Evolutionary and homeostatic changes in morphology of visual dendrites of Mauthner cells in Astyanax blind cavefish

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Abstract

Mauthner cells are the largest neurons in the hindbrain of teleost fish and most amphibians. Each cell has two major dendrites thought to receive segregated streams of sensory input: the lateral dendrite receives mechanosensory input while the ventral dendrite receives visual input. These inputs, which mediate escape responses to sudden stimuli, may be modulated by the availability of sensory information to the animal. To understand the impact of the absence of visual information on the morphologies of Mauthner cells during developmental and evolutionary time scales, we examined the teleost Astyanax mexicanus. This species of tetra is found in two morphs: a seeing surface fish and a blind cavefish. We compared the structure of Mauthner cells in surface fish raised under daily light conditions, in surface fish raised in constant darkness, and in two independent lineages of cave populations. The length of ventral dendrites of Mauthner cells in dark-raised surface fish larvae were longer and more branched, while in both cave morphs the ventral dendrites were smaller or absent. The absence of visual input in surface fish with normal eye development leads to a homeostatic increase in dendrite size, whereas over evolution, the absence of light led to the loss of eyes and a reduction in dendrite size.

KEYWORDS

adaptation, evolution, fish, homeostasis, neuron

1 | INTRODUCTION

Startle responses are rapid reactions that move an animal away from perceived danger ( Peek & Card, 2016 ). These behaviors are found in many species and are typically mediated by fast-acting neural circuits characterized by large diameter neurons and reduced numbers of synapses ( Eaton, DiDomenico, & Nissanov, 1988 ; Eaton & Hackett, 1984 ). Mauthner cells are a pair of large neurons found in the hindbrain of teleost fishes that mediate escape responses ( Bartelmez, 1915 ; Bullock, 1978, 1984 ; Eaton, Lee, & Foreman, 2001 ). These neurons receive sensory input and project contralaterally descending axons onto primary motor neurons in the spinal cord ( Kimmel, Sessions, & Kimmel, 1981 ). Activation of a Mauthner cell results in rapid contraction of the contralateral musculature of the body trunk ( Faber, Fetcho, & Korn, 1989 ), leading to a stereotypical evasive maneuver known as a C-start ( Eaton & Emberley, 1991 ).

Each Mauthner cell has two large-diameter primary dendrites and each dendrite receives sensory information of different modalities. Acoustic and mechanosensory information targets the lateral dendrite (LD; Zottoli, 1977 ; Eaton et al., 1988 ) while visual and somatosensory information is routed to the ventral dendrite (VD) ( Canfield, 2003 ; Faber & Korn, 1978 ; Zottoli, Bentley, Prendergast, & Rieff, 1995 ; Zottoli, Hordes, & Faber, 1987 ). Visual inputs mediated by the VD reliably produce rapid escape responses across teleost species ( Canfield, 2006 ; Dunn et al., 2016 ; Preuss, Osei-Bonsu, Weiss, Wang, & Faber, 2006 ; Zottoli et al., 1987 ).

Troglobitic species throughout the world have convergent adaptations to life in caves, often including dramatic reductions of visual systems. Evolution in continual darkness has led to the degeneration...
of the visual system, and as animals become more cave-adapted, their eye structures are reduced and eventually vanish completely (Barr, 1968; Culver & Pipan, 2015; Fong, Kane, & Culver, 1995; Protas & Jeffery, 2012; Soares & Niemiller, 2013). The Mexican tetra fish, Astyanax mexicanus (Figure 1), is extant in two morphs: an ancestral, river-dwelling morph (surface fish) and various derived, cave-dwelling morphs. Each lineage has unique adaptations to troglobitic life and is named after the cave in which they are endemic (Figure 1a).

There are two main lineages of fish that contain eyeless fish. The older lineage includes Astyanax cavefish from the Subterráneo, Pachón, and Chica populations (Bradic, Teotónio, & Borowsky, 2013; Dowling, Martasian, & Jeffery, 2002; Gross, 2012; Porter, Dittmar, & Pérez-Losada, 2007). The younger lineage includes cavefish from the Los Sabinos, Curva, and Tinaja populations. These two lineages are morphologically distinct (Dowling et al., 2002; Figure 1b).

Although adult Astyanax cavefish lack eyes, eye development is nevertheless initiated during embryogenesis (reviewed in Jeffery, 2001 and Jeffery, 2009; Krishnan & Rohner, 2017; Emam, Yoffe, Cardona, & Soares, 2020). To examine the impact of the loss of visual input on neurons that mediate visually triggered escape responses, we compared the morphologies of Mauthner cells in surface fish and in two lineages of cavefish. Interestingly, we found that the VD that receives visual inputs was diminished or missing in both lineages of cavefish. To determine whether the loss of the VD is caused by homeostatic mechanisms, we examined the morphologies of Mauthner cells of surface fish larvae raised in complete darkness. The VDs of dark-reared surface fish larvae are larger and more branched that of those reared under a daily light cycle. This result shows that in this case, evolutionary and homeostatic mechanisms operate in opposite directions.

2 | MATERIALS AND METHODS

2.1 Animal care

Fish larvae originated from a breeding colony and were kept at 20°C water temperature in either a 12 hr:12 hr light–dark cycle (12L:12D, surface fish) or constant darkness (24D, surface and cavefish). Breeding was induced by gradually increasing the water temperature from 20 to 25 °C over 2 days, and fertilized eggs were collected in meshed containers on the bottom of the tanks, based on the procedures of Dr William Jeffery laboratory (University of Maryland). Animal husbandry and experimentation were covered under the Rutgers University IACUC protocol #201702685. NJIT is under Rutgers-Newark IACUC oversight.
2.2 Retrograde labeling of Mauthner cells

We injected dye into Mauthner cell axons in larvae at 4 days postfertilization (dpf) by adapting previous protocols for backfilling reticulospinal neurons in teleosts (Fetcho & O’Malley, 1995; Orger, Kampff, Severi, Bollmann, & Engert, 2008). We used borosilicate capillary glass needles (thick-walled, with internal filaments, Cat 1B150F-4, VWR), pulled with a Flaming/Brown micropipette puller (Model P-97, Sutter Instruments). We filled injection needles with 62.5 μg/μl of Dextran-TexasRed, 3,000 molecular weight (Cat D3328, Invitrogen) or 20 μg/μl Dextran-Alexa488, 10,000 molecular weight (Cat D22910, Invitrogen). We broke the needle tips by gently swiping against the sharp open end of a Vannas-Tübingen Spring Scissor (Cat 15003-08, Fine Science Tools) to produce a jagged-edged opening.

Larvae were anesthetized before injection in normal Ringer’s solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, 5.0 mM HEPES, and pH 7.2; Westerfield, 2000) containing 0.3% ethyl 3-aminobenzoate methanesulfonate (Tricane; MS-222 [Cat E10521, Sigma-Aldrich]). Anesthetized larvae were placed onto an agar-coated 35 mm Petri dish, and the injection needle was inserted laterally into the spine with a micromanipulator (M3301R, World Precision Instruments) under a dissecting light microscope (Olympus MVX10).

We injected caudal to the anal pore, approximately halfway between the anal pore and the end of the tail and took care to avoid injecting into blood vessels. To eject the dye from the pipette tip, we used a PicoPump (PV820, World Precision Instruments) with no more than 20 PSI of regulated house-air pressure. We let larvae recover in 24-well dishes in fresh fish water for at least 24 hr to allow for retrograde filling of reticulospinal cells. We screened larvae at 5 dpf, under an Olympus MVX10 stereoscope to confirm labeling of Mauthner cells before fixation.

2.3 Fixation

We transferred 5 dpf larvae to 1.5 ml centrifuge tubes (Eppendorf). All fish water was aspirated and replaced with 1.0 ml 4% paraformaldehyde (Electron Microscopy Sciences) in 1X phosphate buffer saline (PBS, Fisher Scientific). We fixed larvae overnight at 4 °C and washed fixative three times with 1X PBS by aspirating and replacing PBS in a centrifuge tube. Fixed larvae can be stored for up to 2 weeks in 1X PBS.

**Figure 2** Neuronal morphology differs among Mauthner cells in surface fish raised in different light conditions, and in Tinaja and Pachón cavefish. Volume rendering of representative right Mauthner cells of (a) surface fish raised in 12L:12D conditions, note the VD and two dendritic tips and (b) Mauthner cell of surface fish raised in 24D conditions, note four dendritic tips on the ventral dendrite. (c) Mauthner cell of Tinaja cavefish, note the ventral dendrite is smaller than those of surface fish. (d) Mauthner cell of Pachón cavefish, note the absence of ventral dendrite. ax, axon; arrowheads, dendritic tips; LD, lateral dendrite, So, soma, VD, ventral dendrite. All neurons from 5 dpf larvae. Scale bar: 20 μm.
2.4 Image acquisition

We dissected the brains of fixed larvae using custom-made tools made of sharpened tungsten wires attached to glass pipettes. We moved each brain to a drop of glycerol in a custom-made coverslip chamber. Briefly, we attached two $20 \times 20$ mm #1.5 coverslips to a $20 \times 40$ mm #1 coverslip with a room-temperature-hardening mixture made of equal weights of petroleum-jelly, lanolin, and paraffin (VALAP), creating a vertical channel. We placed fixed brains in the channel, covered it with another $20 \times 20$ #1.5 coverslip and used VALAP to seal the channel. To image whole brains we used a laser scanning confocal microscope (Leica SP8; microscope: DM6000CS; objectives: Leica $\times 20$/NA0.70 HC PL APO, Leica $\times 40$/NA1.30 HC PL APO oil or Leica $\times 63$/NA1.40 HC PL APO oil, with lateral resolutions of 414, 223, and 207 nm, respectively; laser lines: 561 nm (for Dextran-TexasRed) and 488 nm (for Dextran-Alexa488)). We collected multiple optical slices (thickness optimized by the confocal software, ranging 0.343–0.345 μm for the $\times 63$ objective, and 0.41–0.422 μm for the $\times 40$ objective, and 0.95 μm for the $\times 20$ objective) that contained the complete dendritic structure of Mauthner cells in each brain.

**FIGURE 3** Mauthner cell ventral dendrites of surface fish increase in length and number of branches when raised in 24D conditions, while cavefish dendrites are smaller or absent. (a) Maximum dendritic length. (b) Number of dendritic tips. (c) Maximum dendritic diameter. (d) Length of primary branch. Three comparisons against the shared control surface (Sur.) 12L:12D are shown in Cumming estimation plots in which the raw data is plotted on the upper axes. On the lower axes, mean differences are plotted as bootstrap sampling distributions, each mean difference is depicted as a dot, and each 95% confidence interval is indicated by the ends of the vertical error bars. The numbers of neurons (animals) are surface (Sur.) 12L:12D: n = 7 (5), surface (Sur.) 24D: n = 6 (3), Tinaja: n = 8 (5), Pachón: n = 7 (4)
2.5 | Image processing and measurements

We processed images and measured features using a dedicated software package (Leica Application Suite X 3D Analysis module, Leica Microsystems). We used the viewer panel of the 3D Analysis module to linearly adjust the brightness and contrast of images of volume-rendered 3D objects. Next, we measured the maximum dendritic length from the edge of the soma to the farthest tip of VD with a line-measurement tool (adjusting the line to the dendrite from at least two orthogonal views), the maximum dendritic diameter, and the distance to the first branching point (i.e., length of the primary branch). We reduced background noise with a white noise reduction filter within the 3D Analysis module and cropped neighboring reticulospinal neurons labeled in the hindbrain with Adobe Illustrator to visually isolate

FIGURE 4  Mauthner cell morphology is variable among morphs and experimental conditions. (a1–a6) surface 12L:12D (b1–b5) surface 24D (c1–c7) Tinaja (d1–d6) Pachón. Scale bar: 20 μm
the Mauthner cells. The branches of the VD grow medially toward the soma at the terminus of the VD. We note that it is a hallmark of the VD in young Mauthner cells that finer neurites protrude laterally away from the soma and along the proximal length of the dendrite. We do not consider the finer neurites as branches for our analysis.

2.6 | Statistical analysis

We compared experimental groups (Surface 24D, Tinaja and Pachón) to the control group (surface 12L:12D) by estimation statistics that computes the effect size of each resample and determines 95% confidence intervals of the mean difference by performing 5,000 bootstrapping resamples (Ho, Tumkaya, Aryal, Choi, & Claridge-Chang, 2019). We used ordered groups analysis of variance test and two-sided permutation t test to determine whether there were significant differences among the means of measurements. We considered an alpha value of .05 to be significant.

3 | RESULTS

We examined Mauthner cells of A. mexicanus larvae (Figure 1) by retrograde labeling of the neurons with dextran-conjugated dyes, fixing and positioning the larvae to view the morphology of the labeled Mauthner cells located in the hindbrain (Figure 2a inset, Video S1). Mauthner cells have two primary dendrites: a LD that receives auditory and lateral line input and a VD that is thought to receive visual and tactile input (Medan, Mäki-Marttunen, Sztarker, & Preuss, 2018). We compared the morphology of Mauthner cells of surface larvae raised under one of two conditions, a daily light cycle (12L:12D) or constant darkness (24D), and of cavefish of two lineages, to determine if morphological differences arise in the VD if visual inputs were removed during development or evolution (Figure 2). The maximum dendritic length to the farthest tip, number of dendritic tips, maximum dendritic diameter, and length of the primary dendrite of VDs in 24D surface fish larvae and in Tinaja and Pachón cavefish were significantly different from those in 12L:12D surface fish larvae (Figure 3). The two cavefish lineages were also significantly different from each other in maximum dendritic length, number of dendritic tips, and maximum dendritic diameter.

VDs of 24D surface larvae were significantly longer (84.35 ± 11.46 μm, p < .0001), while VDs of Tinaja larvae were significantly shorter (22.44 ± 19.62, p = .0028) than 12D:12L surface larvae (54.76 ± 8.67; Figure 3a). Since Pachón cavefish larvae did not have VDs (Figures 2d and 4d1–d6), their length was recorded as 0 (p < .0001 compared to 12L:12D surface fish and p = .009 to Tinaja cavefish; Figure 3a).

In 12L:12D surface larvae (Figures 2a and 4a1–a6), VDs began with a 2.98 ± 0.5 μm diameter primary branch with several finer processes protruding medially (Figure 3c). The primary branch branched once after 32.86 ± 7.83 μm into two terminal branches (Figure 3b,d). In 24D surface larvae (Figures 2b and 4b1–b5), VD primary branches were thicker and longer than controls (4.5 ± 0.89 μm, p = .02198; 43.09 ± 9.95 μm, Figure 3c,d). They often arborized into three or more terminal branches (Figure 3b) and also displayed fine medial processes off the length of the dendrite. In Tinaja cavefish, the neurons...
appeared overall smaller than in surface fish (Figures 2c and 4c1-c7) with occasional branching of VD (Figure 3b) that appeared thin (diameter 1.73 ± 1.36 μm; Figure 3c) and truncated with fine medial processes. In the three Tinaja larvae in which the VD did branch, the primary branches were 19.78 ± 5.07 μm long, shorter than the primary branches of 12L:12D surface larvae (p < .0001).

The number of dendritic tips is a measure of terminal branching (Figure 3b). VDs of 24D surface larvae were more arborized than the stereotypic bifurcated VD of 12L:12D surface larvae (p = .0008), while VDs of Tinaja larvae were less arborized (p = .0082). Since Pachón cavefish larvae did not have VDs, their numbers of dendritic tips were recorded as 0 (p < .0001 to control-raised surface fish; Figure 3b).

Furthermore, we measured the total length and thickness of LDs from each of the four groups. The lengths of LDs of the four groups were not significantly different from each other; 37.37 ± 11.08 μm for 12L:12D surface larvae, 50.35 ± 15.28 μm for 24D surface larvae, 30.42 ± 10.71 μm for Tinaja larvae, and 41.67 ± 12.52 μm Pachón larvae (Figure 5a). In contrast, the lengths of 12L:12D surface larvae were thinner than those of 24D surface larvae (6.00 ± 1.12 μm and 8.07 ± 0.98 μm, respectively; p = .022), and thicker than those of Pachón (4.43 ± 0.88 μm; p = .0132), but not different than the LDs of Tinaja larvae (5.48 ± 1.47 μm) (Figure 5b).

4 | DISCUSSION

One of the most intriguing questions in Biology is how evolution changes phenotypes and how evolutionary pressure interacts with homeostatic changes that occur on a much shorter time scale. Do homeostatic responses provide a mechanism for evolutionary modifications? Or conversely, are evolutionary modifications the outcomes of generations of unbalanced homeostatic responses? The latter seems to be a simple and straightforward explanation of how adaptation could be influenced by homeostatic mechanisms. However, here, we have found at least one example of a homeostatic response that leads to the opposite outcome than an evolutionary adaptation.

To examine the influence of the visual environment on the developing circuit, we raised surface morph larvae of A. mexicanus in constant darkness. We predicted that in these fish, the VD would be smaller, but instead, the dendrite was not only longer, but also much more branched than daily light (control) raised fish. This is interesting because it is consistent with the work of Shtanchaev, Mikhailova, Dehtyareva, Kanokova, and Moshkov (2008), where they showed morphological plasticity of the VD of the Mauthner cell in the goldfish depending on the level of stimulation. In those fish, there is a negative correlation between size and level of stimulation. We have shown that A. mexicanus surface fish display the commonly found Mauthner cell morphology, which is shared among fishes and amphibians. However, the cavefish morphs have either much smaller or absent VDs, depending on the duration of the cavefish lineage isolation in the darkness of a cave.

Astyanax as a model animal allowed us to study two different time scales for changes in the morphology of a dendrite involved in visually guided behavior: early development and evolution. Conceptually, homeostatic mechanisms hinder changes, whereas evolutionary processes drive them. Here, the evolutionary changes in larval Astyanax were caused by adaptation to troglobitic life, including the loss of eyes and concomitant changes in neural control networks that rely on vision. Homeostatic regulation was caused by rearing surface fish in complete darkness, eliminating visual signals during development. We compared each of these groups to the basal morphology exhibited by surface fish raised in alternating light and dark epochs like those that the fish experience in nature.

We expected both time scales to follow the same path, and our main findings show that cave adaptation is correlated with a decrease in the size of the dendrite in an evolutionary time scale of tens of thousands of years, but during development occurring over days, darkness has the opposite effect, increasing the size and complexity of the visual dendrite. A possible outcome would have been that cavefish maintained their visual dendrites after cave adaptation, and these dendrites were co-opted by another sensory modality. Another reasonable outcome would have been that dark reared surface fish lost, or at least decreased, visual dendrites. We found neither of these outcomes in Astyanax. Instead, reduced input during the time scale of development resulted in an increase in the morphological complexity of VDs, an opposite result from the evolutionary time scale.

It has been a long-standing question why in rivers with so many species ofteleosts, only Astyanax has been the one to colonize caves successfully, and multiple times (Bilandžija et al., 2020; Yoffe et al., 2020). Here, we added neuromorphological homeostasis to the body of evidence that argues for Astyanax as a particularly plastic species, which is advantageous for dispersal. Accordingly, homeostatic mechanisms are under natural selection, providing malleability for adaptation to constant darkness.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Zainab Tanvir, Daphne Soares, Kristen E. Severi, and Gai Haspel: Conceived the project. Zainab Tanvir, Daphne Soares, and Daihana Rivera: Collected and analyzed the data. All the authors discussed the project throughout and wrote the paper.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
REFERENCES


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