

11-15-2007

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Regeneration of retinotectal projections after optic tectum removal in adult newts

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Purpose: When injured, the adult newt possesses the remarkable capability to regenerate tissues and organs with return of function and physiology. One example is the newt eye, in which regeneration can restore normal vision if the retina or lens has been removed. We wanted to examine how the retinotectal projections regenerate after removal of the brain's optic tectum and establish this animal as a model for retinal projection as well as a central nervous system regeneration model.

Methods: A major portion of the left optic tectum was removed in several adult newts, and the animals were monitored postoperatively for eight months to observe regeneration and innervation. Cell proliferation was examined by histological methods and by BrdU incorporation.

Results: We observed that adult newts have the capability to the excised optic tectum. As indicated by horseradish peroxidase staining, 80% of the retinotectal projection area was regenerated eight months after the operation, even though the wound closed much earlier. Our study provides the first quantitation of regeneration of the retinotectal projections. The ependymal cells that line the ventricle were the most likely source of the regenerated tectum. After removal, cell proliferation was detected only in the ependymal cells layer. Double staining of proliferating cells and neurons was limited, indicating that direct transition of ependymal cells is a possibility.

Conclusions: The retinotectal projections after removal of the adult newt optic tectum can be readily re-established. Thus, this model can become indispensable for the study of vision restoration and neurogenesis.

Regeneration of the central nervous system is quite limited among vertebrates. Studies have indicated neurogenesis is possible in the mammalian brain [1-4]. The mammalian brain does not show any regenerative ability when parts of it are removed. Some fish and amphibians, including the newt, though, show quite remarkable regeneration of the brain [5]. Of all the vertebrates, the adult newt is considered the champion of regeneration. It can readily replace lost limbs, retina, lens, heart, tail (with spinal cord), and other tissues [6,7]. We wanted to establish the newt as a brain and central nervous system regeneration system model. We were especially interested in examining the re-establishment of the retinotectal projections after part of the optic tectum was removed. Previous studies have investigated regeneration in the newt brain, but there has not been a detailed quantitative study of regeneration of the retinotectal projections [8-12]. We employed horseradish peroxidase staining and developed a quantitative method to evaluate and correlate regeneration of the retinotectal projections with regeneration of the wounded (removed) optic tectum.

METHODS

Animals: Adult Japanese newts, *Cynops pyrrhogaster*, were purchased from Hamamatsu Seibutsu Kyouzai (Shizuoka, Ja-

pan) and fed frozen porcine liver pieces. The average size was 11-12 cm long from head to tail.

Excision of the midbrain of the newt: Figure 1 presents newt brain anatomy and histological sections taken from the different areas of the newt brain. Newts were anesthetized by placing them for 20 min in a solution of 0.1% MS222 (Sigma, St. Louis, MO), and held in a special apparatus that we designed and built, which has a head holder so that the newt's head can be held tight. that we designed and built. A 2x6-mm region of the scalp and skull just above the whole brain was cut out with a wheel cutter. After the meninges above the midbrain were peeled off with fine forceps, a portion of the optic tectum of the left midbrain was aspirated with a glass capillary (inside diameter, 0.7 mm) using a vacuum pump. The midbrain was excised with fine scissors, and the scalp and skull (previously removed and preserved in the newt saline solution that consists of 115 mM NaCl, 3.7 mM KCl, 3 mM CaCl₂, 1 mM MgCl₂, 18 mM D-glucose, 5 mM HEPES, pH 7.5 adjusted with 0.3 N NaOH) were re-attached. Each operated animal was placed on wet paper towels. Under these conditions newts were virtually inactive for more than one month. The wound was heal closed naturally. To avoid bacterial infection during the operation, we included 500 µg/ml kanamycin sulfate and 50 µg/ml tetracycline (Invitrogen, Carlsbad, CA) into the anesthetized solutions. After complete wound closure, the newts were returned to the tap water.

Detection of retinotectal projection pattern with horseradish peroxidase: As depicted in Figure 2 we injected horseradish peroxidase (HRP) on the ventral side of both eyes. Such

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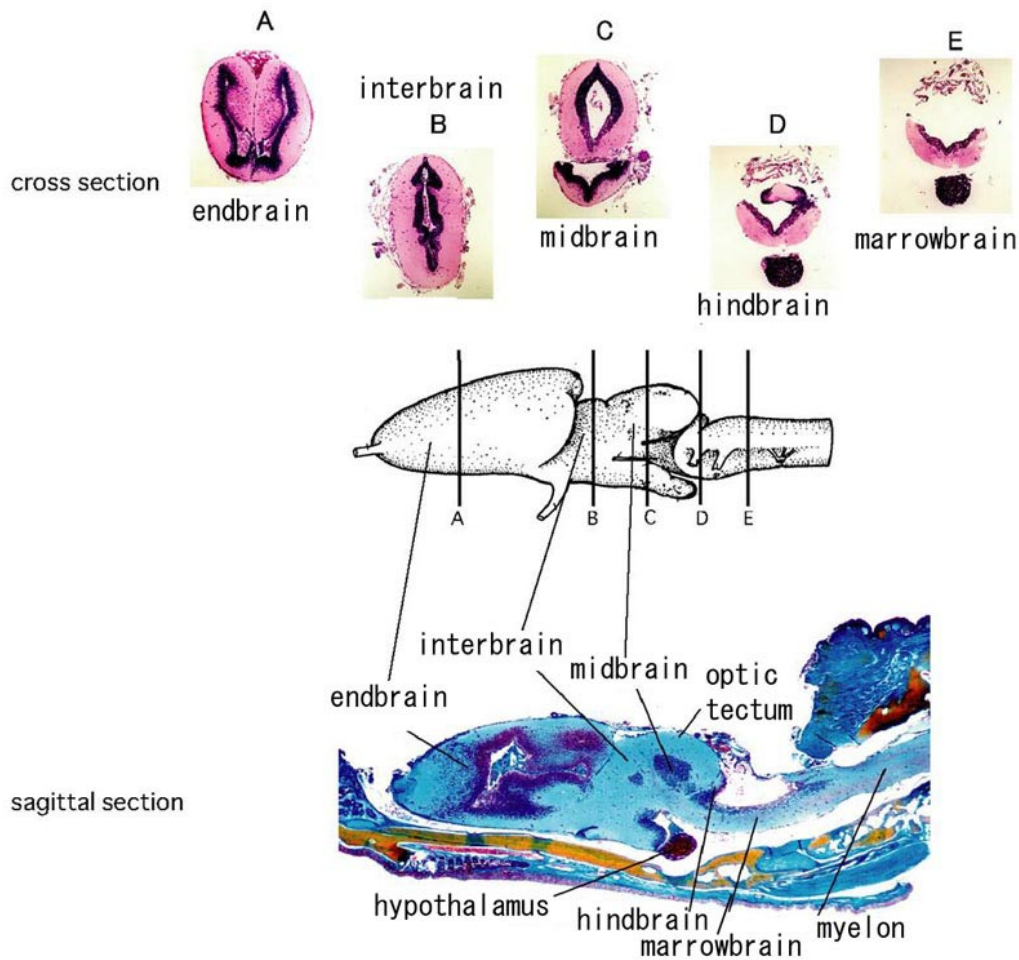


Figure 1. Histological sections and anatomy of the newt brain. This figure shows the basic anatomy of the newt brain and aims to orient the reader in the sections of the brain that were analyzed. In the middle of the panel we can see the overall anatomy of the newt brain. On the top there are cross sections (A-E), which correspond to the different parts of the brain (corresponding to lines A-E in the middle of the panel). On the bottom there is a sagittal section showing also the corresponding parts. A: endbrain, B: interbrain, C: midbrain, D: hindbrain, E: marrowbrain.

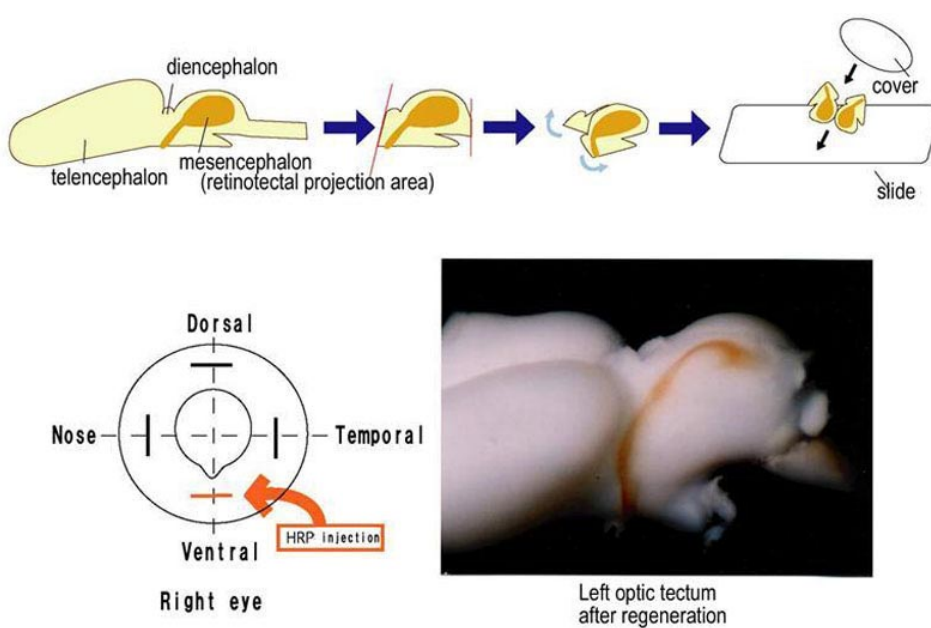


Figure 2. Method for detecting regeneration of the retinotectal projections. The top panel shows the method we devised for quantitation of regeneration of the retinotectal projections. The area that contains the regenerated tectum was excised from the brain. The brain was then divided in two parts by a slit in the ventral side. The right part was the uninjured part, while the left part was the injured and regenerating part. The flattened sides were placed on a glass slide and the photo prints were used to measure the area of the optic tectum that was innervated by the axons (see Methods). The lower panel on the left shows the injection of horseradish peroxidase (HRP) on the ventral site and on the right it shows how the HRP has been taken by the nerve fibers to indicate innervation in the regenerated optic tectum.

an injection labels all the nerve fibers extending from the retina ganglion cells. To measure and quantitate the retinotectal projection area, we developed a new method. For this purpose, mid and inter brain were isolated from the whole brain, and a slit was made in the ventral side dividing the brain in the right part (uninjured) and the left part (injured and regenerating). Then both flattened sides were placed on a glass slide with the dorsal sides up (Figure 2). The photo prints of the flattened samples were treated with computer software to measure the area of the optic tectum that was innervated by the axons.

Detection of cell proliferation and immunofluorescence:

Brain samples were collected at different times from 5-40 days post operation, fixed and embedded in paraffin. Paraffin sec-

tions from the midbrain were stained with H&E and examined for mitosis. Cell proliferation was also detected by BrdU incorporation. BrdU was injected intraperitoneally, starting at day 8 after excision of the optic tectum. We injected BrdU every other day for five times. After 1-2 months, the operated brains were isolated and examined for BrdU incorporation. Deparaffinized sections were treated with mouse anti-NeuN (1:300; Ab Cam, Cambridge, MA) for 1 h at room temperature (RT). After rinsing, the sections were treated with secondary antibody Alexa at 1:100 dilution (Molecular Probes, Carlsbad, CA) for 1 h at RT. After rinsing, 1:10 diluted BrdU antibody was added for 1 h at RT. Then sections were treated with 1:500 diluted anti-rat Cy3 secondary antibody for 1 h at

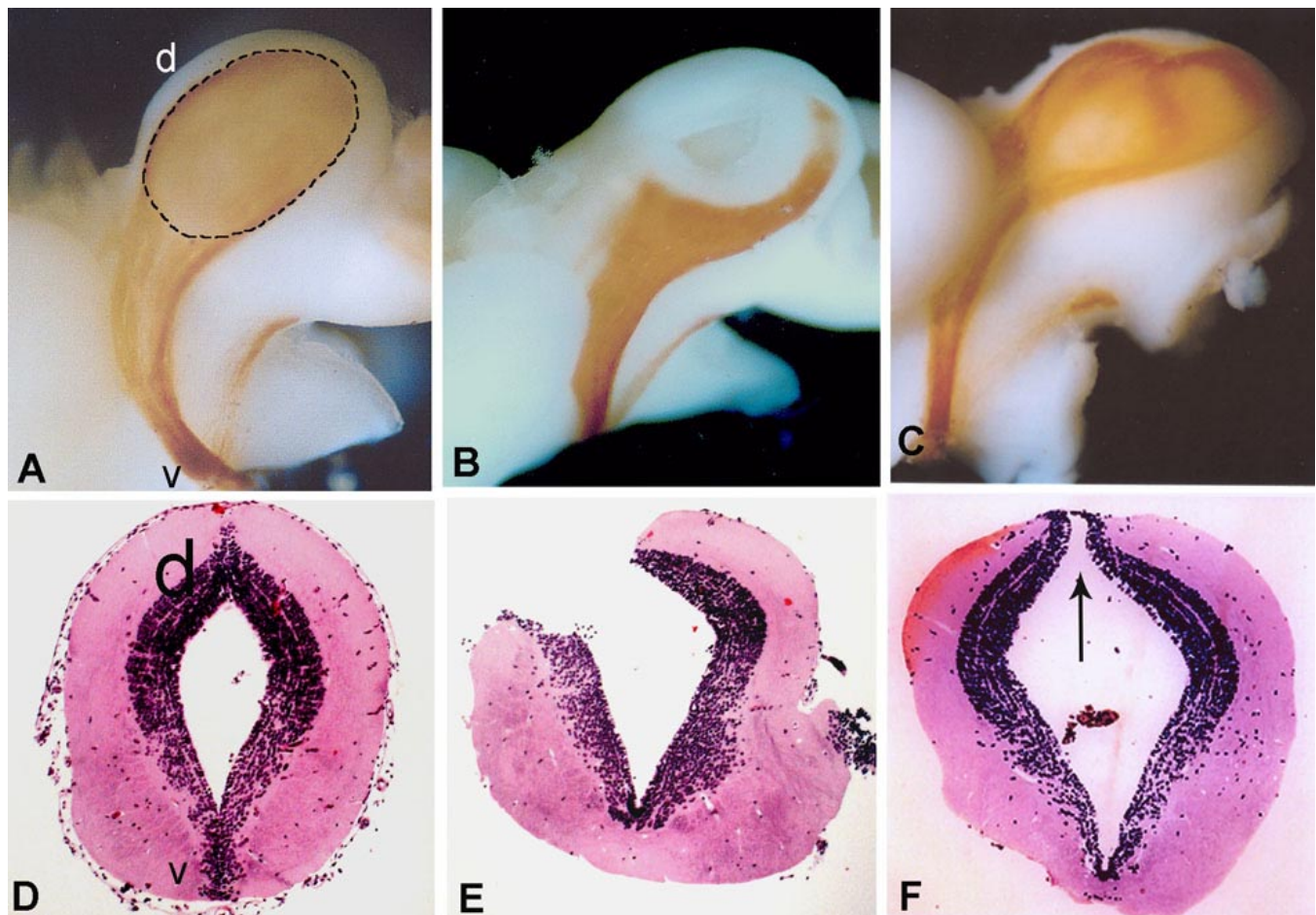


Figure 3. Regeneration of the retinotectal projections. Regeneration of the retinotectal projections pattern after excision of the left optic tectum (OT) of the midbrain (mesencephalon). **A-C**: Visualization of the retinotectal pattern in the midbrain and interbrain (diencephalon) by horseradish peroxidase staining of optic nerve fibers. **A**, Un-operated left OT showing the normal patterns of labeled retinotectal projections. Dotted line defines the OT. **B**, Left OT just after excision with apparent loss of the retinotectal projections due to the operation. **C**: Regenerating left OT six months after operation, with recovery of the retinotectal projections, as indicated by the staining with HRP. Compare the similarity in the staining patterns with **A**. **D-F**: Shown are hematoxylin and eosin-stained transverse sections of the midbrain that correspond to figures in **A-C**, respectively. **D**: Unoperated midbrain. **E**: A cross section through the excised tectum (corresponding to **B**) showing a missing large area due to the removal of the tectum tissue. **F**: A cross section through a regenerating tectum (corresponding to **C**) six months after excision. Note that the tissue has been regenerated and that it looks considerably similar to un-operated brain shown in **D**. Arrow indicates the remnant of the wound in this sample. The top part of the tissue in **A-C** and of the sections in **D-F** is the dorsal (d) and the bottom is the ventral (v) side of the midbrain. The minor tract of labeled axons outside the left optic tectum is the tractus opticus accessory [15]. These tracts are located at the interbrain region. According to the orientation of the specimens in **A-C** the endbrain is located on the left of the stained tracts and the hindbrain on the right (see also illustration in Figure 1).

RT. After rinsing, the sections were stained with DAPI, covered with Permflow medium and observed.

RESULTS & DISCUSSION

We followed the process of the retinotectal projection recovery patterns for eight months following excision of the left optic tectum. Figure 3A shows the projection pattern of the



Figure 5. The coronal section of the optic tectum at one-month post surgery. This figure shows histological features of wound healing in the brain. This sample was taken one month after excision of the left tectum. The arrowhead indicates the joint of the left and the right optic tectum (OT), and the arrow indicates the wound closure. Wound closure of the lesion seemed to occur earlier than brain tissue regeneration.

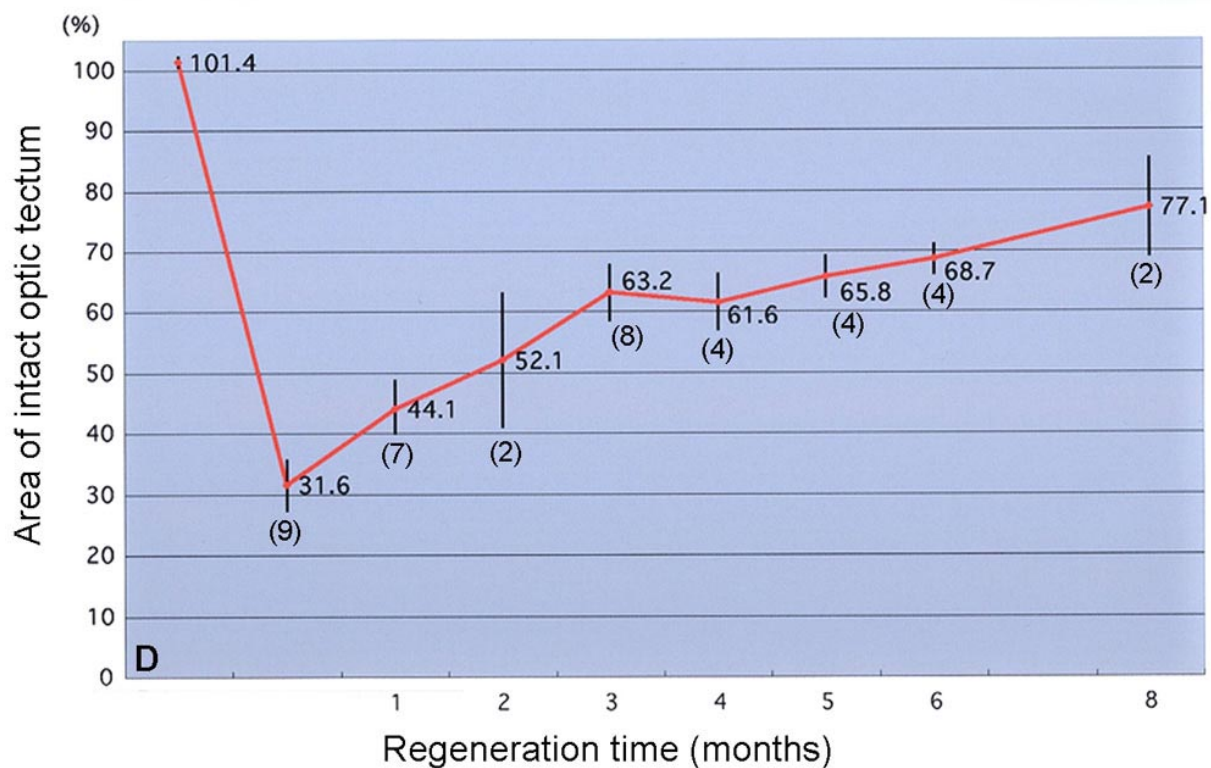
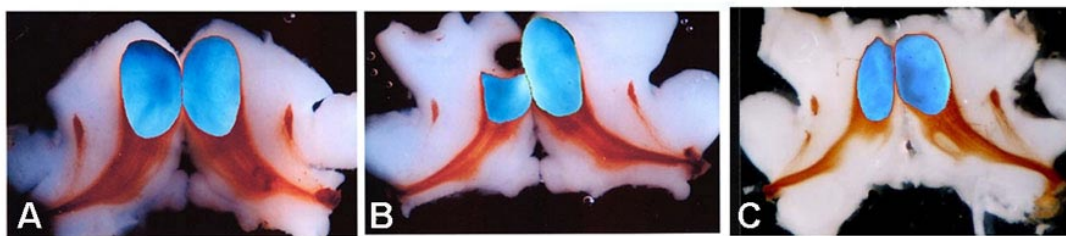


Figure 4. Rate of regeneration of the retinotectal projections during a period of eight months after optic tectum excision. **A-C:** The blue colored areas indicate the left and right optic tectum in the brain samples processed for quantitation as described in the Methods. **A:** Unoperated control. **B:** A sample taken immediately after the excision of the left tectum. Almost 70% of the left OT was excised. **C:** The OT after eight months. The structure of the original midbrain appeared to have recovered at this stage. **D:** The recovery rate during eight months. The unoperated right OT, which served as the standard, was compared to the operated left OT, and the recovery rate was observed. The rate gradually increased to about 80% by eight months. Numbers in parentheses indicate the number of newts for each time point, and bars represent mean±SE. The value 101.4% for the un-operated left OT is because we assume as 100% the mean value of the un-operated right OT. Evidently the right OT was slightly smaller.

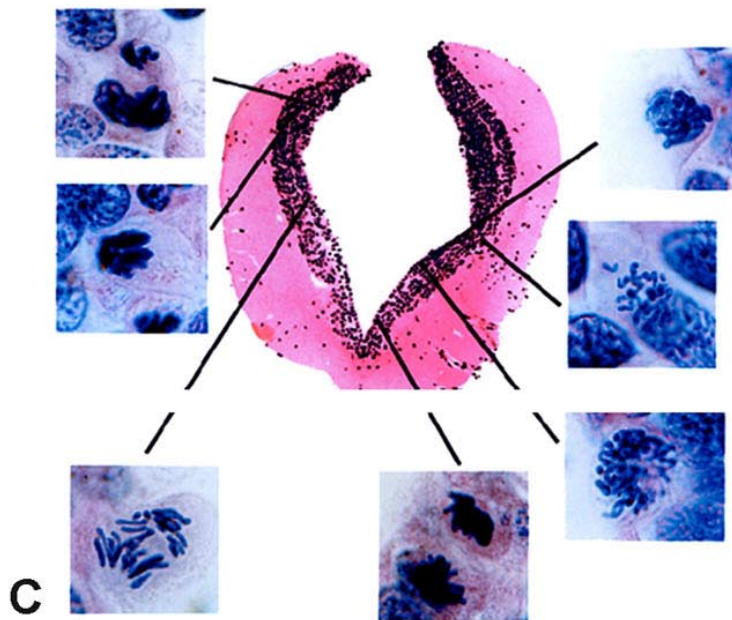
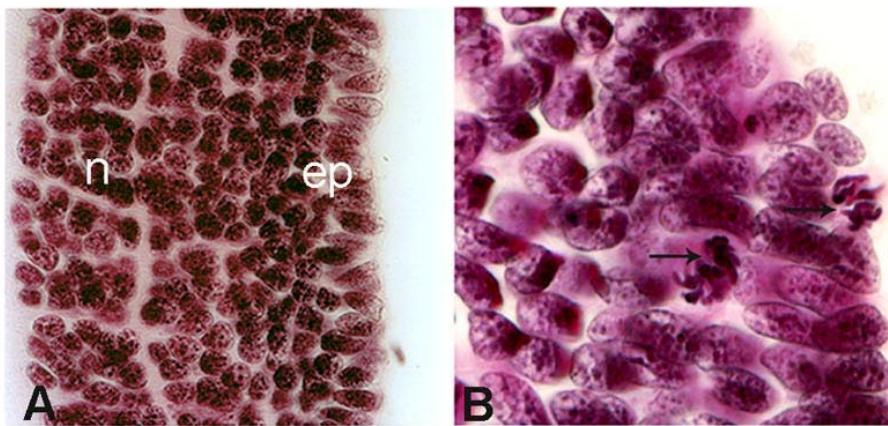
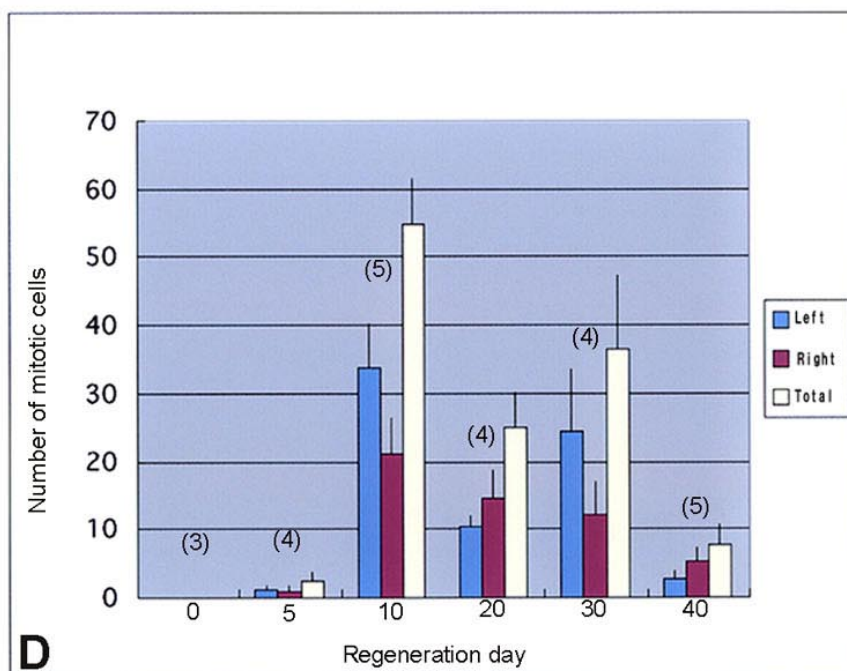


Figure 6. Cell proliferation in the mid-brain during early regeneration stages. **A:** A normal midbrain coronal section. The marker n indicates the neuronal cell layer and ep marks the ependymal cell layer. The layer of nerve fibers is to the left of the neuronal cell layer and the ventricle is on the right side of the ependymal cell layer. **B:** At ten days postoperative, mitotic figures can be seen only in the ependymal cell layer. Unoperated control ependymal cells are composed of a single layer of cells. However, probably due to cellular growth, they form a multilayer by ten days post surgery (compare with A). **C:** A typical section of the midbrain at ten days post surgery. Many mitotic figures can be seen in the ependymal cell layer facing the ventricle. The pink stained area is the nerve fiber region. The neuronal cell layer and ependyma are stained violet. **D:** The change of the mitotic cell numbers counted in serial sections of the midbrain during early stage of regeneration. Blue represent cells in the left part of the brain, red represent cells on the right portion, and white is the total number. Mitotic cells peak at day postoperative 10 and gradually decrease by postoperative day 40. Day 0 is un-operated control. Numbers in parentheses indicate the number of newts for each time point, and bars mark mean±SE.



right optic nerves on the left optic tectum of the normal mid-brain. In the newt, all optic nerves derive from a right eye projection to the left optic tectum through the optic chiasm and vice versa for the optic nerves from the left eye. Figure 3B presents the projection pattern just after excision of the left optic tectum of the midbrain. In the transverse sections, the control and the excised portion of the left optic tectum are clearly evident (Figure 3D,E). Six months after the operation, the retinotectal projection pattern had recovered considerably (Figure 3C,F). As mentioned in the Methods section, we quantitated the innervation of the tectum by following the process for eight months (Figure 4). As shown in Figure 4D, 70% of

the left optic tectum was surgically removed. By postoperative eight months (the last time point in our experiments) nearly 80% of the projection area had been recovered, and it appeared it would take several more months for regeneration to complete.

We must stress here that the wound recovered much sooner. A large wound such as the one shown in Figure 3B,E usually within 1 month (Figure 5). However, as seen in Figure 4D, the projection area was only recovered by 10%. So it seems that regeneration of the retinotectal projections follows with a considerable delay when compared to wound closure.

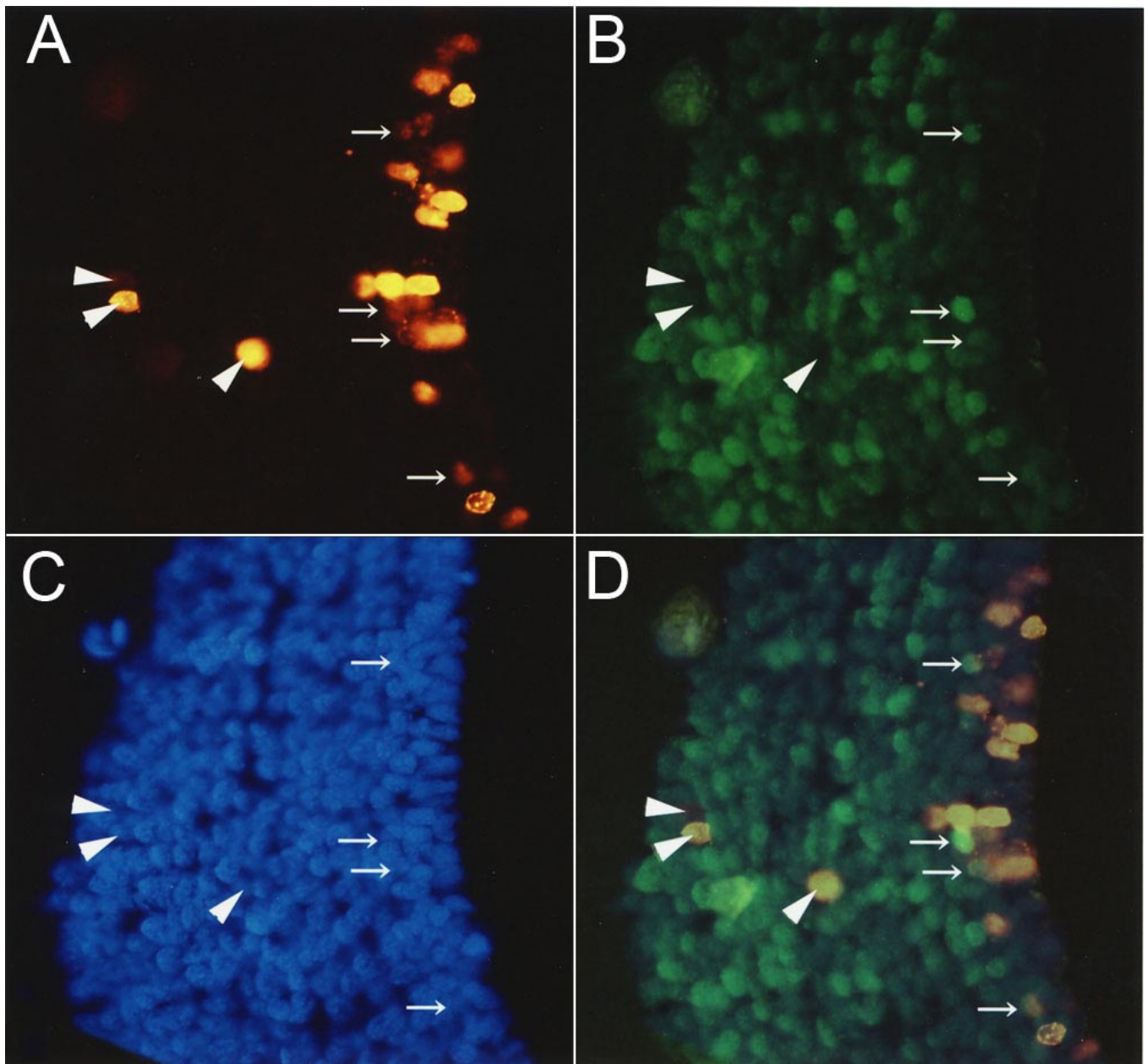


Figure 7. BrdU incorporation in cells of the regenerating brain two months after surgery. **A:** Cells staining positive for BrdU were mainly present in the ependymal cell layer. **B, C:** NeuN (neuronal marker) staining and DAPI staining, respectively. **D:** Shown is an overlay of **A, B** and **C**. Arrows point to NeuN-positive cells, and arrowheads mark NeuN-negative cells. NeuN-negative and BrdU-positive cells could be another cells type, such as astrocytes.

We also examined cell proliferation in the brain after removal of the tectum. For this we counted mitotic figures in histological serial sections of the midbrain. The results are shown in Figure 6. The newt midbrain is mainly composed of three parts: the innermost ependymal cell layer lining the ventricular wall, the neuronal cell layer composed of neurons and glia cells, and the outermost nerve fiber layer (Figure 6A). Figure 6B shows mitotic figures in the transverse sections of the midbrain at postoperative day 10. The mitotic figures were present at the ependymal cell layer. There were a few mitotic cells evident at postoperative day 5, but they dramatically increased around postoperative day 10. The numbers peaked around postoperative month 1, then dropped around postoperative day 40 (Figure 6C). In a comparison with other brain regeneration models [5,11,12], we observed that mitotic figures in our model were not limited to the lesion area, but could be observed in the unoperated right portion of the midbrain. In addition, we observed mitosis in neighboring regions, such as diencephalons and metencephalon. It is not known at the present time if ependymal cells from the un-operated area contribute to regeneration. Special labeling techniques are required to answer this question. We also confirmed such proliferation patterns with BrdU incorporation (Figure 7). However, we were unable to detect many BrdU-positive cells in the neuronal layer. One explanation for this might be that dividing ependymal cells replenish the ependymal cell layer, while non-dividing ones differentiate to neurons and the other cells of the brain. Another explanation could be that one daughter cell (of the labeled ependymal cell) migrates out, but most of them die. It is possible these cells represent differentiated cells that originated from ependymal cells, which, however stain weakly for BrdU because they have gone through many cell divisions and weakly for NeuN because they are just beginning to differentiate into neurons. Such possibilities remain to be examined when newt markers and more sophisticated labeling technology become readily available. Also, it could be that sustained presence for longer times might be necessary for BrdU uptake by the newt cells [14]. However, this line of research was beyond the scope of the present work. In the present study, we wanted to follow and quantitate for the first time (and over long periods of time) regeneration of the retinotectal projections during brain regeneration in the adult newt. Our results clearly show considerable regeneration of the visual projections, demonstrating the adult newt system can be an indispensable model for research in this area.

ACKNOWLEDGEMENTS

We thank Dr. Masami Watanabe for valuable discussions and reading of the manuscript, and we thank Kazuji Suzuki and Tadashi Masuda for their contribution in developing the head holder apparatus. This work was partially supported by the Center for Tissue Regeneration and Engineering at the University of Dayton.

REFERENCES

1. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999; 97:703-16.
2. Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 1999; 96:25-34.
3. Gage FH. Neurogenesis in the adult brain. *J Neurosci* 2002; 22:612-3.
4. Stocum DL. *Regenerative Biology and Medicine*. Academic Press (2006). San Diego, CA.
5. Goss RJ. *Principles of Regeneration*. Academic Press (1969). New York, NY
6. Tsonis PA. Regeneration in vertebrates. *Dev Biol* 2000; 221:273-84.
7. Alvarado AS, Tsonis PA. Bridging the regeneration gap: genetic insights from diverse animal models. *Nat Rev Genet* 2006; 7:873-84.
8. Endo T, Yoshino J, Kado K, Tochinal S. Brain regeneration in anuran amphibians. *Dev Growth Differ* 2007; 49:121-9.
9. Minelli G, Del Grande P. Localization and quantitative analysis of the elements leading to the regeneration of the optic tectum in the adult *Triturus cristatus carnifex*. *Z Mikrosk Anat Forsch* 1974; 88:209-24.
10. Minelli G, Franceschini V, Del Grande P, Ciani F. Newly-formed neurons in the regenerating optic tectum of *Triturus cristatus carnifex*. *Basic Appl Histochem* 1987; 31:43-52.
11. Del Grande P, Franceschini V, Minelli G, Ciani F. Mitotic activity of the telencephalic matrix areas following optic tectum or pallial cortex lesion in newt. *Z Mikrosk Anat Forsch* 1990; 104:617-24.
12. Minelli G, del Grande P, Franceschini V, Ciani F. Proliferative response of the mesencephalic matrix areas in the reparation of the optic tectum of *Triturus cristatus carnifex*. *Z Mikrosk Anat Forsch* 1990; 104:17-25.
13. Fujisawa H, Watanabe K, Tani N, Iyata Y. Retinotopic analysis of fiber pathways in amphibians. I. The adult newt *Cynops pyrrhogaster*. *Brain Res* 1981; 206:9-20.
14. Parish CL, Beljajeva A, Arenas E, Simon A. Midbrain dopaminergic neurogenesis and behavioural recovery in a salamander lesion-induced regeneration model. *Development* 2007; 134:2881-7.
15. Herrick CJ. *The brain of the tiger salamander*. The University of Chicago Press, Chicago, IL, 1948.