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RESEARCH ARTICLE

Quantitative analysis of crypt cell population during postnatal development of the olfactory organ of the guppy, *Poecilia reticulata* (Teleostei, Poecilidae), from birth to sexual maturity

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SUMMARY

Crypt cells are one of three types of olfactory sensory neuron, differing from ciliated and microvillar cells in shape, localization and number, and found only in fish. Although crypt cells are morphologically well characterized, their function remains unclear. They were hypothesized to be involved in reproductive behaviours by detecting sex pheromones, but electrophysiological investigations revealed sensitivity to only amino acids. However, the number of crypt cells in adult guppies is not the same in the two sexes. In this study, we compared the size of the crypt cell population in juvenile guppies during the first 90 days after birth. The purpose of our study was to clarify whether a correlation exists between sex and the number of these olfactory neurons. The data show that guppies reach adult crypt cell density when they become sexually mature. Despite a constant increment in volume during development of the olfactory organ, the minimum density of crypt neurons occurs at ~45 days. Moreover, in the early weeks, the density of crypt neurons is greater in males than in females because in females the total number of cells decreases significantly after just 7 days. In adults, however, crypt neurons are found in higher density in females than in males. These findings suggest that the number of crypt cells is sex specific, with independent developmental dynamics between males and females. A role in pheromone detection could explain such a difference, but the early appearance of crypt cells in the first days of life is suggestive of other, not sexually related, functions.

Key words: crypt cells, olfactory organ, quantitative analysis, S100, guppy.

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INTRODUCTION

Despite the fact that a wide variety of anatomical arrangements in fish olfactory organs from flat structures to multilamellar structures lined with olfactory mucosa (Hansen and Zielinski, 2005) has been described, the histological organization of the pseudostratified epithelium is similar among all fish subclasses. The olfactory sensory epithelium consists of sustentacular cells, a germinative layer of basal cells and bipolar olfactory neurons that are divided into three subtypes, distinguished by morphology and localization. The ciliated olfactory cells, with their apical dendrite knob bearing cilia, occupy the lower third of the sensory epithelium. The microvillar neurons, with a shorter dendrite ending in microvilli, reside in the upper layer. Crypt neurons, devoid of the dendrite and characterized by a crypt-like invagination containing both cilia and microvilli, are localized in the apical portion of the olfactory epithelium. While ciliated and microvillar neurons are common to all vertebrates, crypt cells are present only in fish olfactory systems including both Chondrichthyes (Ferrando et al., 2007) and Actinopterygii (Hansen and Finger, 2000). Crypt neurons were reported to express the G-proteins $G\alpha_o$ and $G\alpha_q$, but their odorant receptor molecules have not yet been established (Belanger et al., 2003; Hansen et al., 2003; Hansen et al., 2004); thus, the role of crypt neurons in chemical perception is still being debated. Electrophysiological studies revealed that a significant percentage of crypt cells appeared to be excited by only amino acids, agonists of the cAMP pathway (Schmachtenberg, 2006; Vielma et al., 2008). However, despite the absence of an identifiable response to pheromones, some indirect observations suggest a possible function in the detection of sexual stimuli. In particular, crypt neurons of *Carassius carassius* seem to project to a region in the ventral olfactory bulb (Hamdani and Døving, 2006) connected with the lateral part of the medial olfactory tract, mediating reproductive behaviour (Weltzien et al., 2003). Moreover, the number of crypt neurons appeared to undergo annual variation with a peak during the spawning season (Hamdani et al., 2008). Accordingly, in fish like guppies, which breed year-round, we did not observe any seasonal variation in the number of crypt cells (Bettini et al., 2009) and little is known about the onset and development of their population. Recently, it was documented that, in *Acipenser naccarii* (Camacho et al., 2010) and zebrafish (Sandulescu et al., 2011), crypt cell differentiation occurs in the early days of embryonic life, but later than differentiation of ciliated and microvillar neurons. In this study, we attempted to evaluate a possible correlation between sex and the growth dynamics of the crypt neuron population, comparing cell densities through the juvenile stage up to sexual maturity in guppy, *Poecilia reticulata*. In the guppy, a model for microsmatic fish (Lazzari et al., 2007), we discovered that adult males and females possess different crypt cell densities (Bettini et al., 2009), a possible confirmation of the hypothesized ability of crypt cells to perceive sexual odorants.

MATERIALS AND METHODS

Animals

Newborn individuals of *P. reticulata* Peters 1859, the offspring of adult fish purchased locally from the Acquario Fossolo (Bologna, Italy), were isolated immediately after birth and raised in aquaria at 25°C on a natural light–dark cycle and fed once daily, 6 days per week, with commercial flake food. All procedures conformed with the guidelines of the European Communities Council Directive (86/609/CEE), current Italian legislation regarding the use and care of animals and the guidelines of the US National Institutes of Health. The Ethic-Scientific Committee of the University of Bologna also approved this study.

Tissue preparation and immunohistochemistry

Sixty guppy fry of both sexes (50:50) were killed at various time points (7, 14, 21, 45 and 90 days after birth). Fish were anaesthetized with 0.1% 3-aminobenzoic acid ethyl ester (MS-222, Sigma Chemical, St Louis, MO, USA) and killed by prompt immersion in a modified Bouin's solution consisting of saturated aqueous solution of picric acid and formalin (ratio 3:1) for 24 h. All fixed animals were repeatedly washed in 0.1 mol l^{-1} disodium phosphate buffer, pH 7.4, and decalcified in 0.25 mol l^{-1} EDTA (Fluka, Buchs, Switzerland) in the same buffer for 1–7 days, depending on specimen age and mineralization. Specimens were finally embedded in Paraplast plus (Sherwood Medical, St Louis, MO, USA; melting point 55–57°C), frontally sectioned (5 μm) with a Leica 2145 microtome (Leica Microsystems, Milan, Italy) and mounted on silanized slides. Sections of testes and ovaries were stained with Haematoxylin and Eosin to determine sex and stage of gonadal development.

We applied the physical disector method (Sterio, 1984) to count crypt neurons: every 4th pair of sections (the disector) of the olfactory lamella was sampled (for details, see Bettini et al., 2009) and immunostained with antibody to S100 (rabbit polyclonal, 1:1000; Z 0311, DAKO Cytomation, Glostrup, Denmark), a family of calcium-binding proteins considered a useful marker for crypt cells (Bettini et al., 2009; Germanà et al., 2004; Germanà et al., 2007). However, anti-S100 also labels other types of olfactory neurons (Bettini et al., 2009; Gayoso et al., 2011), although much more weakly in adult guppy. The sections were de-paraffinized, rehydrated, immersed in 1% H_2O_2 (Sigma Chemical) to saturate endogenous peroxidase and submitted to microwave antigen retrieval in citrate buffer at pH 6.0 for 10 min at 750 W. Non-specific binding sites were blocked with 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) before primary antibody incubation overnight at 4°C. The sections were finally washed and incubated for 1 h 30 min in peroxidase-labelled goat anti-rabbit IgG (1:100; Vector Laboratories) followed by 3,3-diaminobenzidine substrate (Sigma Chemical). Negative controls were obtained by replacing the primary antibody with 10% NGS. Sections of rodent (mouse and rat) olfactory mucosa were chosen as positive controls, because of the known reactivity of mammalian olfactory ensheathing glia to the antiserum used in this work.

Image acquisition and statistical procedures

Images were visualized using an Olympus BH-2 microscope (Olympus Italia, Segrate, Italy) and photographed using a BEL BlackL. 5000 digital camera (BEL Engineering, Monza, Italy). Figures were assembled using Adobe Photoshop (CS3, Adobe Systems, San Jose, CA, USA), resized, rotated and adjusted for brightness, contrast and colour balance, but not altered in content. We used the image analysis software ImageJ (version 1.38r) with Cell Counter plug-in (v.2) to calculate epithelial area (A_e) in each lamella and to count S100-positive cells. The volume estimate (V) of the olfactory organ was obtained using Cavalieri's principle (Gundersen and Jensen, 1987), while the density of crypt cells was calculated as follows:

$$N_v = \frac{\sum Q}{\sum V_{dis}}, \quad (1)$$

and expressed as number of cells in 1×10^5 μm^3. N_v is the numerical density of crypt cells, Q is the number of counted crypt cells in the disector and V_{dis} is the volume of the olfactory epithelium in the disector (A_e × section thickness). The estimated total number of particles (N) was obtained by multiplying N_v with V.

The data collected are reported in the graphs as means ± s.e.m. Comparisons among the five age groups for each variable (V, N and N_v) were evaluated using one-way ANOVA (with LSD *post hoc* test), while Student's *t*-test was used for testing differences between sexes in each group.

RESULTS

Gonad histology

In order to assess gonadal maturation, histological sections were microscopically examined. The presence of spermatozeugmata in cysts of the testis and oocytes in late vitellogenic phase (type IV) [see Rocha et al. (Rocha et al., 2011) for a detailed description of the various types] in the ovary were used as an index of complete gonadal development. At 90 days after birth, ~50% of male guppies were sexually mature (Fig. 1A) while ~66% of females were still immature (Fig. 1B,C). At previous stages no specimens of either sex presented fully developed gonads.

S100 immunohistochemistry

The identification of mature crypt cells was based on their shape, localization and intensity of immunohistochemical reactivity. Some S100-positive crypt cells were already visible 7 days after birth in both male and female guppies (Fig. 2A,B). Most of them were localized close to the epithelial surface, but some ovoid immunostained cells, similar to crypt neurons, were present in lower layers (Fig. 2C). We considered these to be migrating immature elements, and counted them as differentiating crypt cells for statistical analysis.

From day 7 to day 90 after birth we observed an intensification of S100 immunostaining in ciliated and microvillar olfactory neurons, even though crypt cells remained easily identifiable (Fig. 2D,E) because of their more intense staining.

Quantification analysis

The volumetric growth of the olfactory organ appeared to follow similar pathways in the two sexes (Fig. 3A). The size of the lamella remained constant from 7 to 21 days after birth, but then it increased in volume over the next 10 weeks by ~3- to 4-fold.

Despite similar volume growth curves, males and females showed differences in the number of S100-positive crypt cells (Fig. 3B). In males, in fact, the population of crypt cells remained invariable until day 21, while in females it decreased significantly after 7 days of life; then, when the lamella started changing volume, the number of crypt neurons also increased.

The crypt cell density permitted a direct comparison between the sexes. The dynamic developments of the crypt cell population in males and females appeared diametrically opposed in the early weeks after birth (Fig. 3C). Male density of S100-positive cells rose slightly in the period between 7 and 21 days, reaching peak density

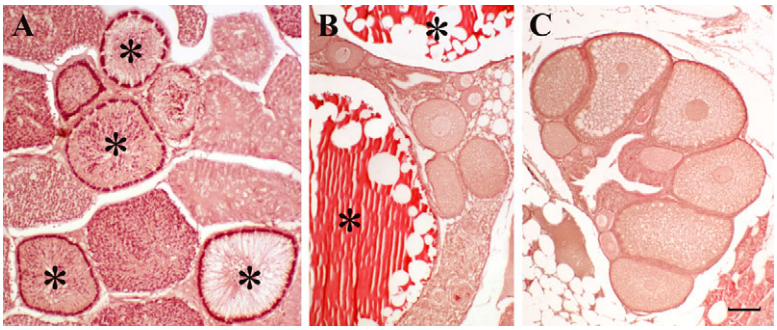

Fig. 1. Gonads of *Poecilia reticulata* at 90 days after birth (Haematoxylin and Eosin). (A) Mature cysts (asterisks) with differentiated spermatocytes in the testis of 50% of males. (B) Mature oocytes (asterisks) characterized by yolk accumulation and large diameter in the ovary of 33% of females. (C) Immature ovary without oocytes at the last vitellogenic phase in 66% of 90 day old females. Scale bar, 100 μm.

at 3 weeks prior to subsequently decreasing. Female crypt cell density, in contrast, progressively decreased just after 7 days, with a trough value at ~21–45 days after birth. Around the first month of life males had a significantly higher crypt cell density than females. However, this situation reversed as female crypt neuron density was higher at 90 days.

In an effort to better understand the mechanisms of development of the crypt cell population, we compared the previous data that included both mature and immature crypt neurons with numbers and densities of fully differentiated crypt cells, i.e. those cells located only in the apical surface of the epithelium. The differences observed in the total number and density of crypt cells appeared generally dependent on the large fraction of mature cells. However, between 14 and 45 days of life, mature crypt cell density in females remained constant in opposition to what was reported for total (mature + immature) values. In this case, the significant decrement seemed to be determined by variations in immature cell amount. The percentage of undifferentiated crypt cells in basal and middle layers progressively decreased during growth and reached a minimum (~10%) in 90 day old guppies.

DISCUSSION

Olfactory crypt cells appear very early in the mucosa, but at different times depending on the species. Crypt cells occur before hatching in *Danio rerio* (at just 2 days post-fertilization) (Sandulescu et al., 2011) and in *Raja clavata* (15 weeks after egg laying) (Ferrando et al., 2007), but after hatching in *Acipenser naccarii* (2–3 days post-hatching) (Camacho et al., 2010) and *Psetta maxima* (at metamorphosis) (Doldán et al., 2011). Initially, immature crypt cells characterized by nuclei segregated in the middle layer are predominant, but their number decreases during growth (Camacho et al., 2010). Also, in *P. reticulata* immature crypt neurons were present in the middle-basal layers, especially at day 7. These crypt cells expressed S100 proteins in early differentiation stages before taking up their definitive position and completing crypt invagination as reported in zebrafish (Sandulescu et al., 2011). As calculated previously (Camacho et al., 2010), the segregation of nuclei is a process that takes 5–21 days to complete. The number of apical crypt cells in guppy olfactory organ at day 7 is elevated in relation to immature crypt cells in the middle layer (~65% in males and ~60% in females), which may suggest that the onset of crypt cell differentiation occurs before birth. It seems probable that crypt cells are involved in some early non-reproductive function, perhaps in association with ciliated and microvillar olfactory cells. Their sensitivity to amino acids (Vielma et al., 2008) might indicate a role in the perception of food-related stimuli.

Our results, however, are also consistent with the hypothesis that crypt cells mediate sexual communication. Even though olfactory crypt cells are assumed to be involved in sex odorant detection (Hamdani and Døving, 2006; Lastein et al., 2006), before the present work no studies had been carried out to establish whether sexual maturation and the development of the crypt neuron population are correlated. We verified in *P. reticulata* that after 90 days of life, crypt cell density was independent of body size and was not significantly different in males and females at this stage or in adults (Bettini et al., 2009). In particular, 90 day old females were smaller

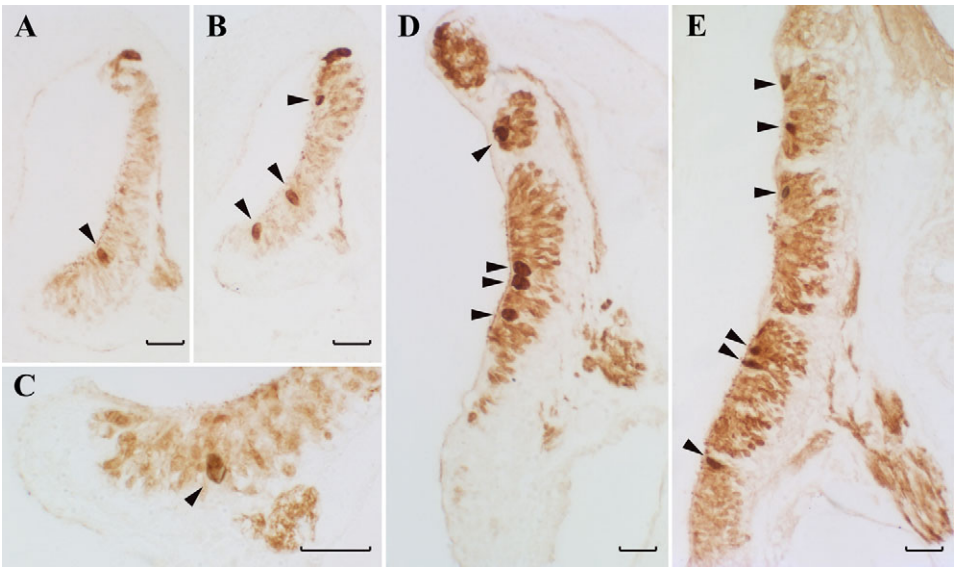

Fig. 2. S100-positive crypt cells in the developing olfactory lamella of *P. reticulata*. (A–C) Crypt cells (arrowheads) in the olfactory mucosa (horizontal sections) of 7 day old guppies. There were fewer crypt cells in males (A) than in females (B). S100-strongly positive cells in the lower layers of the mucosa (C) were counted as immature crypt cells. (D,E) At 90 days crypt neurons reached their definitive density both in males (D) and in females (E). The immunostaining pattern of other S100-positive elements was stronger than at 7 days. Scale bars, 20 μm.

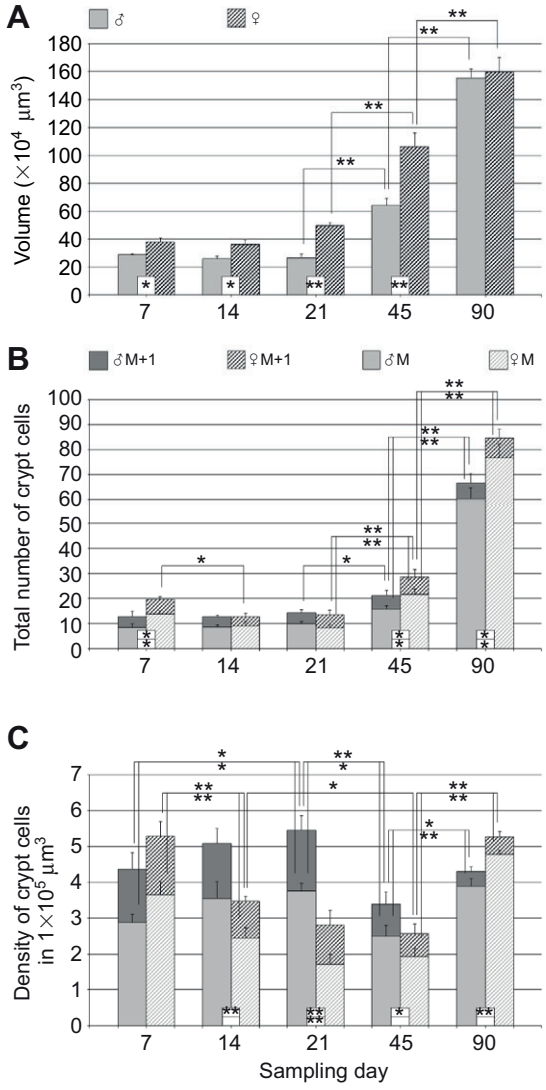

Fig. 3. Developmental dynamics of olfactory organ and crypt cell population in the guppy. (A) The growth of the lamella volume is similar in males and females and starts at 21 days. Significant differences between stages are indicated by asterisks above the brackets, and those between sexes for each sampling time by asterisks superimposed on the bars. (B) In both sexes the total number of crypt cells increases after 21 days in relation to volume growth. However, the total number of crypt cells in the first 3 weeks after birth remains constant in males, while in females it significantly decreases after 7 days. The number of apically located (mature) crypt cells follows a similar trend. (C) The density of crypt cells is subjected to opposing fluctuations in the two sexes before 45 days, increasing in males and decreasing in females. While in males these variations are substantially dependent on mature cell density, the decrement observed in 14–45 day old females seems to depend only on immature elements. In B and C, significant differences between stages are indicated by asterisks above the brackets for mature+immature crypt cells (M+I), and below the brackets for mature crypt cells (M). Differences between sexes for each sampling time are indicated by asterisks superimposed on the bars, with M+I in the upper half and M in the lower half. *P<0.05, **P<0.01.

than 6 month old fish. At 3 months, gonads in both males and females are completing their development (Houde, 1997), although for females the process appears slightly more protracted because only 33% of female guppies showed fully developed oocytes. We can affirm that the establishment of definitive crypt cell density and sexual maturation occurs at the same time. However, in the first weeks of life we observed a fluctuation in the population of crypt neurons, suggesting a substantial reorganization with a crucial stage at ~21–45 days after birth. Examination of the density and total number of crypt cells revealed sex-related differences in the time scale of development. Moreover, the developmental pathway progresses through a transient reduction in the total amount of crypt cells only in females. Except for the decrement in crypt cell number observed at 14 days in female olfactory organs, the process of reorganization appears to be mainly determined by variations in the amount of apical mature crypt neurons, rather than by changes in the proliferation rate of immature elements. It is possible that some mechanism(s) controlling mature crypt cell degeneration could be involved. This instability in the number of crypt neurons was described for the first time in adult *C. carassius* (Hamdani et al., 2008), whose crypt cell population undergoes seasonal regression in winter and renewal during the summer spawning months. However, in *P. reticulata* such changes occur only in the early juvenile stages, while in adult life crypt cell density remains invariant (Bettini et al., 2009) as guppy breeding season is year round (Houde, 1997). Recently, a similar rearrangement was observed during the first days of zebrafish embryonic development, perhaps as a consequence of the transition from endogenous to exogenous feeding (Sandulescu et al., 2011). Analogously, in guppy fry, we can speculate about which event, involving hormone modulation, might influence olfactory neurogenesis. One example could be the differentiation of secondary sex traits due to increasing levels of androgens and oestrogens during gonadal development. In male guppies, testosterone is known to regulate display coloration, gonopodium development and the last phase of spermatogenesis, while the initial development of the testes is under the sole control of pituitary gonadotrophins (Pandey, 1969). The assessment of sexual dimorphism could also explain the observed time difference in crypt cell density decrement between sexes. Male guppies begin to show distinguishing morphological features at about 5–6 weeks of age, while females do this some weeks before (Houde, 1997). Further investigations are needed to clarify whether a relationship between the molecules involved in sexual differentiation and the development of the olfactory crypt cell population really exists. A possible experimental approach could be to compare the data reported in this work with crypt cell number and density measured in guppy fry treated with sex hormones, e.g. testosterone and 11-keto-testosterone, two of the most well known and abundant fish androgens.

Furthermore, the higher crypt cell density observed in juvenile males than in females between 7 and 21 days and the reverse situation in adults suggests that crypt neurons are involved in different sex-linked behaviours during guppy growth. Olfaction mediates various activities concerning intraspecific recognition with sex-specific characteristics. Lastein and colleagues demonstrated that intermediate neurons of crucian carp olfactory bulb respond in sexually distinct ways to pheromone exposure (Lastein et al., 2006). Neurons from females of *C. carassius*, unlike those in males, do not discriminate between four different sex pheromones as a consequence of either differently tuned olfactory receptors or less distinct convergence patterns of olfactory receptor cells onto secondary neurons. Analogously, it is possible that the higher number of crypt cells in sexually mature female guppies than in males reflects a diverse involvement of olfaction in some conspecific interactions. Males use olfaction to distinguish receptive females (Guevara-Fiore et al., 2009), whereas females are more selective in mate choice than males and use both visual and olfactory cues (Shohet and Watt, 2004). Furthermore, the female anti-predator

defence strategies also include schooling preferences for other mature female guppies (Griffiths and Magurran 1998), because being in proximity to brightly coloured males may place them at a greater risk of predation (Shohet and Watt, 2004). Unfortunately, we are unable to speculate on the reason for the higher density of crypt cells in 21 day old male fry because no studies on sex-specific behaviours guided by olfactory cues in juvenile guppy are available in the literature.

In summary, we have revealed that in *P. reticulata* olfactory crypt cells reach their adult density at the end of gonad development. Moreover, males and females differ in the number of crypt neurons and in their development dynamic, suggesting possible control by sex hormones over the growth and establishment of this cell population. However, the early appearance of crypt cells in the first days of life suggests a role not strictly connected with sexual behaviour. We cannot exclude the possibility that crypt cells constitute a functionally heterogeneous population.

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