Identification of Neurotransmitters in Cavefish Brain

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Abstract

I examined differences in the chemical compounds (neurotransmitters) in the brain and retinas of the fish, *Astyanax mexicanus,* sampled from cave and surface habitats. Information was collected by dissecting the tissue, cryostat sectioning, probing sections with antibodies to known neurotransmitter systems in these regions (i.e., GABA and dopamine), and analyzing sections under a microscope. GABA was identified in the surface population's optic tectum and retinas, and TOH (a marker for dopamine-containing neurons) was found in the surface population's optic tectum. Data suggests GABA was negative in the cave population tectum. This research will increase our understanding of the evolution of *A. mexicanus* as it moved from the surface to the cave by identifying potential differences in nerve cell function.

Introduction

The optic tectum is made up of some of the most developed parts of the brain of teleosts, or fish, and is found in the mesencephalon. The optic tectum forms a major portion of the vertebrate midbrain, composed of multiple layers that depend on the species. The optic tectum of fish, and fish brains in general, tend to be more elongated. Cells in the optic tectum are organized into distinct layers. Sensory systems contact the superficial layers, which receive input from the eyes. As a result, the optic tectum functions as the fish's visual processing center. Deep layers are related to motor abilities. Intermediate layers are related to sensory and motor properties. Every tectal layer has a topographic map of the world in retinotopic coordinates. Neurons are activated at specific points on the map (Meek, 1983). The tectum helps evoke responses in vertebrates, such as swimming. Some neurotransmitters that are usually found in the optic tectum of fish are GABA, dopamine, glutamate, and acetylcholine. The typical layers found in many teleost optic tecta can be seen in Figure 1 (Ishikawa *et al.*, 2001).

Like the vertebrate brain, the vertebrate retina is also composed of layers. The layers found in vertebrate retinas can be seen in Figure 2 (Caceci, 2001). In the outer retina, horizontal cells provide inhibitory inputs to the photoreceptor-to-bipolar cell synapse. In the inner retina, amacrine cells modify bipolar-to-ganglion cell connections, and provide information like stimulus contrast and direction of movement. The retina of vertebrates is made up of ten layers. Nine of those layers have origins from the inner wall of the primitive optic cup. The pigment cell layer makes up the tenth outside layer. Portions of the retina are light-sensitive, and light passes through eight layers before a nervous signal is formed. The pigment epithelium layer (PE) makes up the outermost layer, and increases the acuity of vision and makes sure light sensitive portions of rod and cone cells are working. As pigment layer cells are renewed, they phagocytose the ends of rod and cone cells, and accumulate lipofuscin. The PE cells contain melanin which, together with lipofuscin, increase contrast and absorb light. Rods and cones are the photoreceptors, which start signal transduction whenever light is detected. The outer limiting layer is the next layer inwards, and is the site of occluding junctions which seal off the light-sensitive outer segments of the rod and cone cells. The junctions are between the plasma membranes of the rod cells and the Muller cell. This layer forms the blood-retina barrier, and isolates the inner retinal layers from harmful material in circulating blood. The outer nuclear layer is the next layer inwards, and is where the nuclei and cell bodies of the rod and cone cells are located. The nuclei of these cells form the outer nuclear layer. The next layer inwards is the outer plexiform layer, where synapses between the rod and cone cells, and processes of integrator neurons are located. When a signal is formed in the rods and cones, it passes through neural elements for processing. Axon terminals of the rod and cone cells are found the outer plexiform layer, which is a region of synapses. Horizontal and bipolar cell dendrites, are also in

this layer. The dendrites of bipolar cells make up most of the outer plexiform layer, and photoreceptors are presynaptic to bipolar cells. Horizontal cells regulate and inhibit input from photoreceptor cells. The next inner layer is the inner nuclear layer, which contains the cell bodies and nuclei of the integrator neurons (especially bipolar, horizontal, and amacrine cells). The next innermost layer is the inner plexiform, which is where bipolar cell processes synapse with the dendritic processes of ganglion and amacrine cells. Amacrine cells laterally regulate input to ganglion cells. The cell bodies of ganglion cells are located in the next deeper layer, the ganglion cell layer. There are fewer nuclei in this layer than in the more dista nuclear layers. Ganglion cells carry signals with their axons, which are bundled into radial tracts. Ganglion cell axons make up the optic nerve and transfer signals to the brain. The deepest layer is the inner limiting membrane, which contains a fusion between foot processes of the Muller cells. This seal blocks harmful materials that may be in the vitreous chamber. There are occluding junctions in this layer that form a barrier (Caceci, 2001). Some neurotransmitters that are usually found in the retina include glutamate, GABA, glycine, dopamine, and acetylcholine.

The fish used in this study are both the surface and cave populations of *Astyanax mexicanus. A. mexicanus* is found in northeast Mexico and south Texas. The surface population is thought to be ancestral and has fully formed and functional eyes. The cave forms show regressive and constructive changes to the eyes. Figure 3 shows the surface form of *Astyanax* (left) and the cave form (right) (*Mexican tetra,* 2007). Since there are not many studies analyzing the structure, basic anatomy, and neurotransmitter distributions of *Astyanax mexicanus*, background information regarding these issues will be drawn from experiments and known information about other fish or vertebrates.

In one species of cavefish, *Astyanax fasciatus*, there are both surface living and cave dwelling forms. The cave dwelling forms developed by becoming trapped in subterranean caves 1 million years ago, and have reduced eyes and skin pigmentation. *Astyanax fasciatus*, like *Astyanax mexicanus*, is also found in waters in Mexico and southern Texas. *Astyanax fasciatus* make up for their lack of eyesight by improving other senses, such as taste, olfaction, and lateral lines. Similarly, cave dwelling *Astyanax mexicanus* may also have improved their other senses, which may cause different neurotransmitters to be released compared to the surface population. Cave dwelling *Astyanax fasciatus* have been found to develop fairly normally up until the third day of development, when the eye degenerates into the sightless adult form. On the second day of development, cell death occurs in all layers of the developing retina. The final "eye" in cave dwelling adult *Astyanax fasciatus* is only 1% the size of the surface fish (Parry *et al*, 2003). Similarly, *A. mexicanus*'s eyes develop normally during the beginning stages of development, when their lenses and optic cups start differentiating. During larval development, the eyes of *A. mexicanus* degenerate and are covered by connective tissue (Yoshizawa and Jeffery, 2008). In a study on another cave fish, *Asytanax hubbusi*, the structure of the blind cave fish was studied for its retinal layers. The photoreceptors of blind *Astyanax hubbusi* contained the same components as other vertebrates. In vertebrates with normal vision, the distal portions had stacks of flatten membranes while the proximal portions are more cellular. In blind *A. hubbusi,* the outer segments were either absent or contained disorientation of lamellae. Muller fibers, with large amounts of glycogen, separated the photoreceptors from one another. In the plexiform layers, synaptic ribbons and vesicles needed for impulse transmission were present. The large amount of glycogen particles in the Muller branches suggests that the retina needs a lot of storage for metabolic processes. With the exception of the disoriented outer segments and the short inner

segments of the photoreceptors, the retina of *Astyanax hubbusi* resembles the retinas of other vertebrates (Yew & Yoshihara, 1977) and most likely of *Astyanax mexicanus*. There are no reports that document retinal layer structure or cell types in *Astyanax mexicanus.* Depending on how similar *Astyanax mexicanus*'s eyes are to other vertebrate eyes, the neurotransmitter levels may vary.

GABA is one of the major neurotransmitters in the nervous system. It is made in the brain through glutamic acid decarboxylase (GAD), and the synthesis of GABA is catalyzed from glutamate. In the adult brain, GABA is an inhibitory neurotransmitter, while in a developing brain, GABA exhibits neurotrophic action. In adult zebrafish, GABA is distributed in the telencephalon, the midbrain, the ventral diencephalon, the tectum stratum, the forebrain, the hindbrain, the retina, and the optic nerve. GABA displays immunoreactivity in the retina, and is found in the optic nerve and inner plexiform layer two days after fertilization in zebrafish. GABA supports neurotrophic factors in synaptogenesis and neural differentiation. In the adult optic tectum, GABA synapses cause the optic tectum to receive a delayed inhibitory input (Kim *et al,* 2004).

In salmon, the optic tectum is the primary visual center, and receives input from the retina through the optic tract. GABA helps with visual processing in the optic tectum of teleosts. In salmon, the optic tectum is a region of the fish brain which integrates visual, lateral line, and tactile sensory information, with the most important role being receiving retinal input. GABA-imunoreactive and glutamic acid decarboxylase are important in the hypothalamus, thalamus, and in pretectal and accessory visual nuclei. The tectal layers that receive retinal input in salmon are the central tectal layers: the stratum opticum, stratum fibrosum et griseum superficial, and the stratum griseum central (Anzelius *et al*, 1995).

One group found that TOH processes were scattered throughout the optic tectum, while also working with zebrafish, while others argue that these processes are restricted to specific tectal layers (Ma, 2003). TOH processes are believed to provide inputs to the tectum (Kaslin and Panula, 2001).

In this experiment, I focused on studying the retinal degeneration of the cavefish. However, in order to understand the conditions of the cave populations, it was necessary to study the condition of the neural retina in the surface population. The surface population served as the control group. Cavefish eyes develop at a rate similar to other teleost eyes, and the overall eye structure is similar. Like teleost eyes, cavefish eyes contain photoreceptors, bipolar cells, and ganglion cells (Strickler *et al.,* 2007). Because there are currently no reports that show retinal layer structure or classification of retinal cell types in cavefish, I relied on studies on the retinal and brain structures of other teleosts to understand the structures found in *A. mexicanus.*

The cavefish's optic tectum is smaller in the cave forms when compared to the surface population (Soares et al., 2004). Although exact cell layers in the brains and eyes of *A. mexicanus* are not known, they are most likely similar to other teleost eyes. Figure 4 shows side-by-side comparisons of the surface and cave forms of *A. mexicanus*. Upon observing the optic tecta (OT or TO in the figure), there is clearly a tectal size difference between the two populations of fish. Similarly, in the fish, *Medaka,* the eyeless form has a smaller optic tectum than the form with fully functioning eyes (Figure 5) (Ishikawa *et al.*, 2001). The size difference of tectal structures may cause different neurotransmitter distributions in both surface and cave populations of *A. mexicanus*.

Materials and Methods

Throughout this experiment, adult surface *A. mexicanus* were in the lab on a 14 hour light and 10 our dark photoperiod, at room temperature. The fish were fed daily. Surface *A. mexicanus* that were needed for the study were euthanized in tricaine and decapitated. Cave *A. mexicanus* were from the University of Maryland, and had already been euthanized. Whole brain and whole eyes were removed and fixed in 4% paraformaldehyde. Tissue was equilibrated in 30% sucrose overnight, embedded in OCT media, and cryostat sectioned. Cryostat sections were probed with antibodies to GABA and TOH and were stained with TRITC and DAPI. The distribution of labeling was examined in both the optic tectum and retina.

Results

Optic tectum labeling

Slides from the brains and retinas of the surface population and the brains of the cave population of *A. mexicanus* clearly consisted of layers. The SM (outer tectal layer) and SPV (inner tectal layer) layers were the most similar to layering found in other teleost retinas and brains, and have been labeled in some of the slides.

Multiple slides of the surface population's optic tecta were probed for GABA and labeled with the nuclear stain DAPI. DAPI was used to label the nuclei of cells, which facilitated identification of tectal layers. The surface population had positive labeling with the GABA antibody, indicating GABA-containing neurons are present in the tectum (Figures 6C, 9B, and 10B). Arrows in Figures 6C, 9B, and 10B point to some of the positive processes. The processes appeared to be mainly in the outer layers because the outer tectal layers had brighter labeling in GABA positive cells, and the labeling became dimmer when moving towards the inner layers. These positive processes appear to be in layer SFGS. The surface population also had positive labeling for DAPI in Figures 6A, 9A, and 10A. The positive slides that tested for

DAPI and GABA had processes that lined up with each other. Figure 9 shows results from a second surface *A. mexicanus*'s optic tectum; Figure 9A shows the results from DAPI labeling and 9B shows the results after probing with the GABA antibody. Positive results can be seen in both images, and the arrows point to the positive processes. Similarly, Figure 10 shows images taken from a third surface *A. mexicanus* specimen, with 10A showing the results for DAPI labeling, and 10B showing the results after probing with the GABA antibody. Both images show positive processes, and arrows are pointing to the positive processes.

Figure 6 shows side-by-side comparisons of surface and cave fish that were probed with anti- GABA and DAPI. The surface fish are the left images (6A and 6C) and the cave fish are the right images (6B and 6D). Two trials were done to test for GABA labeling in the cave population. No labeling was clear in any of the trials of testing for GABA in the cave population. The images from Trial 1 are shown in 6B and 6D. TOH positive structures were found in the optic tectum for the surface population (Figure 7). These structures appeared to be distributed throughout the tectum. Arrows in Figure 7 point to the positive processes.

Retinal labeling

The retinas of the surface population tested positive for GABA, with GABA labeling located on horizontal and amacrine cells. The retinas of the cave population could not be tested; the retinas were too regressed and appeared to only consist of lipids. Figure 8 shows the results for surface retinas that were probed with the GABA antibody . Arrows point to some of the positive processes.

Discussion

Layers were clearly seen in the optic tectum of both the surface and cave populations, and these layers resembled layers found in the optic tectum and retinas of other teleosts. GABA and

TOH were present in the optic tectum of the surface population. GABA tested negative in both trials of the cave population when testing the optic tectum. More testing may be necessary to confirm that GABA is indeed present or absent from the cave population's optic tectum, though it is possible that GABA-containing cells and processes are not present optic tecta of the cave isoform. In the surface population, GABA-labeling was only observed in the outer tectal layers, and this was the case with all of the surface fish that were tested for GABA.

TOH processes were present throughout the optic tectum in the surface population, and not solely restricted to one layer. The way in which the TOH processes were scattered across layers was similar to the results for zebrafish tested for TOH in optic tectum layers (Kaslin and Panula, 2001). The cave population's optic tectum was not tested for TOH.

Retina samples from one surface fish were tested, and positive processes labeled with the GABA antibody were observed. The processes were found in both the inner and outer retina, and were identified on horizontal and amacrine cells. Retina sections could not be taken from the cave population because the eyes had regressed completely.

The locations of the neurotransmitters show some possible purposes of the neurotransmitters. Cells with labeling may start in the optic tectum and leave the tectum to go elsewhere. It is possible that the signals enter the optic tectum from elsewhere, or that the signals stay in a local circuit in the tectum.

The labeling patterns fit known tectal organization because outer layers are related to sensory inputs while inner layers are related to motor outputs. It is possible that blind *A. mexicanus* do not possess these motor functions because their vision has completely degenerated. Because GABA only appears in the outer layer of the optic tectum, the results could mean the blind cavefish are able to receive sensory information, and that information is

subsequently modified by inhibitory GABA inputs, possibly resulting in reduced, or absent, motor outputs. In TOH positive cells, it is possible that pretectal nuclei receive retinal input from the optic tectum, in a similar manner to zebrafish from the Kaslin and Panula study in 2001. Further testing would need to be done to confirm exactly how signals enter or leave the optic tectum.

If this experiment were to be continued, the next step would be to finish testing TOH in the cave population, and retesting GABA in the surface retinas to confirm the positive results. If this experiment were to be repeated, it would have been better to use fish from the cave population that were still undergoing development. During development, the cave population's eyes develop normally at the beginning, and then regress. All of the cave fish used in this experiment had eyes that had completely regressed, and the eyes were unusable.

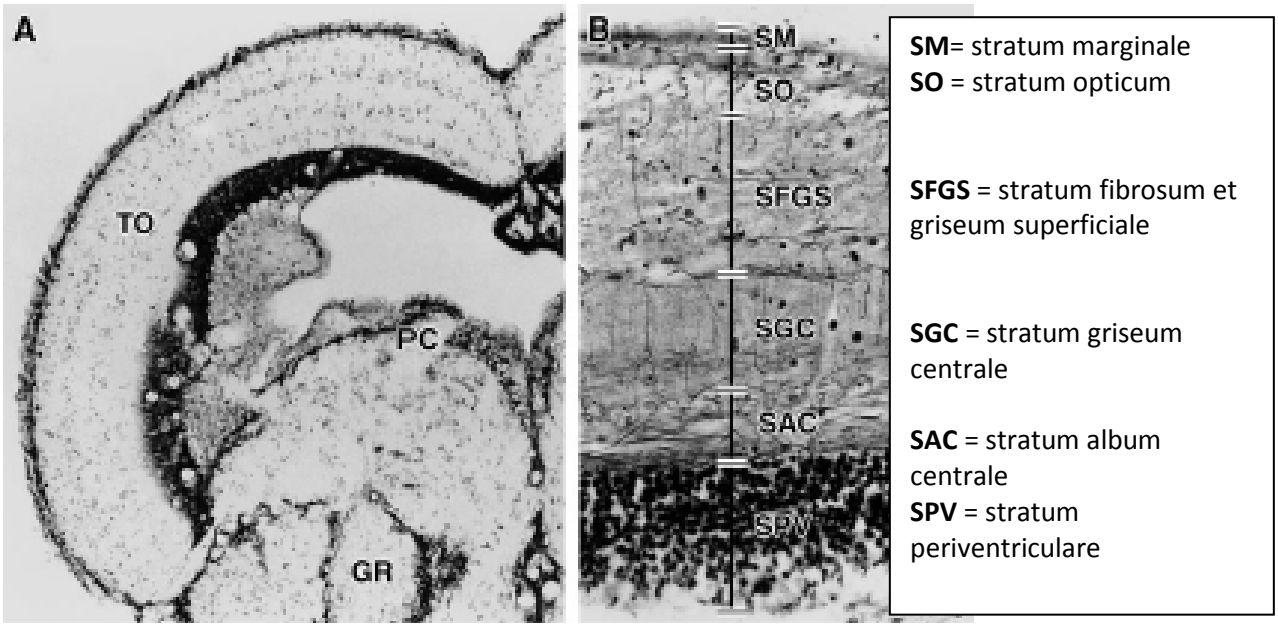


Figure 1: Optic tectum in *Medaka*. (A) The optic tectum (TO) is under low magnification. (B) The tectum is under higher magnification and has the layers indicated (Ishikawa *et al.*, 2001).

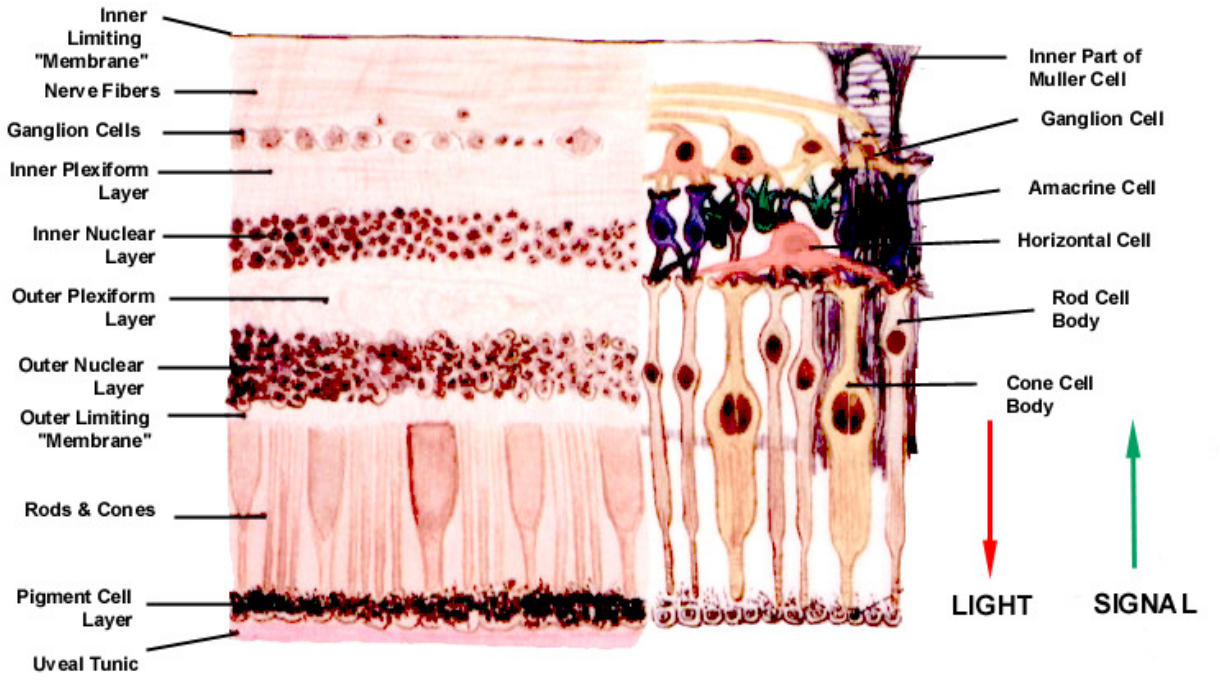


Figure 2: The vertebrate retina, with retinal layers labeled at the left and specific cell types identified at the right (Caceci, 2001).

Figure 3: The surface form of *Astyanax* (left), and the cave form of *Astyanax mexicanus* (right). The right cavefish has regressed eyes (*Mexican tetra,* 2007).

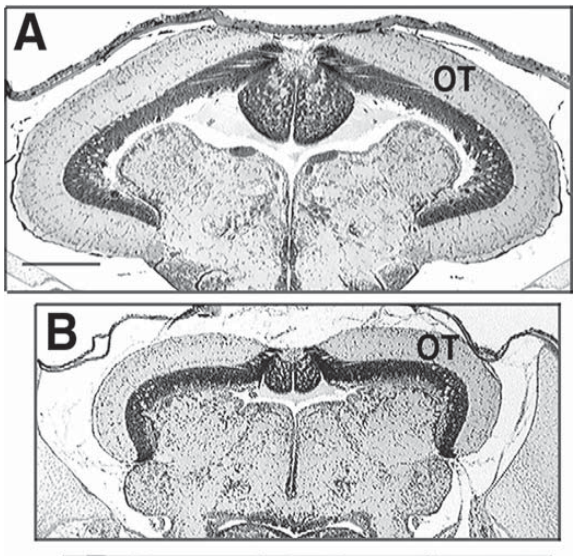


Figure 4: Comparison of the optic tectum in surface and cave forms of *Astyanax* mexicanus. (A) The optic tectum (OT) from surface fish. (B) The optic tectum of cave fish (Soares *et al.*, 2004). A difference in the sizes of the optic tecta can be seen and the tectum is clearly larger in the surface fish.

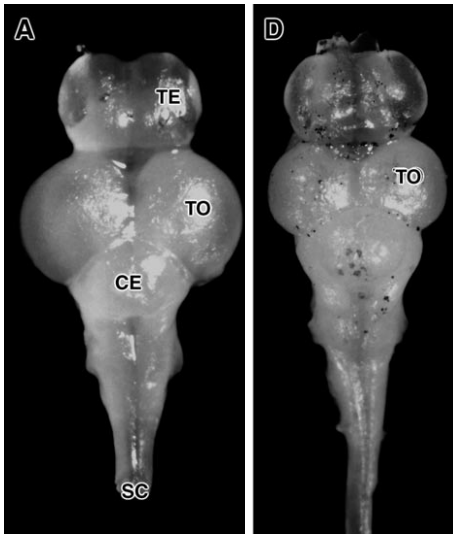
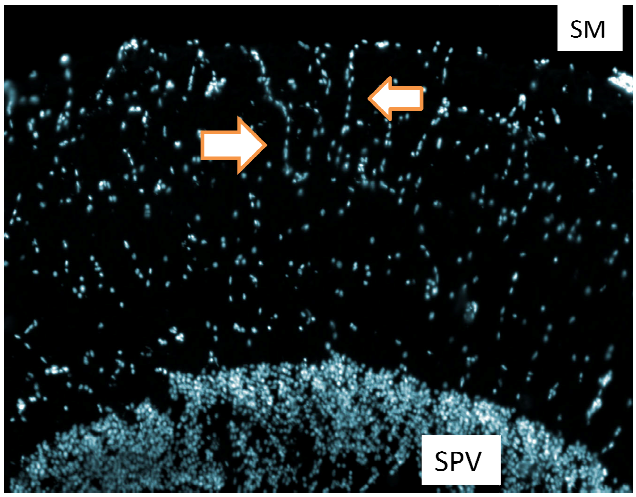


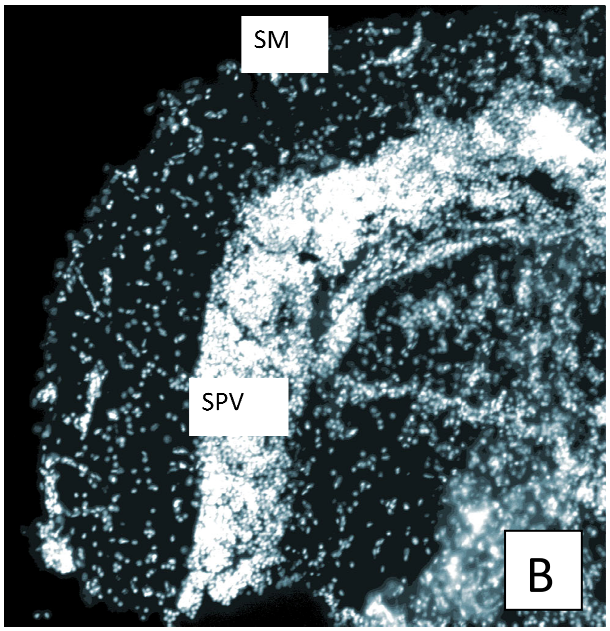
Figure 3: Images of Medaka brain. (A) The brain of *Medaka* with fully functioning eyes. (D) The brain of the eyeless form (Ishikawa *et al.*, 2001). Note that the tectum is smaller in the *eyeless* form, similar to changes in tectal size observed in blind cavefish brains.

Surface

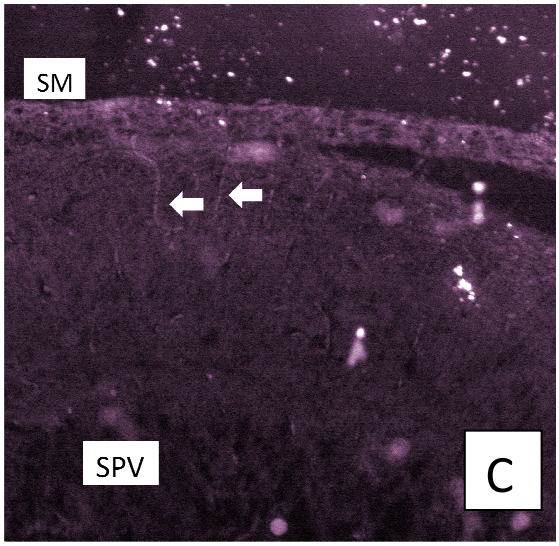


Labeled with DAPI (nuclear stain)

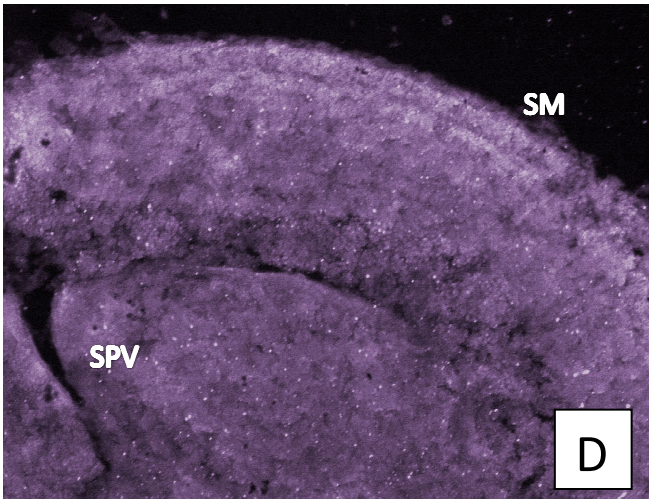
Cave



Surface



Cave



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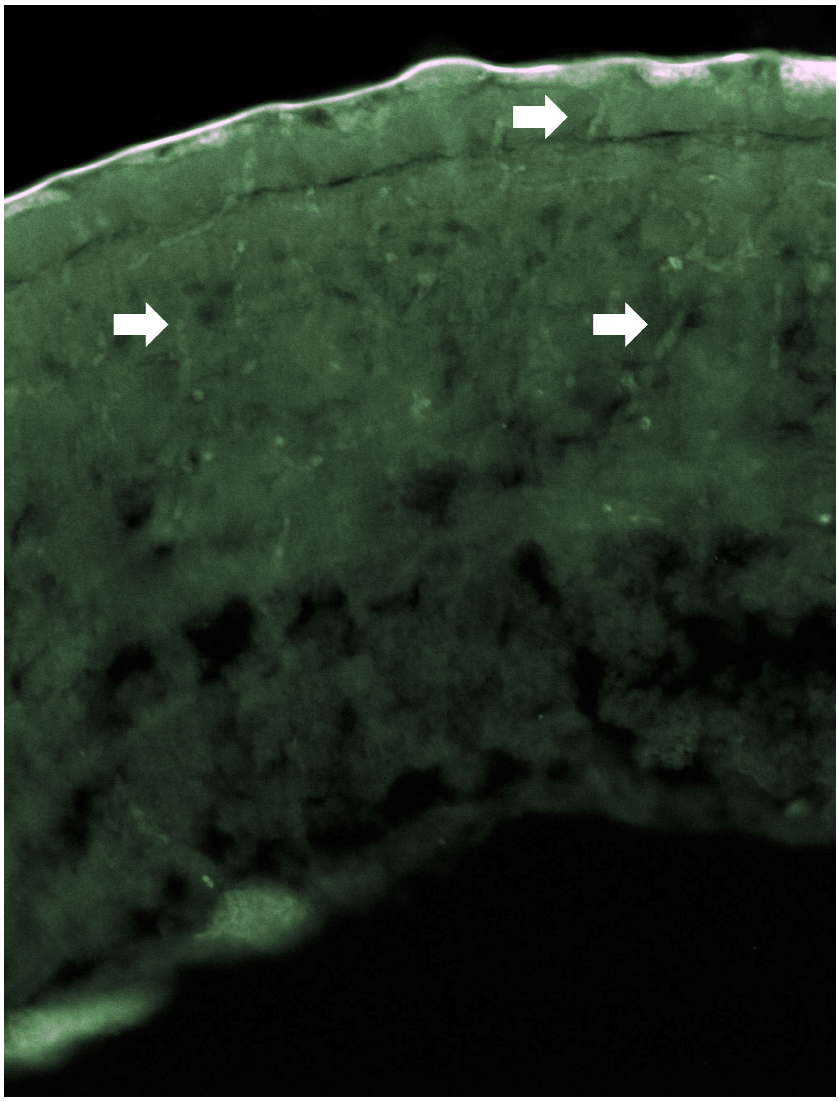
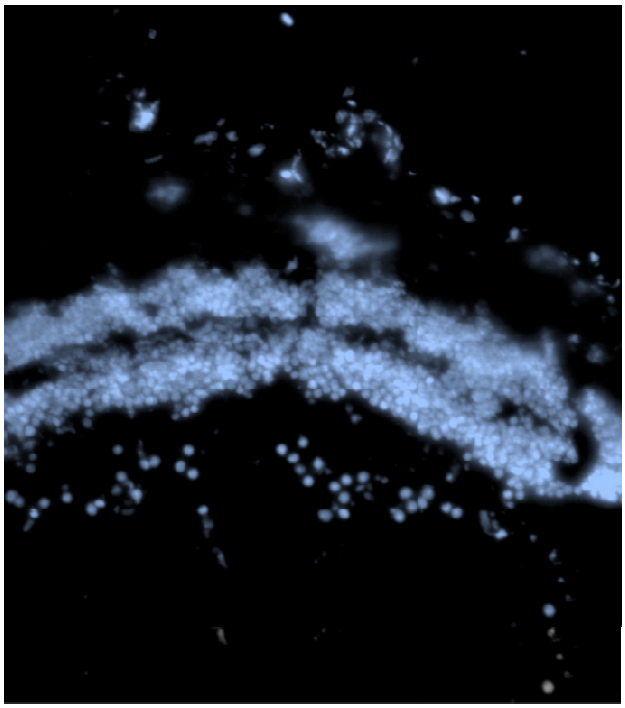
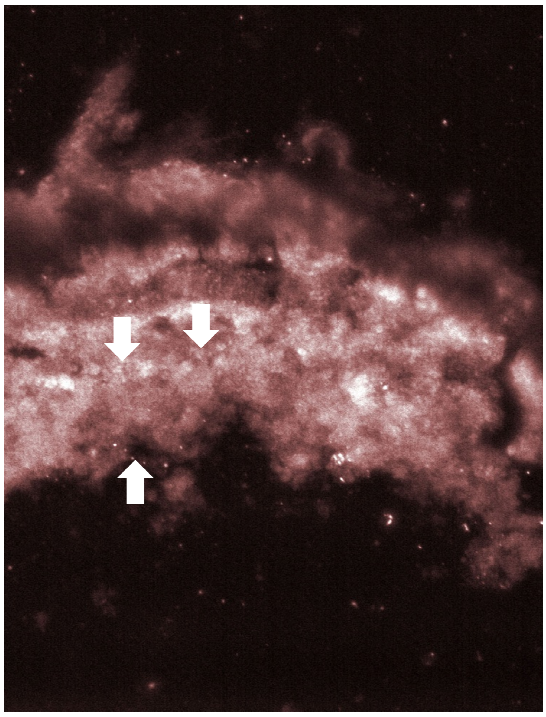


Figure 5: Optic tectum of surface *A. mexicanus*, contains TOH-positive processes. Arrows point to the positive processes, which are distributed throughout the tectal layers



Labeled with DAPI



Labeled with antibody to GABA

Figure 6: These images depict the same retinal section labeled with the nuclear stain DAPI (left) and probed with the GABA antibody (right) in the surface population. Arrows are pointing to positive processes, which are presumed amacrine cells.

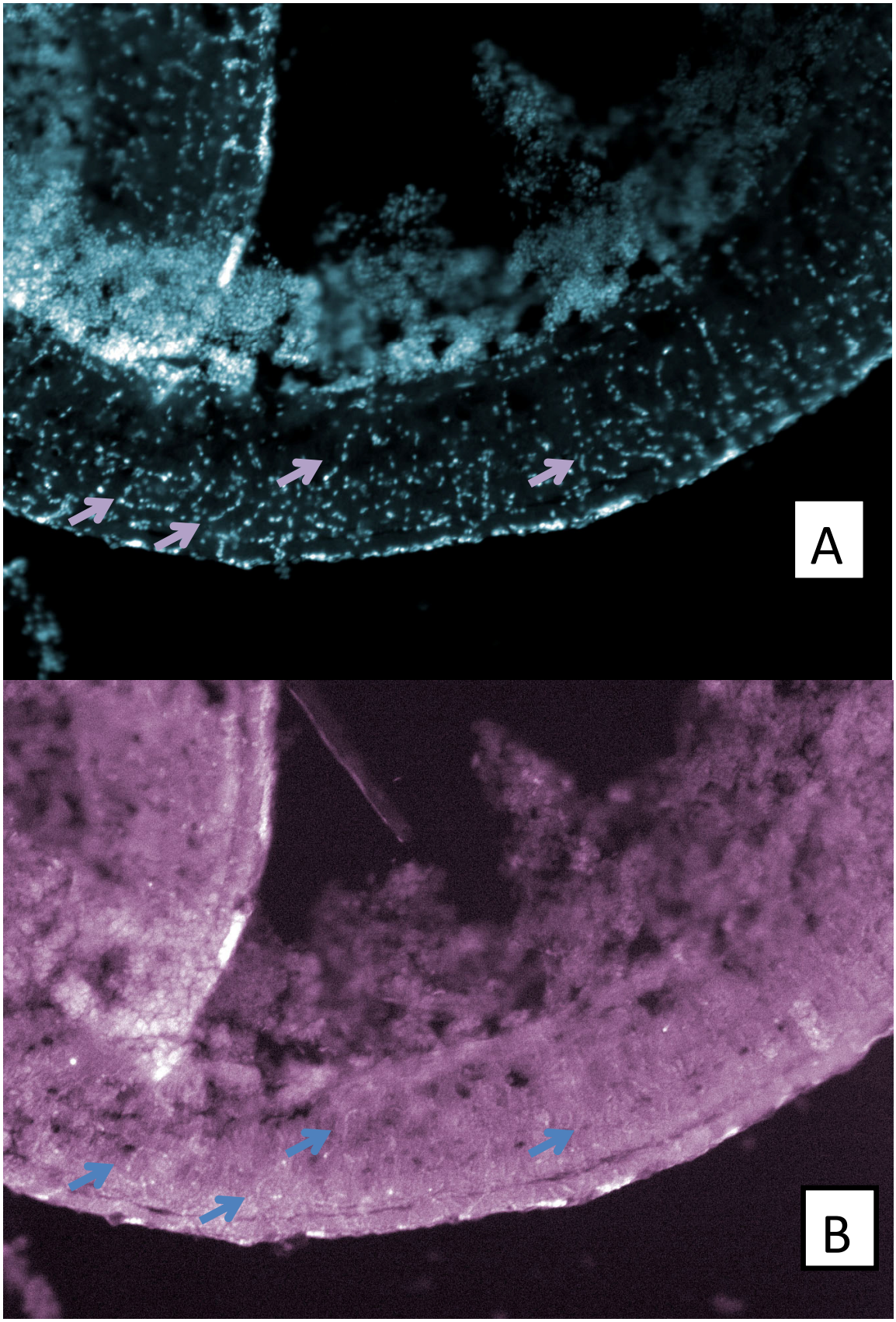


Figure 7: The optic tectum of surface *A. mexicanus*. This section was labeled with DAPI (A) and the GABA antibody. (B) From a surface fish's optic tectum. Arrows are pointing to positive processes, located primarily in the outer tectal layers.

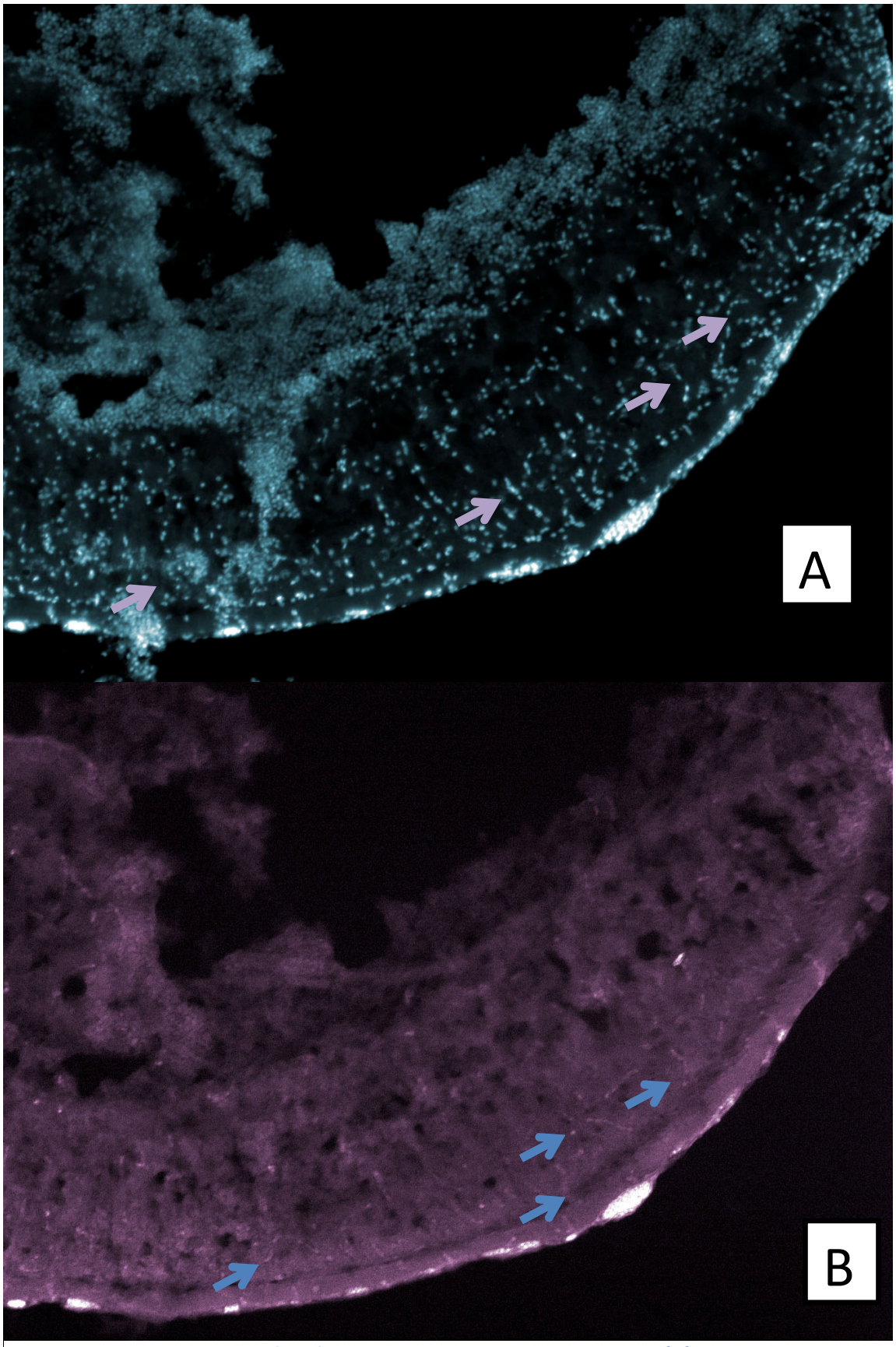


Figure 8: The Optic tectum of surface *A. mexicanus* labeled with DAPI (A) and the GABA antibody (B). Arrows are pointing to the positive processes, located in the outer tectal layers (as observed in Figure 10).

References

Anzelius, M., Ekstrom, P., Mohler, H., & Richards, J. G. (1995). Immunocytochemichal localization of GABA/Benzodiazepine receptor B2/B3 subunits in the optic tectum of salmon. *Journal of Receptors and Signal Transduction, 15*, 413-425.

Caceci, T. (2001, March). *The retinal tunic*. Retrieved from http://education.vetmed.vt.edu/Curriculum/VM8054/EYE/RETINA.HTM

Ishikawa, Y., Yoshimoto, M., Yamamoto, N., Ito, H., Yasuda, T., Tokunaga, F., . . . Wakamatsu, Y. (2001, November). Brain structures of a Medaka mutant, el (eyeless), in which eye vesicles do not evaginate. *Brain Behavior and Evolution*, 173-184.

Kaslin, J., & Panula, P. (2001). Comparative anatomy of the histaminergic and other aminergic systems in zebraﬁsh (Danio rerio). *The Journal of Comparative Neurology*, 342-377.

Kim, Y.-J., Nam, R.-H., Yoo, Y. M., & Lee, C.-J. (2004). Identification and functional evidence of GABAergic neurons in parts of the brain of adult zebrafish (Danio rerio). *Neuroscience Letters, 355*, 29-32.

Ma, P. M. (2003). Catecholaminergic systems in the zebrafish. IV. organization and projection pattern of dopaminergic neurons in the diencephalon. *The Journal of Comparative Neurology, 460*, 13-37.

Meek, J. (1983). Functional anatomy of the tectum mesencephali of the goldfish, an explorative analysis of the functional implications of the laminar structural organization of the tectum. *Brain Research Reviews, 6*, 247-297.

Mexican tetra [Photograph]. (2007, August 11). Retrieved from http://en.wikipedia.org/wiki/

Parry, J. W., Peirson, S. N., Wilkens, H., & Bowmaker, J. K. (2003). Multiple photopigments from the Mexican blind cavefish, Astyanax fasciatus: A microspectrophotometric study. *Vision Research, 41*, 31-41.

Soares, D., Yamamoto, Y., Strickler, A. G., & Jeffery, W. R. (2004, June). The lens has a specific influence on optic nerve and tectum development in the blind cavefish Astyanax. *Developmental Neuroscience*, 308-317.

Yew, D. T., & Yoshihara, H. M. (1977). An ultrastructural study on the retina of the blind cave fish (Asyanax hubbusi). *Cytologia, 42*, 175-180.

Yoshizawa, M., & Jeffery, W. R. (2008). Shadow response in the blind cavefish Astyanax reveals conservation of a functional pineal eye. *The Journal of Experimental Biology*, 292-299.