Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Developmental evolution and developmental plasticity of the olfactory epithelium and olfactory skills in Mexican cavefish

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ARTICLE INFO

Keywords:
Olfaction
Organ size
Proliferation
Neurogenesis
Olfactory sensory neuron

ABSTRACT

The fish *Astyanax mexicanus* comes in two forms: the normal surface-dwelling (SF) and the blind depigmented cave-adapted (CF) morphs. Among many phenotypic differences, cavefish show enhanced olfactory sensitivity to detect amino-acid odors and they possess large olfactory sensory organs. Here, we questioned the relationship between the size of the olfactory organ and olfactory capacities. Comparing olfactory detection abilities of CF, SF and F1 hybrids with various olfactory epithelium (OE) sizes in behavioral tests, we concluded that OE size is not the only factor involved. Other possibilities were envisaged. First, olfactory behavior was tested in SF raised in the dark or after embryonic lens ablation, which leads to eye degeneration and mimics the CF condition. Both absence of visual function and absence of visual organs improved the SF olfactory detection capacities, without affecting the size of their OE. This suggested that developmental plasticity occurs between the visual and the olfactory modalities, and can be recruited in SF after visual deprivation. Second, the development of the olfactory epithelium was compared in SF and CF in their first month of life. Proliferation, cell death, neuronal lifespan, and olfactory progenitor cell cycling properties were identical in the two morphs. By contrast, the proportions of the three main olfactory sensory neurons subtypes (ciliated, microvillous and crypt) in their OE differed. OMP-positive ciliated neurons were more represented in SF, TRPC2-positive microvillous neurons were proportionately more abundant in CF, and S100-positive crypt cells were found in equal densities in the two morphs. Thus, general proliferative properties of olfactory progenitors are identical but neurogenic properties differ and lead to variations in the neuronal composition of the OE in SF and CF. Together, these experiments suggest that there are at least two components in the evolution of cavefish olfactory skills: (1) one part of eye-dependent developmental phenotypic plasticity, which does not depend on the size of the olfactory organ, and (2) one part of developmental evolution of the OE, which may stem from embryonic specification of olfactory neurons progenitor pools.

1. Introduction

The olfactory epithelium (OE) of vertebrates is the external sensory organ devoted to the sense of smell. During development, the OE derives from a thickening called the placode that is formed at the end of gastrulation (Grocott et al., 2012; Whitlock, 2004). The non-neural ectoderm (at the border of the neural plate) or neural ectoderm (inside the neural plate) origin of the placode is currently debated. During neurulation and after, the placode cells undergo migrations and morphogenesis, transforming a thickened sheet of ectoderm into a multilayered pit, in which proliferation and neurogenesis occur (Breau and Schneider-Maunoury, 2014; Maier et al., 2014; Torres-Paz and Whitlock, 2014). Differentiated olfactory sensory neurons of the OE then project their axons onto the glomeruli of the olfactory bulbs, in the telencephalon, progressively establishing the path of the olfactory nerve (Koide et al., 2009; Shao et al., 2017; Whitlock and Westerfield, 1998). Each olfactory sensory neuron expresses a single GPCR olfactory receptor among the

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https://doi.org/10.1016/j.ydbio.2018.04.019
Received 31 January 2018; Received in revised form 19 April 2018; Accepted 24 April 2018

species' repertoire of odorant receptors and is specialized to detect a single odorant (Korsching, 2009; Shao et al., 2017; reviewed in Miyasaka et al., 2013). The olfactory system is used to detect, discriminate and identify odorant molecules in relation with a variety of adaptive behaviors such as foraging, communication, reproduction or predator avoidance.

Although different species show markedly different odor detection capacities, or olfactory specialization, not much is known about the factors determining olfactory sensitivity. Among mammals for example, differences of 5–6 orders of magnitude in the capacity to detect certain odorants have been reported, but no direct correlation between olfactory sensitivity and numbers or densities of olfactory sensory neurons, or the size of olfactory structures, has been reported (reviewed in Wackermannova et al., 2016). On the other hand, some birds such as the nocturnal New Zealand kiwi for which olfaction is of strong behavioral relevance possess enlarged olfactory structures (Corfield et al., 2014), and some fish such as sharks with exceptional olfactory skills present an extra-large OE surface and large olfactory bulbs (Collin, 2012; Tricas et al., 2009). The developmental origin(s) of these sensory specializations, in terms of both olfactory system anatomy and function, are mostly unknown.

Here, we addressed the question of the developmental origin of olfactory sensitivity using the comparison between the two morphs of the fish *Astyanax mexicanus*. The blind and depigmented cave-dwelling morphs (cavefish, CF) and their river-dwelling conspecifics (surface fish, SF) have markedly different olfactory capacities and are unique models to study this question. In fish, olfactory responses to relevant odorant cues (amino-acids, nucleotides, pheromones, alarm substance) can be recorded through electro-olfactograms, functional imaging or behavioral analyses (Behrens et al., 2014; Caprio et al., 1989; Friedrich and Korsching, 1997; Hara, 1994, 2006; Keller-Costa et al., 2015; Miyasaka et al., 2013; Tricas et al., 2009; Vitebsky et al., 2005; Wakisaka et al., 2017; Whitlock, 2006; Yoshihara, 2008). In an olfactory assay performed in the lab, CF larvae originating from the Pachón cave are able to detect and show attractive response to concentrations as low as 10^{-10} M of amino-acid, whereas SF larvae can only detect 10^{-5} M ranges (Hinaux et al., 2016). In the wild, blind CF inhabiting the Subterráneo cave respond to food odors whereas eyed hybrid fish phenotypically resembling SF do not (Bibliowicz et al., 2013). Interestingly, CF from these two caves have larger OEs and nostrils than SF. In Pachón embryos and larvae, the larger sensory organ results from early developmental evolution during gastrulation and neurulation, due to CF-specific modulations of midline signaling from organizer centers (Hinaux et al., 2016; Pottin et al., 2011; Yamamoto et al., 2004). Here, in search for the developmental origins of the enhanced olfactory skills of cavefish, we investigated the relationship between the size of the olfactory organ and olfactory capacities in *Astyanax mexicanus*. We also analyzed other developmental processes, including developmental plasticity due to loss of vision, and changes in neurogenesis control influencing the neuronal composition of the OE. The data are presented in a "results and discussion" format.

2. Materials and methods

2.1. Fish

Laboratory stocks of *A. mexicanus* SF and CF (Pachón population) were obtained in 2004 from the Jeffery laboratory at the University of Maryland, College Park, MD, and were since then bred in our local facility. Fish are maintained at 23–26 °C on 12:12 h light: dark cycle and they are fed twice a day with dry and live food. The breeding colonies spawn two or three times per week and generate a highly variable quantity of embryos (a few dozens to a few thousands). Spawning induction and larval care are described elsewhere (Elipot et al., 2014b). Embryos and larvae were raised at 24 °C in embryo medium (EM). SF raised in the dark were kept in thermostatically-controlled light-proof tanks. Feeding and daily EM changes were done in the dark. The F1 hybrids used in this paper were the progeny of a female SF x male Pachón cross. Animals were treated according to the French and European regulations for use of animals in research. SR's authorization for use of animals in research including *Astyanax mexicanus* is 91–116. Paris Center-Sud Ethic Committee authorization numbers are 2012-0055, 2016-36 and 2017-04.

2.2. Behavioral testing

Behavioral tests were performed as previously described (Hinaux et al., 2016) in a specially constructed sound- and light-proof room that includes a main compartment for testing and a second computer work station compartment from which recording of the tests was performed with minimal disturbances. All fish were fed 24 h prior to the test with two day-old Artemia and then food was withheld until testing in order to standardize their feeding state. Four one-month-old juveniles were placed in behavioral testing boxes (see Fig. 1C; 9 cm wide x 13 cm long) containing 150 mL embryo medium (EM) and let acclimatize for two hours prior to the test at a temperature of 24 °C and in the dark. SF and CF (or experimental and control animals) were always tested in parallel. Boxes were placed on top of an infrared light box (ViewPoint S.A.). Each test was initiated by simultaneously opening the Luer stoppers of medical solution administration tubing (Baxter, U.K.) to perfuse solutions at 5 mL/min from two reservoirs containing 60 mL of either amino-acid containing EM or EM alone (control). On the EM-perfused side, the flow generated was identical to the flow on the amino acid perfused side. Tests were recorded for 7 min on a Dell work station using ViewPoint imaging software and a DragonFly2 camera equipped with an infrared filter (PointGray).

Utilizing a colorimetric test for the quantification of amino acid concentrations (Hinaux et al., 2016), we defined four quadrants of the boxes in which the amino acid concentration was very high, high, low or zero over the duration of the test. Each of these quadrant was attributed a coefficient to calculate a Preference Index Score (PIS) reflecting the attraction of fish (or absence of attraction) to the amino acid source. The PIS for each time point (at 30 s intervals) was the cumulative score of the four fish in the box, where the position of each fish was scored with the values of − 3 (quadrant furthest away from amino acid source), − 1, 1, or 3 (quadrant closest to amino acid source). Thus the maximum and minimum PIS scores are + 12 and − 12, respectively, for a given time point. In order to correct for the initial position of the fish when the amino acid first enters the box at 1.5 min after the start of the experiment, the PIS was reset at zero for this time point and the subsequent PIS values were corrected by subtracting of the initial raw score at 1.5 min. Experiments in which the initial PIS score was > 6 or < −6 were discarded because the correction for initial position of the fish lead to artefactual "false attraction" or "false repulsion" at subsequent time points (Hinaux et al., 2016). Statistical significance of replicate tests was calculated using the non-parametric Mann-Whitney test and was performed using the StatView software. In all figures, n = 1 corresponds to one test, i.e., the cumulative score of 4 fish.