to the intact tectum, persisted until 4 days after injury (P < 0.01; Fig. 4E–H,K). By 5 days after injury, the numbers of PH3-positive cells in the injured tectum had decreased and were indistinguishable from the uninjured tectum (P = 0.13) (Fig. 4I,J,K). Within the injured tectum, the numbers of proliferating cells increased significantly between days 1 and 2 (P < 0.01) and decreased significantly between days 2 and 3 (P < 0.01); however, the numbers of proliferating cells are not significantly different between days 3, 4, and 5.

As an independent means to detect and quantify cell proliferation, we labeled proliferating cells in S phase by exposing injured animals to the thymidine analog IdU for 2 hours immediately following surgery or at either 24 or
48 hours after surgery and compared the IdU labeling between injured and intact tectal lobes in the same animal. Animals were fixed after the 2-hour exposure period and their brains were processed for anti-IdU immunofluorescence and imaged as whole-mounts. Images were analyzed as individual confocal sections and are presented in Figure 5 as Z-projections of the entire tectum. Two hours after surgery the tectal lobes showed relatively little IdU labeling and the extent of labeling was comparable between the injured and intact tectal lobes ($P = 0.4$; Fig. 5A,D). By contrast, 1 day after surgery IdU incorporation was significantly greater in the injured tectal lobe compared to the intact contralateral tectal lobe ($P < 0.01$; Fig. 5B,D), indicating that the injured tectal lobe has a higher rate of cell proliferation. The increase in cell proliferation persisted such that 2 days after surgery, a 2-hour exposure to IdU resulted in a significantly greater number of IdU-labeled cells in the injured tectal lobe compared to the intact contralateral tectal lobe ($P < 0.01$; Fig. 5B,D), indicating that the injured tectal lobe has a higher rate of cell proliferation. The increase in cell proliferation persisted such that 2 days after surgery, a 2-hour exposure to IdU resulted in a significantly greater number of IdU-labeled cells in the injured tectal lobe compared to the intact contralateral tectal lobe ($P < 0.01$; Fig. 5B,D), indicating that the injured tectal lobe has a higher rate of cell proliferation. The increase in cell proliferation persisted such that 2 days after surgery, a 2-hour exposure to IdU resulted in a significantly greater number of IdU-labeled cells in the injured tectal lobe compared to the intact contralateral tectal lobe ($P < 0.01$; Fig. 5B,D), indicating that the injured tectal lobe has a higher rate of cell proliferation.

Proliferating cells are neural progenitors

To determine the identity of the cells that proliferate in response to injury, we tested whether cells that incorporate CldU are neural progenitor cells. We had previously shown that neural progenitor cells in the developing optic tectum are Msi1-immunoreactive radial glial cells (Sharma and Cline, 2010; Bestman et al., 2012). Msi1 is an RNA binding protein that is enriched in neural progenitor cells and is both necessary and sufficient for maintenance and proliferation of the neural progenitor pool (Kaneko et al., 2000; Sharma and Cline, 2010). To determine whether the proliferating cells are neural progenitor cells, we exposed injured animals to CldU 2 days after surgery, when we observe the peak increase in dividing cells (Figs. 4, 5). Following a 2-hour exposure to CldU, animals were fixed, sectioned, and immunostained for CldU and Msi1. Consistent with the data presented in Figures 4 and 5, we find a significant increase in CldU-labeled cells in the ventricular proliferative zone in the injured tectal lobe compared to the intact contralateral tectal lobe (Fig. 6A–C). Sections through the injured tecta had about 4 times as many CldU-labeled cells compared to the uninjured tecta ($18.5 \pm 3.4$ CldU-labeled cells/section in injured tecta compared to $4.7 \pm 1.3$ CldU-labeled cells/section in the contralateral tecta, $P < 0.01$, $n = 21$ animals). Furthermore, $93.3 \pm 2.5\%$ of the CldU-labeled cells in the injured tectum are Msi1-immunoreactive ($12.7 \pm 1.1$ CldU+/Msi1+ cells/section out of $13.6 \pm 1.2$ total CldU+ cells/section, $n = 7$ animals). These experiments indicate that the majority of proliferating cells in the injured tectum at 48 hours after injury, when we observe the largest increase in proliferation, are Msi1-immunoreactive neural progenitor cells.
Injury-responsive neural progenitor cells generate neurons

We next investigated whether the injury-induced neural progenitors differentiate into neurons. We exposed injured animals to CldU in their rearing solution for 2 hours starting 48 hours after injury and then transferred animals to fresh rearing solution for 2 days, after which they were fixed and their brains were sectioned. To identify the fate of CldU-labeled progeny of neural progenitor cells, we double-labeled sections with antibodies to CldU and the neuronal marker N-β-tubulin, and counted the numbers of immunolabeled cells. We found that 74.1 ± 4.6% of the CldU-labeled cells were also immunoreactive for N-β-tubulin (Fig. 7A–C), indicating that the majority of the injury-induced proliferating cells differentiate into neurons within 2 days. In fact, significantly more of the CldU-labeled progeny in injured tectal lobes expressed the neuronal marker N-β-tubulin compared to the contralateral intact tectum (74.1 ± 4.6% in injured tecta vs. 58.1 ± 9.0% in contralateral tecta, P < 0.01; Fig. 7A–C), suggesting that injury increases the rate at which cells differentiate into neurons. Together, these data indicate that injury increases proliferation of neural progenitor cells preferentially in the injured tectum and that injury affects the fate of neural progenitor cells in the injured tectum so that they generate more neuronal progeny in response to the injury.

To test whether newly-generated cells in the injured tectum differentiate into neurons that extend dendritic arbors, animals were injured in the right tectum and then both tecta were immediately electroporated with the pSox2bd::gal4UASturboGFP expression vector (Bestman et al., 2012). The pSox2bd::FP construct drives gene expression in Sox2-expressing neural progenitor cells because protein expression requires endogenous Sox2/Oct3-4 transcription factors to bind to Sox2/Oct3-4 binding site in the miniFGF4 promoter. Once expressed, the turboGFP persists in the cells and permits visualization of the structure of neuronal progeny. Animals were imaged over 7 days after electroporation and injury. The day after injury, turboGFP (tGFP)-expressing cells can be seen in both the intact and injured tecta (Fig. 8A,E). Two days after injury, labeled cells in the uninjured tecta have both radial glia morphology, characteristic of neural progenitors (Bestman et al., 2012), and neuronal morphology, identified by elaborate dendritic arbors (Fig. 8B), whereas the majority of tGFP-expressing cells in the injured tectum have radial glial neural progenitor morphologies (Fig. 8F). Images collected 4 days after injury show that tGFP-expressing cells have differentiated into neurons with elaborate dendritic arbors (Fig. 8C,G). The same neurons can also be seen in images collected 7 days after injury (Fig. 8D,H). Although the labeling method does not distinguish between injury-induced neurogenesis and ongoing neurogenesis, these data indicate that neural progenitor cells labeled at the time of injury generate mature neurons with complex dendritic arbors over the
period corresponding to the time course of behavioral recovery.

Cell proliferation promotes recovery of visual behavior after injury

Recovery of the visual avoidance behavior after injury could result from reorganization of remaining circuitry or from the generation of new neurons and their integration into the tectal circuit. The data presented above indicate that new neurons are generated in response to injury. To test whether cell proliferation is necessary for recovery from injury, injured animals were exposed to a cell division blocker cocktail of hydroxyurea and aphidicolin (CDBs). This combination of cell division blockers has been shown to block cell proliferation in intact Xenopus tadpoles at earlier stages (Harris and Hartenstein, 1991). We find that exposure of stage 47 tadpoles to cell division blockers for 24 hours in their rearing solution decreases PH3-immunolabling by 74% on average compared to stage-matched control animals exposed to the drug vehicle, 0.5% DMSO, alone. The total number of PH3+ cells per tectal lobe is 28 ± 3.1 for DMSO control vs. 7.2 ± 0.73 for CDB-treated animals, P < 0.01, n = 8 animals (16 tectal lobes) for each condition. Animals were treated with cell division blockers for 24 hours at the peak of cell proliferation, 48–72 hours after injury, and then tested for the visual avoidance response over the next week (Fig. 9A). Exposure to cell division blockers for 24 hours had no effect on the visual avoidance response in uninjured animals, suggesting that blocking cell proliferation and the generation of new cells for a brief period does not interfere with visual circuit function with respect to the visual avoidance behavior (Fig. 9A, blue line). Importantly, blocking cell division after injury resulted in a significant delay in behavioral recovery (Fig. 9A, red line) compared to recovery of injured animals treated with the drug vehicle, DMSO (Fig. 9A, green line). Injured animals treated with cell division blockers for 24 hours from 2–3 days after injury eventually recover the visual avoidance behavior by the end of the experiment. Because the cell division blockers act globally, either extending treatment time or delaying the period of treatment killed the animals, partially due to an effect on hematopoiesis. Nonetheless, these data indicate that tectal cell proliferation and the generation of new neurons promote behavioral recovery from injury.

Previous work has shown that maintaining stage 47 tadpoles in the dark for 48 hours increases cell proliferation in the optic tectum and that subsequent visual experience drives newly-generated cells to exit the cell cycle and differentiate into neurons (Sharma and Cline, 2010). To test whether the visual deprivation-induced increase in cell proliferation can enhance the rate of recovery from injury, injured animals were deprived of visual experience by placing them in the dark for 48 hours immediately after surgery (red) or were put on the standard 12-hour light/dark conditions (green). Injured animals with visual deprivation show an accelerated recovery compared to injured animals housed in a normal 12-hour light/dark cycle. All animals recover behavior by the end of the 7-day experiment, regardless of treatment. **P < 0.01. Data are average ± SEM.

Figure 9. Regulation of neural progenitor cell proliferation affects rate of recovery of visual behavior. Stage 47 tadpoles were assayed for baseline avoidance behavior and subjected to injury (Day 0), then assayed for recovery. A: Animals were treated with cell division blockers (CDBs, red) or DMSO vehicle (green) for 24 hours from Day 2 to Day 3, then assayed for recovery of visual avoidance behavior. Injured animals treated with CDBs show a delayed recovery compared to injured animals treated with DMSO vehicle alone. CDBs have no effect on visual avoidance behavior in intact animals (purple). Control uninjured animals (blue) exposed to DMSO. B: Animals were deprived of visual experience for 48 hours by placing them in the dark immediately after surgery (red) or were put on the standard 12-hour light/dark conditions (green). Injured animals with visual deprivation show an accelerated recovery compared to injured animals housed in a normal 12-hour light/dark cycle. All animals recover behavior by the end of the 7-day experiment, regardless of treatment. **P < 0.01. Data are average ± SEM.
DISCUSSION
Nature of the injury and reactive neurogenesis

Brain injuries induce a variety of cellular responses depending on the type of injury, the region and areal extent of injury, the age at injury, and species studied (Stahel et al., 1998; Cernak, 2005; Endo et al., 2007; Zupanc, 2009; Covey et al., 2010; Xiong et al., 2010; Liu and Guthrie, 2011). Nonmammalian vertebrates, including amphibia and fish, can regenerate brain tissue after damage and in some cases can recover behavior after injury (Srebro, 1957, 1959; Jordan, 1958; Filoni and Gibertini, 1969; Filoni and Margotta, 1971; Minelli et al., 1987, 1990; Gong and Shipley, 1995; Zhang et al., 2000; Chernoff et al., 2003; Yoshino and Tochinai, 2004; Endo et al., 2007), suggesting that identification of mechanisms for recovery from injury in nonmammalian species may inform studies in mammalian systems. Zebrafish have been particularly valuable for studies of regeneration in the adult CNS (Zupanc, 2009). Here we tested the potential contribution of neurogenesis and the integration of newly-generated neurons in the recovery of behavior in the developing visual system. Although neurogenesis is known to occur in response to injury in Xenopus (Endo et al., 2007; Ferretti, 2011), studies have not tested whether cell proliferation and differentiation of new neurons are required for recovery of visual function after injury. We used vacuum aspiration to remove a fraction of the tectal cells while avoiding the ventricular layer where neural progenitor cells are located. By inserting the needle perpendicular to the tectal surface, we minimized damage to the retinotectal axons which laminate the superficial layers of the tectum. This caused minimal bleeding and swelling and appears to produce injury principally by removal of tectal neurons. This injury paradigm may be analogous to penetration injuries or surgical removal of part of the brain (Stahel et al., 1998; Spencer and Huh, 2008; Kroehne et al., 2011; Kishimoto et al., 2012), for instance in treatment of epilepsy (Spencer and Huh, 2008). Animals that received closed needle penetration into the optic tectum without removal of cells, which is similar to a stab-wound injury, had no deficit in visual behavior, highlighting the value of a behavioral assessment of injury and recovery. We see no signs of “secondary injury,” where an initial injury triggers swelling, cell death, and tissue deterioration, frequently seen in response to contusive or blunt force injuries (Stahel et al., 1998; Morganti-Kossmann et al., 2001). We see no evidence of a glial scar in the tectum, consistent with previous observations that little scarring occurs in Xenopus tadpoles (Gibbs et al., 2011). We also find no evidence of an immune response to injury, as reported in mammals (Nimmerjahn et al., 2005; Davalos et al., 2005; Harry and Kraft, 2012) and in Xenopus tadpoles in response to optic nerve injury (Goodbrand and Gaze, 1991), likely because of the relatively immature immune system in young Xenopus tadpoles (Hsu and Pasquier, 1984; Kim and Szele, 2008). By contrast, a penetrating injury to the telencephalon of adult zebrafish, which produced significant axonal injury, resulted in secondary edema and reactive proliferation of immune cells at the site of injury, and neurogenic proliferation in the telencephalic ventricular zone (Kroehne et al., 2011; Kishimoto et al., 2012). Finally, the tectal injury in Xenopus tadpoles induces a local proliferative response in the injured tectum, consistent with studies demonstrating a focal signal for proliferation (Zupanc and Ott, 1999; Kroehne et al., 2011), but different from a recent study in Xenopus tadpoles where amputation of the tail induced a widespread increase in sox2 expression in the CNS (Gaete et al., 2012).

Mechanisms for recovery from injury

Two types of mechanisms may contribute to recovery from brain injury: The circuit may regain function as a result of reorganization of neural components remaining after damage (Xiong et al., 2010). An alternative, but not mutually exclusive, mechanism is that newly-generated neurons integrate into the damaged circuit and result in recovery of function. Animals in which injury induces neurogenesis may allow assessment of the relative contribution of circuit reorganization versus neurogenesis for recovery of behavior. Increased cell proliferation is seen in many experimental models of brain injury (Kleindienst et al., 2005; Covey et al., 2010; Kernie and Parent, 2010; Xiong et al., 2010; Kim et al., 2011). Although the increased cell proliferation seen after injury is thought to promote recovery of function following injury (Kernie and Parent, 2010), not all conditions that increase neurogenesis restore lost brain function (Wang et al., 2011). For instance, induced apoptosis of cortical neurons in adult mice triggers proliferation of endogenous cortical neural progenitors and differentiation of newly-generated cells into neurons (Magavi et al., 2000; Chen et al., 2004); however, animals did not show recovery of function. By contrast, a recent study suggested that blocking injury-induced neurogenesis in adult mouse hippocampus intensified brain injury-induced behavioral deficits in hippocampal-dependent tasks (Blaiss et al., 2011). Recently, grafts of neural progenitors transplanted into a spinal cord injury site were shown to generate neurons and lead to partial recovery of function (Lu et al., 2012). Our studies support the idea that the generation of new neurons enhances recovery from damage. We showed
that blocking cell division during the peak of the injury-induced proliferation slows the rate of recovery from injury, and that visual deprivation for 2 days, which increases proliferation of neural progenitors (Sharma and Cline, 2010), improves the rate of behavioral recovery. Animals treated with drugs to block cell proliferation are still able to recover the visual avoidance behavior, possibly through reorganization of remaining neurons in the visuomotor circuit or a delayed increase in cell proliferation after removal of the cell proliferation blockers. Similar mechanisms may operate in the spinal cord where damage-induced proliferation of Sox2-expressing progenitors appears to be required for regeneration and recovery of swimming behavior (Gaete et al., 2012).

One concern might be that the behavioral deficit arises from damage to retinotectal axons. Clearly, severing RGC axons would disrupt the visual circuit and affect behavior. By inserting the needle perpendicular to the tectal surface, we minimized damage to the retinotectal axons which lamine the superficial layers of the tectum. Our imaging experiments show that retinotectal axon arbors do not appear damaged in injured tecta. Finally, cell division blockers would not be expected to affect recovery from local damage to retinotectal axons. Taken together, these data indicate that the RGC axons remain largely intact in our injury model and the injury is successfully targeted to the postsynaptic tectal cells within the visual circuit.

Injured animals treated with DMSO appear to have an increased rate of recovery compared to injury-only controls (Table 2). It has been reported that DMSO exhibits beneficial effects in other injury models; however, the beneficial effects of DMSO are not seen consistently across studies and there is little consensus about how DMSO might act in the injured brain (Jacob and de la Torre, 2009; Julien et al., 2012). Importantly, our data show that the cell division blockers inhibit the rate of recovery despite the facilitative effects of the DMSO vehicle in which the drug is delivered (Fig. 9A; Table 2).

Despite the increase in both proliferation of neural progenitors and the rate of their differentiation into neurons following injury, the injured tectum never fully recovers its original shape or volume. These data indicate that behavior can recover without complete restoration of gross brain structure that was present before injury. This suggests that the tectal circuit may not fully regenerate and furthermore that the newly-generated neurons efficiently integrate into and enhance the function of the remaining tectal circuit. We have previously shown that newly-generated tectal neurons integrate into the visual circuit and participate in visual responses (Chiu et al., 2008; Bestman et al., 2012), indicating significant plasticity of the circuit. The data presented here suggest that the damaged circuit retains sufficient plasticity to integrate newly-generated neurons, resulting in functional recovery of visually-guided behavior. It is also interesting to note that while the tectal injury was performed unilaterally, animals fail to avoid stimuli approaching either eye, ipsilateral or contralateral to the damage. This suggests that intertectal communication that is required for the behavior is disrupted by the injury and subsequently recovers.

Injury-induced change in fate of neural progenitors

Neural stem cells can divide symmetrically to generate progeny which are both progenitors, or they can divide asymmetrically to generate a progenitor and another cell that will differentiate into a neuron (Gotz and Huttner, 2005). Our in vivo time-lapse imaging of neural progenitors in the Xenopus optic tectum demonstrated that radial glial neural progenitors undergo both symmetric regenerative divisions and asymmetric neurogenic divisions. Furthermore, progeny with radial glial morphology can remain proliferative or differentiate into neurons with a delay of up to 2 days before the radial glial cells show morphological evidence of neuronal differentiation. This temporal delay in establishing a neuronal fate suggests that cell fate may be susceptible to extrinsic determinants for a limited time. Indeed, our previous work has shown that providing animals with visual experience promotes tectal neuronal differentiation (Bestman et al., 2012). In this study, our data demonstrate that injury produces another external trigger regulating neural progenitor fate: injury transiently increases the number of proliferating neural progenitor cells, suggesting that quiescent progenitor cells change their fate in response to an injury signal by reentering the cell cycle. In addition, we find that a higher proportion of the ClbU-labeled neural progenitor cells differentiate into neurons, suggesting that injury induces a second type of fate change in the neurogenic cells in which progeny are more likely to differentiate into neurons. The data presented here show that quiescent progenitor cells can be triggered to proliferate and differentiate in response to injury, suggesting that there is homeostatic regulation of cell number in the developing visual system. It is possible that injury induces a signal that releases the negative homeostatic control of proliferation by changing progenitor cell fate. The molecular mechanisms that negatively regulate cell proliferation in the healthy brain and then initiate it in response to injury remain largely unknown. Nitric oxide (NO) negatively regulates cell proliferation in the brain (Packer et al., 2003) and blocking NOS activity in Xenopus optic tectum increases cell proliferation (Peunova et al., 2007). We have previously shown that decreasing brain activity by...
visual deprivation also increases proliferation (Sharma and Cline, 2010; Bestman et al., 2012), and here we show that deprivation-induced proliferation facilitates recovery from injury. While it is not clear if the signal controlling progenitor cell fate is the same in the injury and visual deprivation paradigms, both experiments demonstrate that neuronal progenitor cells change fate in response to different physiological stimuli.

In summary, we used a visual avoidance behavior in Xenopus laevis tadpoles to assess functional recovery of the retino-tectal-spinal cord circuit following injury to the tectum. Neurogenesis in the injured tectum promoted recovery of visual behavior and increasing endogenous neurogenic activity by decreasing visual system input facilitated recovery from injury. Together, these experiments show that engaging activity-dependent mechanisms that regulate endogenous neurogenesis facilitates functional recovery from local CNS injury. Although neurorestorative strategies to increase neurogenesis, synaptogenesis, circuit remodeling, and angiogenesis are all promising therapies in the treatment of brain injuries (Xiong et al., 2010), understanding the unique capacity of lower vertebrates to engage activity-dependent mechanisms that regulate progenitor cell fate is the same in the injury and control conditions is an important step toward understanding the mechanisms underlying the activation of quiescent neural progenitor cells and the integration of newly-generated neurons into the remaining circuit may facilitate treatment of brain injury.

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CONFLICT OF INTEREST

We have no known or potential conflict of interest including any financial, personal, or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: HTC, CRM. Acquisition of data: CRM, PS, HES. Analysis and interpretation of data: CRM, PS, HES, HTC. Drafting of the article: CRM, PS, HTC. Critical revision of the article for important intellectual content: CRM, PS, HES, WS, HTC. Statistical analysis: CRM, PS. Obtained funding: HTC. Contributed analytic tools: WS. Study supervision: CRM, HTC.

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