Developmental Biology 330 (2009) 200–211

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

Pleiotropic functions of embryonic *sonic hedgehog* expression link jaw and taste bud amplification with eye loss during cavefish evolution

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ARTICLE INFO

Article history:
Received for publication 26 November 2008
Revised 26 February 2009
Accepted 4 March 2009
Available online 11 March 2009

Keywords:
Sonic hedgehog
Oral and jaw development
Taste bud development
Eye degeneration
Pleiotropy

ABSTRACT

This study addresses the role of *sonic hedgehog* (*shh*) in increasing oral–pharyngeal constructive traits (jaws and taste buds) at the expense of eyes in the blind cavefish *Astyanax mexicanus*. In cavefish embryos, eye primordia degenerate under the influence of hyperactive Shh signaling. In concert, cavefish show amplified jaw size and taste bud numbers as part of a change in feeding behavior. To determine whether pleiotropic effects of hyperactive Shh signaling link these regressive and constructive traits, *shh* expression was compared during late development of the surface-dwelling (surface fish) and cave-dwelling (cavefish) forms of *Astyanax*. After an initial expansion along the midline of early embryos, *shh* was elevated in the oral–pharyngeal region in cavefish and later was confined to taste buds. The results of *shh* inhibition and overexpression experiments indicate that Shh signaling has an important role in oral and taste bud development. Conditional overexpression of an injected *shh* transgene at specific times in development showed that taste bud amplification and eye degeneration are sensitive to *shh* overexpression during the same early developmental period, although taste buds are not formed until much later. Genetic crosses between cavefish and surface fish revealed an inverse relationship between eye size and jaw size/taste bud number, supporting a link between oral–pharyngeal constructive traits and eye degeneration. The results suggest that hyperactive Shh signaling increases oral and taste bud amplification in cavefish at the expense of eyes. Therefore, selection for constructive oral–pharyngeal traits may be responsible for eye loss during cavefish evolution via pleiotropic function of the Shh signaling pathway.

Introduction

Cave animals have evolved novel morphological, developmental, physiological, and behavioral phenotypes during the relatively short time since they diverged from surface-dwelling ancestors (Culver, 1982). The Mexican tetra *Astyanax mexicanus*, which consists of a sighted surface-dwelling form (surface fish) and a series of blind cave-dwelling forms (cavefish), is an emerging model system for studying development and evolution of cave-adapted phenotypes (Jeffery, 2008). Like many other cave-adapted animals, *Astyanax* cavefish have lost their eyes and pigmentation during evolution in perpetual darkness. In concert with regressive evolution, constructive traits have also evolved, including additional gustatory organs (taste buds) and changes in feeding behavior (Schemmel, 1967, 1980; Hüppop, 1987; Jeffery, 2001), which are probably adaptive and subject to enhancement by natural selection in the cave environment. It has been postulated that non-visual sensory systems were improved to compensate for loss of vision during cavefish evolution (Voneida and Fish, 1984; Teyke, 1990; Jeffery et al., 2000; Jeffery, 2001) but the responsible molecular causes have not been identified. Genetic studies have revealed overlapping quantitative trait loci (QTL) governing eye size and increased gustatory organs (taste buds), which could be explained by pleiotropic tradeoffs (Protas et al., 2008). Here we address the possible pleiotropic function of *sonic hedgehog* (*shh*) in linking the gain of oral and gustatory constructive traits to the loss of eyes in blind cavefish embryos.

Despite the absence of functional eyes in adults, small eye primordia with a lens and optic cup are initially formed in cavefish embryos but subsequently arrest in development, degenerate, and sink into the orbits, where they are covered by connective tissue and epidermis (Cahn, 1958; Langecker et al., 1993; Jeffery and Martasian, 1998). As a first step in eye degeneration, the cavefish lens undergoes apoptosis (Jeffery and Martasian, 1998; Yamamoto and Jeffery, 2000). Later in cavefish development, the dysfunctional lens fails to induce the anterior eye chamber, iris, and cornea, although a normally layered retina initially develops from the optic cup. Photoreceptor cells are formed in the layered retina but subsequently degenerate (Langecker

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doi:10.1016/j.ydbio.2009.03.003

et al., 1993; Yamamoto and Jeffery, 2000). The surface fish lens can restore eye development, including the cornea, iris, and retina with photoreceptor cells, after transplantation into the cavefish optic cup (Yamamoto and Jeffery, 2000), indicating that the lens has a fundamental role in sustaining eye development (Strickler et al., 2007a). Several factors have been discovered that may induce apoptosis in the cavefish lens. Two of these are the antiapoptotic factor αA-crystallin, which is downregulated in the cavefish lens (Strickler et al., 2007b) and maps near an *Astyanax* eye loss QTL (Gross et al., 2008), and the putative proapoptotic factor Hsp90α, which is upregulated during cavefish lens development (Hooven et al. 2005). A third is *shh*, which probably induces lens apoptosis indirectly following its overexpression in surface fish embryos (Yamamoto et al., 2004).

To investigate the molecular basis of eye degeneration, we previously compared the expression of eye regulatory genes in cavefish and surface fish embryos (Strickler et al., 2001; Yamamoto et al., 2004; Jeffery, 2005). These studies pointed toward genes in the Shh midline-signaling system as regulators of cavefish eye regression. First, we observed that the bilateral eye domains of *pax6* expression in the cavefish neural plate are reduced and separated by a larger gap along the dorsal anterior midline. Second, we showed that *shhA* and *shhB* (formerly *tiggy winkle hedgehog*) expression is increased along the anterior midline (prechordal plate) in early cavefish embryos. Third, we found that expression of downstream genes in the Sonic Hedgehog (Shh) signaling pathway, such as the receptor *patched*, *nkx2.1a* in the neural plate, and *pax2a* and *vax1* expression in the optic vesicles, is also amplified, implying Shh hyperactivity along the cavefish anterior midline. Vertebrate optic vesicles are patterned by reciprocal transcriptional repression between *pax6* and *pax2/vax1* (Schwarz et al., 2000; Take-uchi et al., 2003), and upregulation of the latter by Shh signals is partially responsible for the small cavefish eye. Together with effects on the lens, *shh* mediated changes in gene expression in the optic cup suggest that the Shh signaling pathway negatively controls cavefish eye development.

Because *shh* is a pleiotropic gene with both positive and negative roles in development (Ingham and McMahon, 2001), in addition to negative effects on eye development, Shh hyperactivity could be related to the evolution of constructive traits, such as taste buds. Taste buds are more numerous in adult cavefish than in surface fish (Schemmel, 1967; Boudriot, and Reutter, 2001; Schemmel, 1980), and this expanded gustatory sense may be beneficial for cave life. Overexpression of *shh* has been previously detected in Shh signaling domains in the developing cavefish brain (Menuet et al., 2007) but oral–pharyngeal structures have not been investigated. Here, we have followed *shh* expression during oral–pharyngeal development to identify features that may be under positive control of pleiotropic Hh signaling. We found that *shh* expression is expanded in the oral–pharyngeal region and is later expressed in taste buds. The results of functional experiments suggest that *shh* amplification is required for increasing taste bud number during the same developmental interval as it inhibits eye development. In addition, genetic crosses revealed an antagonistic relationship between eye size and taste bud number in *Astyanax*. The results support the possibility that increased oral and gustatory development may have occurred at the expense of eyes during cavefish evolution via pleiotropic effects of the Shh signaling pathway.

Materials and methods

Animals and embryos

Laboratory colonies of *Astyanax mexicanus* were derived from surface fish collected at Balmorhea Spring State Park, Texas and cavefish collected at Cueva de El Pachón, Tamaulipas, Mexico. Embryos were obtained by temperature induced spawning and reared at 25 °C (Jeffery and Martasian, 1998; Jeffery et al., 2000).

In situ hybridization

RNA probes were generated from surface fish *shh* (AY661431), *nkx2.1a* (AY661435), and *pax2a* (AY661436) cDNA sequences as described previously (Yamamoto et al., 2004). Embryos or larvae were fixed in 4% paraformaldehyde–PBS (pH 7.2; PFA). *In situ* hybridization was done using digoxygenin-labeled RNA probes as described previously (Strickler et al., 2001; Yamamoto et al., 2004). Following *in situ* hybridization the specimens were post-fixed in PFA, dehydrated through an ethanol series, embedded in polyester wax, and sectioned at 10 μm. *In situ* hybridized specimens were viewed as whole mounts or sections and photographed.

Quantitative real time RT-PCR

Total RNA was extracted from 3-day post-fertilization (dpf) larvae with Ribopure kit (Ambion, Austin, TX) according to the manufacturer's protocol. Extracted RNA was quantified and its integrity verified using the UV absorbance (260/280) bioanalyzer (Agilent Technologies, Palo Alto, CA). Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) was used to create cDNA from 1 μg of RNA according to the Invitrogen protocol using an oligo (DT) primer (5′-CGGAATTCTTTTTTTTTTTTTTTTTTV-3′, Sigma Genosys, The Woodlands, TX). Blank cDNA was also created with total RNA as described, but with no reverse transcriptase, to serve as a negative control for genomic contamination. mRNA levels were measured by quantitative real time RT-PCR (RT-qPCR) using 2 μl of diluted cDNA (1:100) in a 20 μl qPCR reaction with SYBR Green ER qPCR SuperMix using an iCycler (Invitrogen, Carlsbad, CA) and analyzed according to the manufacturer's protocol with the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Primers were designed using Primer Express (v 2.0, Applied Biosystems) and either a known *A. mexicanus* sequence (see below) or the homologous region between zebrafish and *Tetraodon nigroviridis* cDNAs (for β-actin). The qPCR products were verified for the appropriate size by dissociation curve analysis and gel electrophoresis. Primers were 18–30 nucleotides in length with a melting temperature between 58–64 °C. The primer sequences were as follows: *shh* (AY661431) forward primer, 5′-AGCGCTTCAAGGAGCTCATC-3′ and reverse primer, 5′-CGTGTTCTCCTCGTCCTTAAAGA-3′; *vax1* (AY661437) forward primer, 5′-TCTACAGGCTGGAGATGGAGTTC-3′ and reverse primer, 5′ TTGAGTTGGCGTGCAAGCT-3′; *pax2a* (AY661436) forward primer, 5′-GCACGACTTCTCCACCCGTAT-3′ and reverse primer, 5′-GATGCCGTTGATGGAGTAGGA-3′; *pax6* (AY651762) forward primer, 5′-TGGCTGCCAGCAATCAGATG-3′ and reverse primer, 5′-CTTCTGAGTCCTCCCCATTTGAG-3′; α-actin (Strickler and Jeffery, unpublished) forward primer, 5′-CACGGCATCATCACCAACTG-3′ and reverse primer, 5′-CCACACGGAGCTCGTTGTAGA-3′, and β-actin forward primer, 5′-CACACMGTGCCCATCTAYGA-3′ and reverse primer, 5′-CRGCARATCCAGACGCAGRAT-3′. The qPCR output provided a Ct value for the threshold cycle, which is representative of fluorescence derived from binding of SYBR green to the double-stranded PCR product. Data were transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to α- and β-actin using the geNorm software and methods (GeNorm v3.4, Vandesompele et al., 2002).

All levels of gene expression were compared using a one-way ANOVA with cavefish and surface fish as the independent variables, and relative mRNA levels as the dependent variable. Values are reported as means ± SE, and $p<0.05$ was required for significance. Statistica v.6.1 (StatSoft, Inc., Tulsa, OK) was used for data analysis and Graphpad was used to construct graphs (Graphpad Prism Version 4.0, Graphpad Software, Inc.).