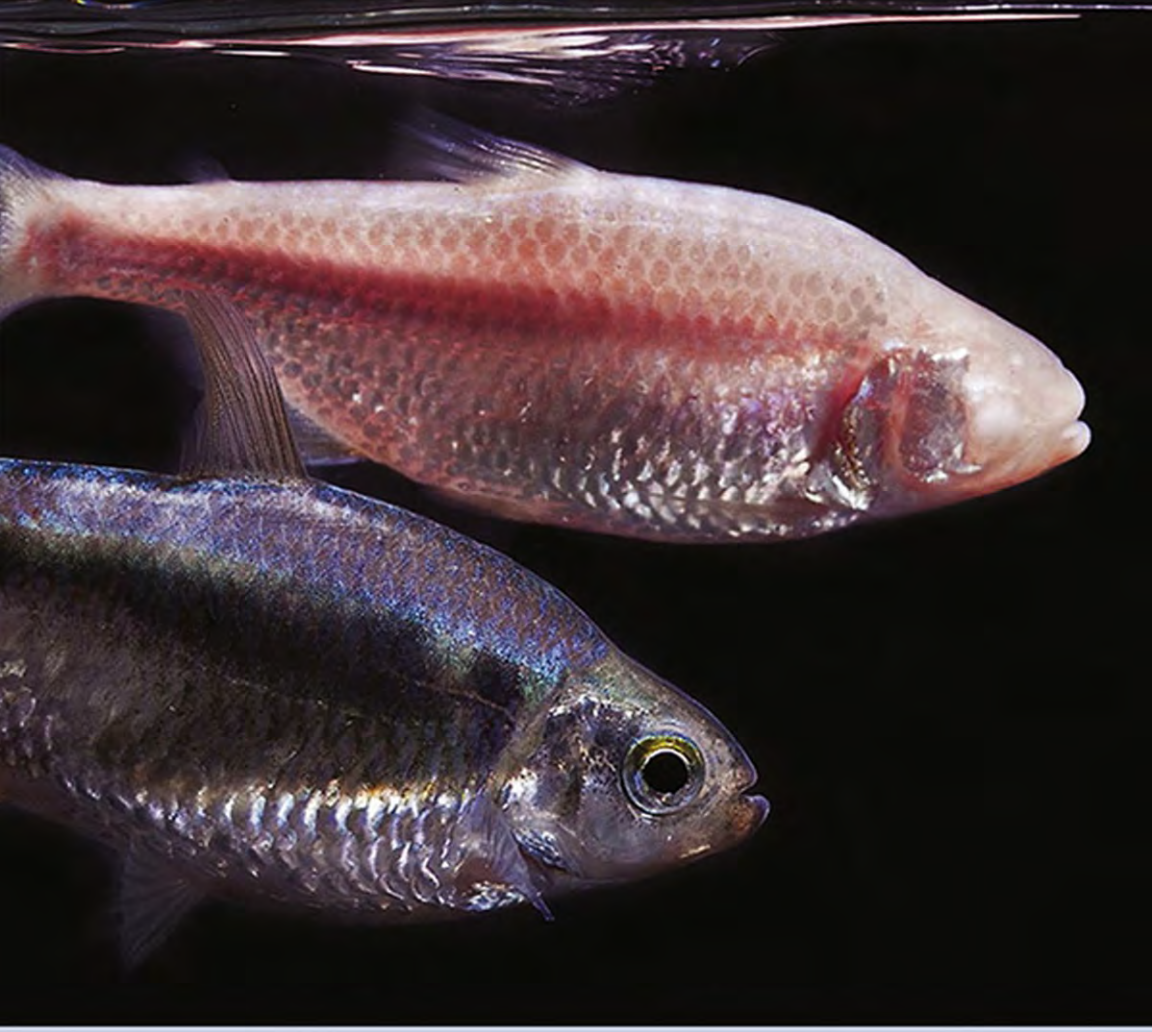

Biology and Evolution of the Mexican Cavefish

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Preface

Evolution has created a spectacular assortment of diversity that has intrigued naturalists for centuries, and more recently, has been used by biologists to investigate basic principles of life on earth. In the same light that biomedical research uses dysfunction such as cancer or neurological disease to better understand function, the extreme cases of evolutionary processes can be used to study the basic principles that govern adaption in response to a changing environment. Perhaps one of the most distinct shifts in environment seen in nature is one of moving from surface to subterranean life.

Throughout the world are examples of cave animals, ranging from salamanders to small insects that have evolved cave-like traits that include albinism and eye loss. The Mexican cavefish, *Astyanax mexicanus*, provides a particularly striking system, because fish evolved in 29 geographically isolated caves over the last ~5 million years. While these fish look dramatically different from their river-dwelling counterparts, they remain interfertile, providing biologists with a tool to investigate the genetic basis for developmental, anatomical, and behavioral evolution.

The interest in cavefish extends well beyond the scientists using this system to those interested in cave exploration, biology, zoology, and evolution. The book is written to provide both historical perspective and a current snapshot of research on these fish. The first investigation of these caves, dating back to the early 1920s, included the heroic attempts by early speleologists to characterize the geology and biology of cave life. This book takes readers from the initial discovery of these caves to early experiments classifying fish through recent advances in genomics and neuroscience. As such, the diverse authors share a variety of perspectives that are pertinent to some of the ongoing discussions and debates about the biology of *Astyanax*.

The decision to write this book largely stems from the unique state of the research community. The recent era of genomics has provided powerful tools for investigating the evolutionary and population history of these fish. A genome for Mexican cavefish was published only last year, and the advent of genome-editing tools may allow for the identification of genes regulating behavior and developmental processes at a resolution previously thought possible only in genetically amenable systems, such as mice, zebrafish, and fruit flies. Therefore, we believe this is an excellent time to review the history of investigation in this field, as opportunities and interest in this system are likely to expand greatly in the future.

Many of the contributors to this book are the titans of the field and responsible for some of the most important discoveries in this system. Included are contributions from Bill Elliot, part of a small team that explored many caves for the first time; Bill Jeffery (University of Maryland) and Cliff Tabin (Harvard), who led work describing biology underlying albinism and eye loss in cavefish; Richard Borowsky (New York University), who has used genomics to trace the evolution of these fish; and Sylvie Rétaux (CNRS, France), who has identified many factors governing changes in brain development and behavior in cavefish.

Also included are contributions from more junior researchers that have recently started their independent research careers. As editors, we fall into this category and are grateful for the support we have received from our senior colleagues. We hope that this book serves as a captivating read and conveys the history and promise of this fascinating biological system.

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Chapter 12

Neural Development and Evolution in *Astyanax mexicanus*: Comparing Cavefish and Surface Fish Brains

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INTRODUCTION

Anybody who visits an *Astyanax* facility hosting surface fish and cavefish in adjacent tanks and who has a good sense of observation will at first have a hard time believing that they truly belong to the same species. Indeed, the two morphs look so different. Usually, the visitor first sees that one is depigmented and albino while the other is nicely colored. Then he rapidly gets a feeling that there is "something wrong" with the head of the cave morph, and he finds that the eyes are missing. These are the two main, obvious morphological differences.

The attentive observer will further compare the two types of fish and he will find much more. He will see that surface morphs swim in the water column and school, while cave morphs have a tendency to occupy the bottom half of the tank, and swim constantly on their own. He will notice that in the surface fish groups, one or two individuals constantly strike at some others, behaving as dominant in the school, and that this does not apply to cave morphs. On the other hand, he will be surprised by how well blind morphs navigate in their tank, almost never bumping on the aquarium walls or into their congeners. If he has the chance to visit the fish facility at feeding time, he will be struck by the fast and furious way the surface fish swim toward food; and he will appreciate the special feeding posture taken by cave morphs, which allows them to clean the food from the bottom of the tanks efficiently within a few minutes. These differences, together with some others that are not obvious at first sight (e.g., reduced sleep/increased wakefulness, attraction to vibrations or olfactory capabilities),

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correspond to major behavioral differences between the two *Astyanax* morphs. They correspond to behaviors that can be classified as various types: (1) sensory; (2) motor; and (3) other, more complex and motivated behaviors, which all are governed by various parts of the nervous system.

What are the developmental and evolutionary mechanisms underlying the above-listed changes in the cavefish nervous system and its associated behaviors? Research in the field has mainly explored two directions that will be reviewed here.

First, comparative neurodevelopment and comparative neuroanatomy studies have revealed quantitative variations in the size of specific regions of the brain or in the number or size of specific sensory organs between cave and surface morphs. This type of variation can be coined as "neural specialization," supposedly in adaptation to environmental changes. For example, in the dark, it is probably advantageous to be "olfactory-oriented" to find food and mates, while in a lighted environment, it is important to maintain visual function. Classical cases of such brain evolutionary specialization come, for example, from nocturnal rodents in which the visual cortex is reduced, but the auditory and somato-sensory cortex is expanded (Campi and Krubitzer, 2010; Krubitzer et al., 2011). Among fishes, similar processes are described in cichlid fishes. In African lakes, very closely related cichlid species with distinct ecological specializations have significantly different brains: rock-dwellers (Mbuna) live in complex environments, engage in complex social interactions, and have a large telencephalon; while sand-dwellers (non-Mbuna) live in a simple environment, essentially use visually driven behaviors, and have a large optic tectum and thalamus. Interestingly, it has been shown that differences between Mbuna and non-Mbuna arise early in development, and that boundaries between brain regions, hence the respective sizes of these brain regions, are set up through antagonisms among signaling systems (Sylvester et al., 2010, 2013). In cave *Astyanax* as well, we will see that natural variations in nervous system patterning occur through early signaling modulations.

Second, some recent evidence suggests evolution of "brain neurochemistry" between cave and surface *Astyanax*. Indeed, even subtle changes in neuromodulatory systems are prone to generate significant variations in complex behaviors, such as motivated or social behaviors. This can be achieved if the number of neurons using a given neurotransmitter is changed (a situation that resembles the possibility discussed above, in which the size of a brain region or a neuronal group varies), or if the intensity or amount of neurotransmission is affected at the level of the synthesis, release, reception, modulation, or transduction of the signal. From a neurophysiological point of view, the behavioral syndrome—which is a correlated suite of behavioral phenotypes across multiple situations (Sih et al, 2004), such as those described above—exhibited by cave *Astyanax* clearly evokes the possibility of such disequilibrium in neuromodulatory transmitters.

ADULT BRAIN ANATOMY AND BRAIN NETWORKS

Figure 12.1 presents the comparative anatomy of adult Pachón cavefish and surface fish brains at the macroscopic level. As described by Riedel (1997), the cavefish brain is "slender and elongated." This impression is mainly due to the difference of shape of the telencephalon (trapezoidal in cavefish, ovoid in surface fish) and to the severe reduction in the width and global size of the optic tectum in cavefish (Figure 12.1(A) and (B)).

Concerning the telencephalon, volumetric studies indicate it is enlarged in the Pachón population, but not in other populations, such as Micos or Chica (Peters et al., 1993). The authors of this study hypothesized that telencephalic enlargement was due to the enhancement of the sense of taste, but this should be confirmed by connectivity studies. Qualitative and quantitative observations done in our laboratory in adults and juveniles also suggest that the olfactory bulbs are larger in cavefish (Figure 12.1(A) and (B); Rétaux and Bibliowicz, unpublished). Concerning olfactory connectivity, Riedel and Krug have documented

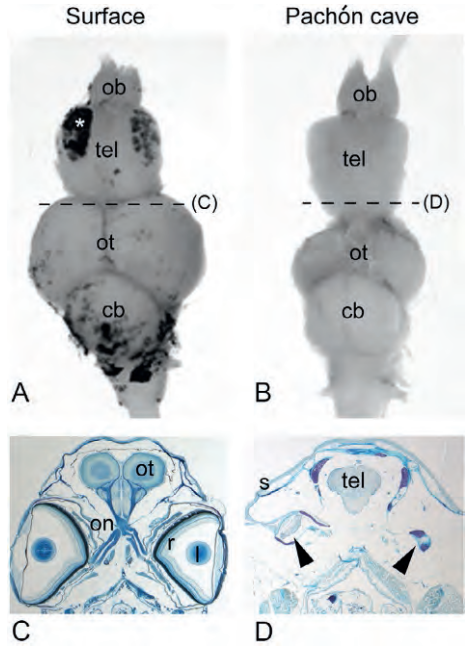

FIGURE 12.1 Comparing adult brains in *Astyanax* surface fish and Pachón cavefish. (A and B) show dorsal views of adult brains after dissection (anterior is up). The two individuals were of identical size (4 cm standard length). The dotted lines indicate the approximate section levels shown in (C) and (D). ob, olfactory bulbs; tel, telencephalon; ot, optic tectum; cb, cerebellum. (C and D) show frontal sections through the head of adult fish, after Klüver and Barrera coloration. The arrowheads on the Pachón picture show degenerated and cystic eye, partially calcified (dark/purple) and covered by skin. r, retina; l, lens; on, optic nerve; ot, optic tectum; s, skin.

that the projections of the olfactory bulb onto the cavefish telencephalon resemble a "simple Bauplan," and they concluded that the telencephalon is not dominated by olfactory inputs (Riedel and Krug, 1997); however, as they only analyzed the cavefish olfactory projections, no comparative surface fish data exists to determine olfactory specialization, or lack thereof, at this level.

At the diencephalic level, the major difference between the two morphs is the absence of eyes in cavefish, and is accompanied by a very severe reduction of the optic nerves (Riedel, 1997; Figure 12.1(C) and (D)). Despite evolution in complete darkness, cavefish have nevertheless conserved their "pineal eye"; the dorsal diencephalic pineal gland (or epiphysis) is structurally intact (Grunewald-Lowenstein, 1956; Herwig, 1976; Langecker et al., 1993; Omura, 1975) and has conserved the ability in larvae to detect light, probably thanks to correct rhodopsin expression (Yoshizawa and Jeffery, 2008). A specific and progressive regression of the regular outer-segment organization of pineal sensory cells nevertheless occurs in 3-, 9-, and 18-month-old cavefish, without affecting other parts of the pineal gland (Herwig, 1976). Interestingly, this regression begins earlier and is more obvious when cavefish are reared in constant darkness than when they are reared in light/dark conditions (Herwig, 1976), and it also occurs in constant light (Omura, 1975). This suggests that part of the degenerative process is attributable to a lack of light-activated neuronal activity.

Caudally, in the mesencephalon, the difference in the size of the optic tectum of the two morphs is striking (Figure 12.1(A) and (B)). This applies to all *Astyanax* cavefish populations examined, including Micos and Chica for which no difference was found with the surface fishes' telencephalon (Peters et al., 1993), or Los Sabinos (our personal observations), and to various extents that seem related to the degree of eye reduction. Logically, tectal hypomorphy has been linked to eye rudimentation (see below). Regarding the connection, as stated above, the optic nerve is greatly reduced, and projections from the retinal cyst are very sparse (Voneida and Sligar, 1976; Figure 12.1(C)). Some residual fibers can be seen in the superficial layers of the medial third of the tectum (as well as in the nucleus opticus hypothalamicus and lateral geniculate nucleus); however, this remnant visual connection is unresponsive to visual cues, and no electrophysiologically detectable signal can be recorded from the optic cyst onto the tectum (Voneida and Fish, 1984). This poses the question of the function of the cavefish "optic" tectum, which does indeed contain efferent pyramidal cells. The only evoked activity that is recordable in the cavefish tectum is generated after somatosensory stimulation (but not lateral line or auditory stimulation, which are invariably evoked in the torus semicircularis), and in a topographical manner (Voneida and Fish, 1984). As it is not unusual to find extravisual modality in the vertebrate tectum, the authors interpreted this finding as a decrease in visual inputs paralleled by an increase in somatic inputs, a situation that is comparable to experimental models following enucleation (e.g., Benedetti, 1992; Chabot et al., 2007; Champoux et al., 2008; Mundinano and Martinez-Millan, 2010). An interesting question would be to know whether the

visual-to-somatic rewiring in cavefish occurs during development as a plasticity phenomenon, in parallel to the progressive degeneration of the eye and the loss of visual innervation, or whether this rewiring is genetically programmed and has already been fixed during evolution in the dark. Comparison with visually deprived surface fish would start answering this question. More generally, cavefish are useful models when studying vision-related and vision-dependent neural plasticity phenomena.

A SPECIAL CASE: DEVELOPMENT AND DEGENERATION OF THE CAVEFISH VISUAL SYSTEM

The genetic mechanisms underlying eye loss in cavefish are reviewed in Chapter 11 of this book (Yamamoto et al.), and the evolutionary forces leading to eye loss have been discussed recently (Rétaux and Casane, 2013). Here, we will only briefly describe the progressive remodeling of the visual system that occurs in cavefish between embryonic and adult stages.

During cavefish early embryogenesis and larval development, an eye is formed from the diencephalic neuroepithelium and its adjacent lens placode. This eye starts forming retinal layers (Alunni et al., 2007) and the proliferative zones of both the retina and the lens are active, although they are smaller than in surface fish larvae (Alunni et al., 2007; Hinaux et al., 2015; Strickler et al., 2002; Figure 12.2(C′) and (D′)). In fact, retinal cells are constantly born and are incorporated into the retina, while concomitantly many retinal cells die by apoptosis. At the end, cell death will win the battle against neurogenesis, and the eye will disappear (Figure 12.1(D)). The initial trigger for eye degeneration in cavefish is thought to be lens apoptosis; transplantation of a surface fish lens into a cavefish optic cup is able to rescue the eye of the cavefish while the reciprocal experiment induces the degeneration of the surface fish eye (Yamamoto and Jeffery, 2000).

The optic tectum, a brain region that derives from the alar plate of the mesencephalon and that constitutes the major retinorecipient structure in the brains of fishes and amphibians, is also patterned and regionalized properly in cavefish. The presumptive optic tectum expresses *Pax6*, *Pax2*, *Engrailed2* in domains of equivalent sizes in the two morphs (Soares et al., 2004; see *Lhx9* in Figure 12.2(A) and (B)). During the first days of development, proliferation is also equivalent in the dorsal mesencephalon of cave and surface larvae (Menuet et al., 2007; Blin and Rétaux, unpublished observations) (see proliferating cell nuclear antigen (PCNA) in Figure 12.2(C) and (D)). When the DiI tracing technique is applied on the initial cavefish retinotectal projection at 36 or 72 h post-fertilization (hpf), it is found that the optic nerve develops from the axons of the first generated retinal ganglion cells, reaches the tectum, and even arborizes on its target (Soares et al., 2004). This may explain why cavefish may be able to see, or at least to have some visual abilities, very transiently, during their first days of life, as suggested by positive electroretinograms and

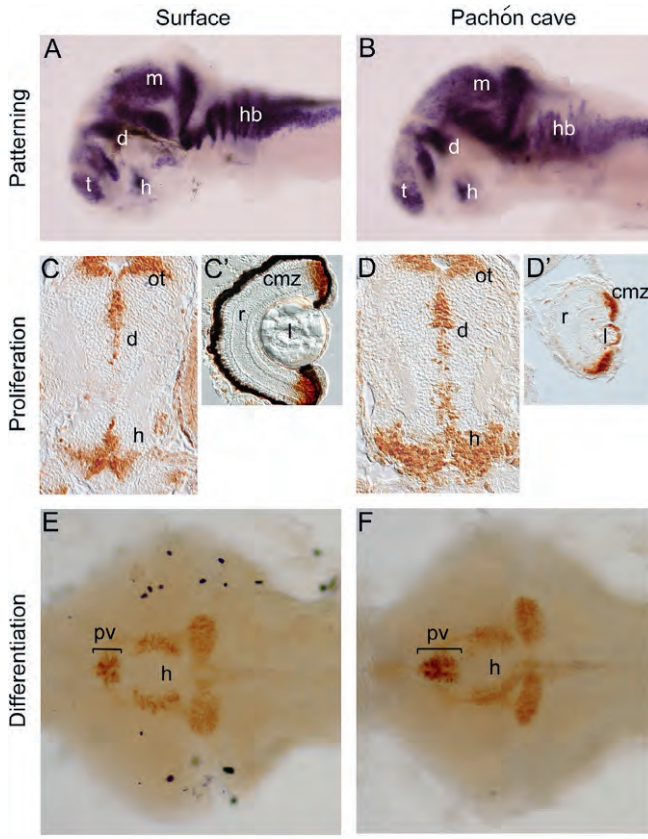

FIGURE 12.2 Comparing larval brain development in *Astyanax* surface fish and Pachón cavefish. (A and B) Patterning of the 36 hpf brain, as observed through the expression of the LIM-homeodomain transcription factor *Lhx9, in toto*, on a lateral view. Anterior is left and dorsal is up. t, telencephalon; d, diencephalon; h, hypothalamus; m, midbrain; hb, hindbrain. (CC′ and DD′), proliferation in the 60 hpf larval brain (C and D) and in the 7 dpf eye (C′ and D′), as viewed through PCNA immunohistochemistry on frontal sections. d, diencephalon; h, hypothalamus; ot, optic tectum; r, retina; l, lens; cmz, ciliary marginal zone. (E and F) Differentiated neurons in the hypothalamus (h) and the paraventricular nucleus (pv) at 7 dpf, illustrated here for the serotonergic system on a ventral view of a dissected brain after serotonin immunostaining, *in toto*. Anterior is left.

prey-catching behavior (Daphne Soares, communication at AIM2009); however, as rhodopsin is apparently not expressed in the cavefish retina around these stages (Yoshizawa and Jeffery, 2008), the underlying visual circuit and visual transduction mechanism is therefore unclear and deserves further studies.

As mentioned above, though, the adult cavefish tectum is much reduced in volume (−50%) and contains less neurons (−20%) than in surface fish (Soares et al., 2004). To our knowledge, cell death (by apoptosis, necrosis, or autophagy) has not been investigated in the cavefish optic tectum during the period of

tectal shrinkage. To investigate directly whether tectal regression was a secondary consequence of eye degeneration, the lens transplantation model was used. In lens-transplanted cavefish with a restored eye (on one side only), the size of the corresponding optic nerve and the extant of contralateral tectal innervation are increased; however, the procedure results in only a slight increase in tectal volume (+13%) and tectal neuron number (+8%) (Soares et al., 2004), which hardly compares to the surface fish situation. Importantly, it seems that lens transplantation in cavefish restores the eye as an organ, but does not restore vision-based response, tested in a phototaxis assay (Romero et al., 2003). Thus, in cavefish that received embryonically a surface fish lens, an eye and a retinotectal projection is present (Soares et al., 2004), but this visual system is probably not active or functional. This data strongly suggests that the reduced size of the optic tectum in cavefish is indeed a secondary consequence of eye degeneration, and indicates activity-dependent mechanisms that are probably lacking at the tectal level to maintain the integrity of the structure.

EARLY EMBRYONIC DEVELOPMENT: THE ORIGIN OF CAVEFISH DIFFERENCES?

Fortunately for EvoDevo studies, surface fish and cavefish embryos develop at the same pace, allowing rigorous comparisons of early embryogenesis and larval stages (Hinaux et al., 2011). At the end of gastrulation, at 9.5-10 hpf, Pachón cavefish embryos have a slightly ovoid shape resembling a rugby ball, whereas surface fish embryos exhibit a rounder shape (Hinaux et al., 2011). According to the literature, such a phenotype suggests a slight change in dorsoventral patterning during early embryogenesis (e.g., Barth et al., 1999; Kishimoto et al., 1997; Neave et al., 1997). *Bmp* and *Wnt* signaling molecules and activities have not yet been investigated significantly and in a comparative manner between cavefish and surface fish embryos, but it is well established that Hedgehog expression (Sonic and Tiggy-Winckle) is expanded at the anterior ventral midline during gastrulation (Pottin et al., 2011; Yamamoto et al., 2004; Figure 12.3(A)). Such Hedgehog hypersignaling from the mesoderm in the cavefish gastrula is indirectly responsible, through unknown mechanisms, for lens apoptosis and subsequent eye loss (Yamamoto et al., 2004). As demonstrated by pharmacological manipulations, Hedgehog hypersignaling also affects the precise onset of expression of other signaling molecules such as *Fgf8*, although it does not apparently change *Fgf3* expression (Pottin et al., 2011; Figure 12.3(A)). In fact, Hedgehog heterotopy (expanded expression) and *Fgf8* heterochrony (earlier expression) at the anterior margin of the cavefish neural plate affects the patterning and morphogenesis of its future forebrain. Both the expression patterns of several transcription factors that prefigure the presumptive territories of the retina and other forebrain regions (*Pax6*, *Lhx2*/*Lhx9*) and the neural plate fate map are slightly modified in cavefish, precisely at midline level where Hedgehog and *Fgf8* signaling are

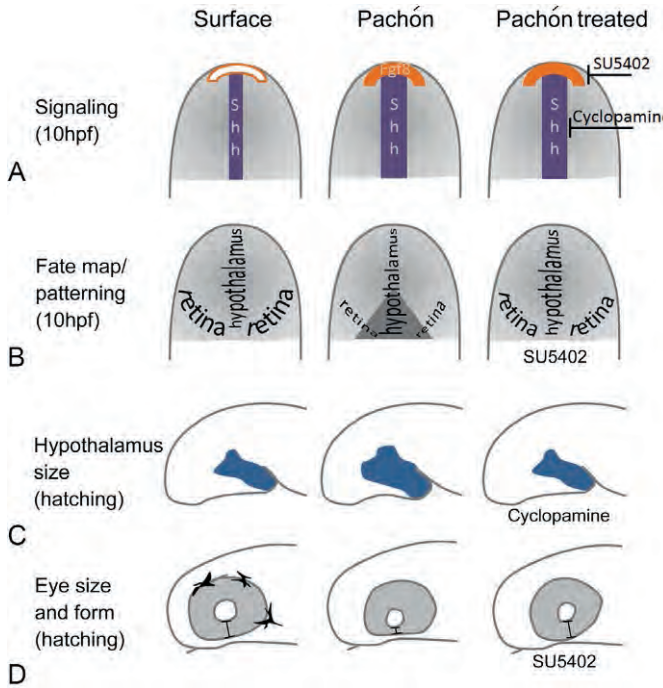

FIGURE 12.3 Comparing the neural plate and embryonic brain morphogenesis in *Astyanax* surface fish and Pachón cavefish. The first and second columns show surface fish and cavefish, respectively. In the third column, the patterning and morphogenetic effects of Hedgehog signaling inhibition by cyclopamine or *FgfR* signaling inhibition by SU5402 on Pachón embryos are depicted and resemble the surface fish phenotype. Schemas are drawn according to experimental evidences from several articles (Menuet et al., 2007; Pottin et al., 2011; Rétaux et al., 2008; Yamamoto et al., 2004). (A) Signaling systems at neural plate stage, schematized on a dorsal view (anterior is up). *Shh* expression (dark/purple at the midline) is larger and *Fgf8* expression (gray/orange at the anterior neural border) is earlier in cavefish. Note: *Fgf8* is not expressed at this early stage in surface fish. (B) Neural fate map and neural plate patterning. The regions of the neural plate fated to become the hypothalamus and the retina are indicated. Several anterior neural plate genes (*Lhx2*, *Lhx9*, *Zic1*, and *Pax6*) show a lack of expression at the cavefish midline (gray). In surface fish embryos, these midline cells are fated to give rise to the ventral quadrant of the retina, which is absent in cavefish. In cavefish, cells located in the equivalent zone of the neural plate (gray triangle) contribute to the hypothalamus or the dorsal retina. (C) Size of the hypothalamus, as labeled by *Nkx2.1a*, on a lateral view of a schematic brain at 24 hpf (anterior is left and dorsal is up). (D) Morphology of the eye. Black brackets indicate the ventral quadrant of the retina, strongly reduced in cavefish and restored after SU5402 treatment. Pigment cells on the surface fish eye are drawn.

changed (Pottin et al., 2011; Strickler et al., 2001; Figure 12.3(B)). We have proposed that medial neural plate cells that are normally fated to become the ventral part of the retina instead contribute to the hypothalamus in cavefish (Pottin et al., 2011). At the end of neurulation, the resulting morphology is an eye with a missing ventral quadrant and a forebrain with an enlarged presumptive hypothalamic territory (Figure 12.3(C) and (D)).

Importantly, *Shh* expression is also expanded in Chica and Los Sabinos embryos, and their *Pax6* medial neural plate pattern is modified the same way as in Pachón embryos (Jeffery, 2009; Strickler et al., 2001; Yamamoto et al., 2004). This tells us that similar developmental processes are modulated in independently evolved cavefish populations, and give rise to the same phenotypes. In fact and more generally, in cavefish embryos from all populations examined so far, the eyes first develop and then regress. This observation can even be extended to other cave vertebrates, including mammals, fishes, and amphibians (reviewed in Rétaux and Casane, 2013). Although this could be viewed as a waste of energy for a developing embryo, we have proposed that optic cup morphogenesis corresponds to a developmental constraint and probably cannot be circumvented (Pottin et al., 2011; Rétaux and Casane, 2013). Indeed, from a morphogenetic point of view, the vertebrate forebrain cannot develop properly without undergoing coordinated cell movements that include the initial formation of the visual organ (e.g., England et al., 2006; Rembold et al., 2006).

LARVAL BRAIN DEVELOPMENT: ESTABLISHING SUBTLE DIFFERENCES

During neurulation and after hatching, cavefish continuously display expanded *Shh* expression in all anterior basal forebrain domains (Menuet et al., 2007). In conjunction with the above described consequences of midline-dependent early morphogenetic events, sustained *Shh* hypersignaling affects neuronal patterning in a subtle manner—and to an extent that is developmentally tolerable, viable, and possibly even adaptive. Indeed, the global regionalization of the cavefish brain remains correct and unaffected, as shown by standard expression patterns of all "developmental" genes investigated (see *Lhx9*, e.g., in Figure 12.2(A)).

In the cavefish ventral telencephalon or subpallium, the expression domains of *Shh*, *Nkx2.1b* (a marker of the medial part of the subpallium and the preoptic region) and the *Nkx2.1*-dependent LIM-homeodomain factors *Lhx6* and *Lhx7* (Grigoriou et al., 1998; Sussel et al., 1999) are enlarged (Menuet et al., 2007). This enlargement appears specific to ventral telencephalic neural components under the control of this particular developmental "cascade," as the *Dlx2* or *Nkx2.2* expression domains are unchanged when compared to surface fish. Interestingly, *Nkx2.1b* and *Lhx6* happen to label a population of GABAergic interneurons that migrate tangentially to populate the olfactory bulbs (Menuet et al., 2007), and that we hypothesized to be the equivalent of the mammalian rostral migratory stream (e.g., Lois and Alvarez-Buylla, 1994). As a positive correlation between the abundance of olfactory bulb interneurons and olfactory performance is reported, it is tempting to speculate that the *Shh*-dependent increase in GABA/*Lhx6*/*Nkx2.1b*-positive migratory stream in cavefish could be advantageous for their life in perpetual darkness.

The cavefish hypothalamus, as labeled with *Shh*, with *Nkx2.1a* and *Nkx2.1b* regional markers, with *Lhx6* subterritory marker, or else as assayed

for proliferation (Figure 12.2(B)), appears larger and actively proliferating compared with surface fish (Menuet et al., 2007; Rétaux et al., 2008). Increased proliferation is observed specifically in the hypothalamic and preoptic territories, but not in the more dorsally located diencephalic or mesencephalic regions (Figure 12.2(B)), and a treatment of cavefish embryos with the *Shh*-signaling inhibitor cyclopamine diminishes hypothalamic proliferation and size. This suggests a region-specific, *Shh*-dependent control of proliferation and possibly neurogenesis in the hypothalamus, opening the interesting possibility that this neuroendocrine brain region, which contains many neuronal groups expressing neuromodulatory transmitters, such as monoamines and neuropeptides, has evolved in cavefish.

Is the entire hypothalamus/preoptic region, then, enlarged in cavefish? Or are specific neuronal groups affected, while others are unchanged? Some recent insights came from the comparative analysis of the serotonin neurotransmitter system (Elipot et al., 2013). First, a 4-h heterochrony exists between the appearance of the first serotonin-expressing neurons in the anterior hypothalamus of cavefish (at 18 hpf) and surface fish (at 22 hpf). This may be related to the differences in proliferation/neurogenesis control discussed above. Second, the resulting serotonergic group is larger and contains more cells in cavefish; however, the size of other, more posterior hypothalamic serotonin neuronal groups is identical in cave and surface larvae (Figure 12.2(C)), showing a finely regulated and group-specific regulation of neuron numbers. Third, the size difference in the anterior group is *Shh*-dependent. And finally, this anatomical variation in serotonin circuits seems to translate into behavioral differences, namely an increase in foraging behavior (Elipot et al., 2013).

Much remains to be investigated in a comparative manner on neural patterning, differentiation, and wiring in the larval cavefish brain. But the few aspects that have been investigated so far indicate that we will probably discover discrete, specific, and multiple variations in neuronal patterning in cavefish that result from early embryonic events that change subtle aspects of behavior, and that illustrate the morphogenetic and functional outcomes of developmental evolution and variations.

SENSORY SYSTEMS

The idea of a sensory compensation for absence of vision in animals living in the dark is “classical” and was proposed by early authors, and has been regularly reviewed since (Barr, 1968; Niemiller and Poulson, 2010; Soares and Niemiller, 2013; Wilkens, 1988). Longer appendages in insects and lateral line modifications in fish were often cited. More recently, the idea of sensory modules that would either be developmentally and genetically independent, or that would interact together, and upon which natural selection could act, has been put forward (Franz-Odendaal and Hall, 2006; Wilkens, 2010). Below, we briefly review available data on the developmental evolution of chemosensation

(gustation, olfaction) and mechanosensation (lateral line) systems in *Astyanax*. Of note, although hearing is an important sense for aquatic organisms, differences in auditory capacities have not been reported for cavefishes, including for *Astyanax* (Popper, 1970).

Chemosensory System

A better chemical sense has long been suggested for cave *Astyanax* (Breder and Rasquin, 1943; Humbach, 1960). Strikingly, according to Humbach's observation, blind cavefish would have a sense of taste 300 times more acute for bitter and 2000-4000 times more acute for salty, acid, and sweet substances than *Phoxinus* (a minnow, cyprinid); however, these early studies did not strictly discriminate between olfaction and gustation.

Olfaction is surprisingly poorly studied. A "classical" development of the olfactory organs and their lamellae from ectodermal placodes was described in *Astyanax* (Schemmel, 1967). Quantification of the continuously increasing number of olfactory lamellae in surface fish and the Pachón and Los Sabinos cavefishes throughout their lives shows no significant difference. Schemmel concluded that the olfactory modality cannot be considered as specialized in cavefish. He noted, however, that nasal capsules are more opened and flattened in cavefish, so that lamellae are more exposed. More recently, using *in situ* recordings in the Subterráneo cave, which hosts a hybrid population of mixed troglomorphic and epigean characters, we have found that troglomorphic fish present significantly larger naris size, and this was associated with a strong behavioral response elicited by food extracts (Bibliowicz et al., 2013), opening the possibility that olfactory abilities might have evolved in cave-dwelling *Astyanax*.

Concerning gustation, Schemmel was also the first to describe an increased number of tastebuds in Los Sabinos, and even more in Pachón cavefish (Schemmel, 1967). He reported a several-fold increase in tastebud numbers in adults (Schemmel, 1974). More precisely, three different types of tastebuds are distributed on the lips and oral cavity of both *Astyanax* morphs, but only cavefish harbor some on their lower jaws (Boudriot and Reutter, 2001). Moreover, the nerve fiber plexuses of type II and III tastebuds contain more axons in cavefish (Boudriot and Reutter, 2001), and there are more sensory receptor cells per tastebud in cavefish (Varatharasan et al., 2009). Such an enlarged and predominantly ventrally spread gustatory area on the skin of the head was interpreted as functionally relevant to localize food situated on the bottom, and considered as a compensatory improvement of the sense of taste. Interestingly, Substance P is found in tastebuds of cavefish, but not surface fish or other teleosts (Bensouilah and Denizot, 1991). These authors have proposed that the presence of this neurotransmitter could modulate the threshold of excitability of the taste cells.

In fact, tastebud number amplification in cavefish is already present in the first days post-fertilization (dpf) (Varatharasan et al., 2009; Yamamoto et al.,

2009), and the rate of tastebud development is accelerated in cavefish larvae; the difference with surface fish larvae is small at 5 dpf, but threefold at 22 dpf (Varatharasan et al., 2009). That these differences are detectable only after the onset of eye degeneration and that they increase during the degeneration process suggests a link between gustatory and visual development, a notion that is supported by functional experiments (Yamamoto et al., 2009): (1) *Shh* hypersignaling in the oropharyngeal region of cavefish embryos is responsible for tastebud number amplification; (2) early conditional overexpression of *Shh* in surface fish induces positive effects on later tastebud development and negative effects on eye development in the same embryos; and (3) there is an inverse relationship between eye size and tastebud number in the progeny of crosses between surface and cave *Astyanax*. This constitutes the only example to date of a direct link between the development of two sensory organs involving a pleiotropic effect of *Shh* and suggesting indirect selection as an evolutionary driving force for eye loss in cavefish.

Lateral Line

It has been long known that cavefish (Pachón, Los Sabinos, Chica) possess more free (superficial) neuromasts in the suborbital region of their face, and more fragmentation of infraorbital canal neuromasts than surface fish (Bensouilah and Denizot, 1991; Jeffery et al., 2000; Schemmel, 1967). More recently, Yoshizawa and colleagues (2010, 2012) have reported that neuromasts found at a high density in the suborbital and eye orbit region of cavefish mediate the vibration attraction behavior (VAB). Indeed, cavefish are specifically attracted by vibrations at about 35 Hz at the surface of the water, a behavior that clearly seems advantageous to find food in the dark (Yoshizawa et al., 2010). Of note, the morphology of cavefish sensory receptors also differ; the cupula (hair stereo-cilia covered by gelatinous case) of their free head neuromasts is up to 300 µm in length, compared to about 42 µm in surface fish (Teyke, 1990; see also Varatharasan et al., 2009), and is also larger, as are the neuromasts themselves (Yoshizawa et al., 2010). As the height and diameter of the cupula regulate sensitivity, these large, free neuromasts are twice as sensitive in young adults than smaller ones (Yoshizawa et al., 2014). This could explain why cavefish neuromasts can detect low frequency stimuli (below 50 Hz) in otherwise calm cave pools.

The cavefish VAB-mediating neuromasts develop late, after 2 months of age, when the eye is completely gone (Yoshizawa et al., 2010), which may explain why they invade the cavefish eye orbit region. Moreover, and contrarily to the case of tastebuds discussed above, experimental induction of eye regression in surface fish via Hedgehog overexpression is insufficient to increase the number of orbital neuromasts or to promote the appearance of the VAB (Yoshizawa et al., 2012). It will, therefore, be crucial to understand the developmental mechanisms controlling the timing of head neuromast organogenesis and the

size of individual sensory organs, as well as to determine the neuronal circuits underpinning the VAB. Considering that some cavefish populations (Pachón, Los Sabinos, or Piedras), but not others (Molino), exhibit a strong form of VAB, this system is ideal to investigate the origin of neural and behavioral novelty during evolution.

CAVEFISH BRAIN NEUROCHEMISTRY

The ensemble of cavefish behavioral modifications described in the introduction is sometimes referred to as a "behavioral syndrome," which would appear quite pathological to a clinician, to whom the cavefish condition would probably evoke disorders involving neuromodulatory and aminergic transmission. Actually, we currently know two genes that are important players in these neurotransmitter systems, and which carry mutations in their coding sequence in cavefish: *Oca2* and monoamine oxidase (MAO) (Elipot et al., 2014a; Protas et al., 2006). Moreover, several relevant genes (such as the 14-3-3 protein YWHAE, the glutamate receptor AMPA2 or the cannabinoid receptor CB1) whose expression is altered in cavefish were recently identified through a microarray study (Strickler and Soares, 2011). These genes play roles in neural networks controlling learning, feeding, or addiction, and could therefore underlie some of the cavefish behavioral phenotypes.

Oca2 (ocular and cutaneous albinism-2) is a transmembrane protein involved in the transport of L-tyrosine, the precursor of melanine, into melanosomes. Its mutation in cavefish (Protas et al., 2006) explains the depigmented phenotype that is reviewed in Chapter 8 of this book (Jeffery et al.). But L-tyrosine also happens to be the precursor of dopamine and noradrenalin, two central monoamines, therefore opening the possibility that an expanded L-tyrosine pool is available as a precursor for dopamine in cavefish. In line with this idea, *Oca2* morpholino knockdown in surface fish embryos increases both L-tyrosine and dopamine levels (Bilandžija et al., 2013b), and dopamine and noradrenalin levels are very high in the brains of young adult cavefish when compared with surface fish (Elipot et al., 2014a). Importantly, *Oca2* carries different loss of function mutations in various *Astyanax* cavefish populations (Protas et al., 2006) and more generally, the first step in melanin synthesis is also affected in other cave-dwelling animals, including insects (Bilandžija et al., 2013a), showing striking convergence on a defect in this particular pathway. Of note, the direct link between a large pool of available L-tyrosine due to the *Oca2* deficiency and the high levels of dopamine in the brain of cavefish remains unclear, because the activity of tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis, is identical in the brains of surface and cave morphs (Elipot et al., 2014a). An interesting research venue may be offered by the fact that the dopamine synthesis activator gene *YWHAE* is up-regulated in cavefish (Strickler and Soares, 2011). Finally and behaviorally, high noradrenalin levels in cavefish probably play a role in reduced sleep/increased wakefulness, as

suggested by sleep rescue after beta-noradrenergic receptor blockade (Duboue et al., 2012). And high dopamine levels may underlie feeding drive and reward-associated responses (Singh, 2014).

MAO is the serotonin-degrading enzyme. It carries a partial loss-of-function point mutation in Pachón cavefish, leading to very high serotonin levels in the brain (Elipot et al., 2014a). Note that there is only one form of MAO in teleosts, whereas mammals have two. Combined with the larger anterior hypothalamic serotonergic group, this mutation could contribute to the cavefish's persistent foraging behavior (Elipot et al., 2013). The MAO mutation is also likely to explain other cavefish behavioral phenotypes; in surface fish treated with deprenyl, a specific MAO inhibitor, serotonin levels (but not dopamine or noradrenalin) are increased, therefore mimicking the cavefish condition (Elipot et al., 2014a), and both schooling behavior (Kowalko et al., 2013) and hierarchical aggressiveness (Elipot et al., 2013) are lost. The serotonergic raphe nucleus in the hindbrain is probably involved in the loss of aggressiveness. Indeed, low raphe serotonin levels are associated with dominant individuals in surface fish groups, and it is possible to elicit some aggressiveness in cavefish by embryonic manipulations that reduce the size of their raphe serotonergic nucleus (Elipot et al., 2013). On the other hand, the serotonin-dependent brain circuits involved in loss of schooling are unknown, but we have proposed that the lack of collective behaviors in cavefish is probably tightly related to their loss of hierarchical and aggressive behavior (Rétaux and Elipot, 2013 and see also this book, Chapter 17).

In sum, we are at the beginnings of our understanding of cavefish brain neurochemistry, but all the data accumulated so far suggest that the subtle equilibrium and neurotransmission homeostasis present in vertebrate brains are unbalanced in more than a few ways in cavefish. Along the same lines, some "general" neurotransmission genes, such as neuroligin or the neurofilament protein M seem up- or down-regulated in cavefish, respectively, according to cross-species (zebrafish) microarray experiments (Strickler and Jeffery, 2009). Neuroligin is a postsynaptic adhesion molecule thought to control the balance between excitatory and inhibitory synapses (Mackowiak et al., 2014) and NF-M is a cytoskeleton component that regulates axonal growth and homeostasis (Yuan et al., 2012). Therefore, such dysregulations might also have general effects on cavefish neural functions, but their origin and their exact impacts have not yet been investigated.

CONCLUSIONS AND PERSPECTIVES

The *Astyanax* model system is now entering the genome era, with the available Pachón genome (McGaugh et al., 2014) and the exciting possibilities offered by transgenesis and genome-editing techniques (Elipot et al., 2014b; see also Chapter 19 by Burgess et al. in this book). Such progress will render possible many new lines of investigations. Comparative genomics will allow the investigation of *cis*-regulatory aspects in the evolution of gene regulation. Loss and gain

of function experiments in cave and surface morphs will decipher the exact roles and effects of mutations identified in cavefish. With the generation of transgenic fluorescent reporter cavefish and surface fish lines, researchers will be able to compare early cavefish brain morphogenesis and growth by 3D live imaging, or to analyze and manipulate neuronal activity *in vivo*. In other words, the cavefish has become a top model for neuroscience research for investigators interested in brain evolution and morphological, functional, and behavioral adaptation.

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