Development of the Olfactory Epithelium and Vomeronasal Organ in the Japanese Reddish Frog, Rana japonica

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ABSTRACT. Histological and ultrastructural development of the olfactory epithelium (OE) and vomeronasal organ (VNO) was investigated in the Japanese reddish frog, Rana japonica. Tadpoles, from hatching to the end of metamorphosis, and adult frogs were examined. In the adult, olfactory cells of the OE were equipped with olfactory vesicles with long cilia, but supporting cells with microvilli. The supporting cells of the OE contained secretory granules, PAS-positive by light microscopy, in their apical cytoplasm. On the contrary, sensory cells of the VNO were equipped with microvilli, and supporting cells of the VNO were equipped with cilia, but without secretory granules. Embryologically, the olfactory cells were indistinguishable from the supporting cells in the olfactory placode (primitive OE) lining the nasal pit, at hatch. The VNO appeared as a concave of the ventral part of the OE at 4 days after hatch. At this time, the olfactory and supporting cells of the OE became distinguishable from each other. Secretory granules were formed in the supporting cells of the OE at 36 days after hatch, and the OE was similar in fine structure to that in the adult. While, the VNO remained immature at 24–36 days after hatch, and did not complete its ultrastructural development at 60 days after hatch, the end of metamorphosis. These findings suggest that the OE may take part in the olfaction earlier than the VNO in ontogeny. — Key words: development, frog, olfactory epithelium, ultrastructure, vomeronasal organ.

In vertebrates can occur at least two different types of olfactory receptors, i.e., olfactory epithelium (OE) and vomeronasal organ (VNO). The OE exists in all vertebrates from fish to mammals [1, 7, 8, 10, 11, 19]. On the other hand, the VNO first appears in amphibians phylogenetically as a diverticulum of the nasal cavity [2, 4, 5, 13]. The degree of the development of the VNO has been hitherto discussed in relation to the life style of a species. The VNO is considered to be well developed in terrestrial species [9, 11, 14, 23–26], but absent in aquatic species [3, 19, 20]. Thus, it is absent in aquatic crocodiles [6], although it is well developed in other reptiles such as lizards and snakes [16, 20]. Among mammalian species, it is similarly absent in cetaceans well adapted to aquatic life style [17]. On the other hand, amphibians generally spend an aquatic life during larval stage and later go ashore to adopt to terrestrial life style. This change in the life style of amphibians from aquatic to terrestrial may be reflected in the fine structure of the OE and VNO during development. There have been published, however, no reports on the ultrastructural development of the OE and VNO in amphibians.

In the present study, therefore, the histological and ultrastructural development of the OE and VNO were chronologically examined in the Japanese reddish frog, Rana japonica, in relation to the change in their life style during metamorphosis to reveal the influence of this change in the amphibian olfaction.

MATERIALS AND METHODS

Clusters of eggs of the Japanese reddish frog, Rana japonica, were collected, together with the adult frogs, from ponds and creeks in the neighbourhood of our university and maintained in the glass aquarium in our laboratory to induce the spontaneous hatching. Average temperature of the aquarium was kept about 20 ± 5°C. At least 4 to 6 larvae were obtained every other day from day 0 to day 46, and every day from day 57 to day 60 (end of metamorphosis) after hatch for light and electron microscopy. Attention was paid to obtain larvae of average size at each sampling day. As there is no normal table of development for the Japanese reddish frog, developmental stages of larvae were described by days after hatch. Major developmental features of larvae were summarized in Table 1 with the corresponding stages of Xenopus laevis described by Nieuwkoop and Faber [18].

For light microscopy, larvae were anesthetized by cooling on ice, sacrificed by decapitation, and the heads were fixed in the FEA solution (5% formalin and 5% acetic acid in 80% ethanol) for 48 hr and embedded in paraffin by the routine procedure. Adult frogs were anesthetized by cooling on ice, sacrificed by cardiac perfusion with the same fixative and processed thereafter as for larvae. Heads from larvae after day 47 and adult frogs were decalcified in the mixture of 10% formalin and 10% formic acid with several changes for 7 to 14 days prior to paraffin embedding. Paraffin sections were cut serially at 5 μm in the frontal plane and stained with PAS-hematoxylin, alcian blue (pH 2.6)-hematoxylin or hematoxylin-eosin.

For electron microscopy, larvae and adult frogs were sacrificed as for light microscopy and fixed in a modified Karnovsky's solution (2% glutaraldehyde and 2.5%
paraformaldehyde in 0.05 M cacodylate buffer, pH 7.3). Following fixation for 2 hr at 4°C, the materials were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 1 hr at 4°C, and embedded in epoxy resin by the routine procedure. Ultrathin sections were cut on a LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Hitachi 700H electron microscope. Semi-thin sections were cut at 1 μm and stained with Mallory's azur II-methylene blue for light microscopic observations.

RESULTS

Topographic relationship of the OE and VNO in the adult: The OE occupied a large area in the dorsal to medial walls of the main nasal cavity (Fig. 1, OE), while the VNO was observed as a pair of diverticula in the ventromedial part of the nasal cavity at the base of the nasal septum (Fig. 1, VNO). The VNO communicated laterally with the nasal cavity by a narrow slit. The epithelium of the VNO was tall in its medial part to represent the vomeronasal sensory epithelium, but gradually decreased in height laterally to transit to the respiratory epithelium of the nasal cavity.

Structure of the OE and VNO in the adult: The OE and VNO were both consisted of three kinds of cells; olfactory (sensory in the VNO), supporting and basal cells. By PAS-hematoxylin and alcian blue-hematoxylin staining, the apical part of the OE was intensely positive to PAS, but negative to alcian blue (Fig. 2). On the contrary, the VNO was negative to both PAS and alcian blue (Fig. 3).

The olfactory cells of the OE were equipped with olfactory vesicles with long cilia on their apical surfaces. These cells contained many mitochondria, numerous neurotubules and basal bodies (Fig. 4, arrows) in the apical cytoplasm, and rER, Golgi apparatus and dense bodies in the perikarya. The supporting cells of the OE were covered with microvilli on their apical surface, and characteristic with a large number of electron-pale vesicles with mucous substance in the apical cytoplasm. They contained sER, rER, Golgi apparatus, mitochondria in their cytoplasm (Fig. 4). The basal cells were small cells scattered on the basement membrane, poor in cytoplasmic organella, and had round or segmented nucleus.

On the other hand, the apical end of the sensory cells of the VNO slightly protruded into the lumen, and weared microvilli. These cells contained centrioles just beneath the free surface. The other ultrastructural features were similar to those of olfactory cells in the OE. Supporting cells of the VNO were covered with cilia on their apical surfaces and lacked vesicles with mucous substance (Fig. 5). Basal cells of the VNO were also similar to those in the OE.

Histological development of the OE and VNO: Metamorphic changes of the Rana japonica and corresponding stages of Nieuwkoop and Faber in Xenopus laevis, were summarized in Table 1.

At hatch, the olfactory placode containing many melanin granules was already invaginated to form the nasal pit (Fig. 6A). At 4 days after hatch, the VNO appeared as a concave of the epithelium on the ventromedial side of the OE (Fig. 6B). The number of melanin granules was smaller in the VNO than that in the OE. The VNO invaginated deeply as the ontogeny proceeded (Figs. 6C-6E). Jacobson's glands (JG) appeared medially to the VNO at 10 days after hatch (Fig. 6C, arrowhead). A large number of Bowman's glands

Fig. 1. Schematic drawing of the transverse section at the level of the naris of the nasal cavity of the adult frog, indicating the olfactory epithelium (OE), vomeronasal organ (VNO), Bowman's glands (BG) and Jacobson's glands (JG). The cartilaginous structure is painted black. N; naris, NS; nasal septum, RE; respiratory epithelium. × 130.
Fig. 2. Adult olfactory epithelium (OE) stained with PAS-hematoxylin (A) and alcian blue-hematoxylin (B). The apical part of the OE is intensely positive to PAS, but negative to alcian blue. × 210.

Fig. 3. Adult vomeronasal organ (VNO) stained with PAS-hematoxylin (A) and alcian blue-hematoxylin (B). The VNO is negative to both PAS and alcian blue. × 210.

Fig. 4. Fine structure of the apical part of the adult olfactory epithelium. Many olfactory vesicles and olfactory cilia of the olfactory cells (OC) are identified. Supporting cells (SU) are covered with microvilli and contain mucous substances in the apical cytoplasm. Arrows; basal bodies. × 6,300.

Fig. 5. Fine structure of the apical part of the adult vomeronasal organ. Sensory cells (SE) are covered with microvilli and supporting cells (SU) with cilia. Arrows; basal bodies. × 6,300.

(BG) of the OE were present at 60 days after hatch (Fig. 6F, arrows).

Ultrastructural development of the OE: At hatch, cells in the olfactory placode (OP) contained a number of melanin granules, about 480 nm in diameter, throughout the cytoplasm (Figs. 7, 8). They also contained lots of yolk materials (Fig. 8, arrows) and lipid droplets (Fig. 8, arrowheads) in perikarya and basal cytoplasm. The olfactory and supporting cells were almost indistinguishable in the OP.

By 4 days after hatch, the majority of yolk materials disappeared (Fig. 9). Melanin granules were still observable throughout the epithelium. At this time, the olfactory cells became distinguishable from the supporting cells. Olfactory vesicles with cilia were formed on the apical end of olfactory
Table 1. Stage criteria during the development of *Xenopus laevis* and *Rana japonica*

<table>
<thead>
<tr>
<th>NF-stage</th>
<th>Rj-age</th>
<th>General survey of development</th>
</tr>
</thead>
<tbody>
<tr>
<td>35/36</td>
<td>0</td>
<td>Hatching, Existence of olfactory placode</td>
</tr>
<tr>
<td>37/38</td>
<td>4</td>
<td>Appearance of vomeronasal organ, Appearance of lung anlage containing thick wall with yolk material and narrow lumen</td>
</tr>
<tr>
<td>39</td>
<td>5</td>
<td>Completion of external gills</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>Beginning of formation of the operculum</td>
</tr>
<tr>
<td>42</td>
<td>10</td>
<td>Segregation of trachea and lung from alimentary canal</td>
</tr>
<tr>
<td>43</td>
<td>15/16</td>
<td>Appearance of Jacobson's gland</td>
</tr>
<tr>
<td>45</td>
<td>19/20</td>
<td>Beginning of decrease in thickness of the pulmonary wall</td>
</tr>
<tr>
<td>46</td>
<td>21/22</td>
<td>Beginning of reduction of external gills</td>
</tr>
<tr>
<td>47</td>
<td>23/24</td>
<td>Beginning of feeding</td>
</tr>
<tr>
<td>48</td>
<td>25/27</td>
<td>Degeneration of the majority of external gills</td>
</tr>
<tr>
<td>51/52</td>
<td>31/33</td>
<td>Appearance of hindlimb</td>
</tr>
<tr>
<td>57</td>
<td>44</td>
<td>Appearance of Bowman's gland</td>
</tr>
<tr>
<td>58</td>
<td>45</td>
<td>Appearance of forelimbs, Ultimate length of larvae</td>
</tr>
<tr>
<td>62</td>
<td>50/52</td>
<td>Beginning of tail shrinkage</td>
</tr>
<tr>
<td>66</td>
<td>60</td>
<td>Completion of metamorphosis</td>
</tr>
</tbody>
</table>

a) Stages in *Xenopus laevis* by Nieuwkoop and Faber [18], b) Days after hatch in *Rana japonica*.

cells (Fig. 9, arrows). The olfactory cells contained many mitochondria and neurotubules in the distal part of the cytoplasm. The supporting cells were covered with microvilli and contained a small number of sER, free ribosomes, and mitochondria. At 8 days after hatch, melanin granules decreased in number in the epithelium (Fig. 11). Some ciliated cells lacked olfactory vesicles and neurotubules, and were regarded as non-sensory cells (Fig. 11, arrow). The basal cells were observed on the basement membrane.

At 24 days after hatch, the olfactory cells had round to oval nuclei with prominent nucleoli and electron-pale cytoplasm, while the supporting cells had elongated nuclei with peripheral chromatin accumulation, and cytoplasm with moderate electron density (Fig. 13). The supporting cells contained a small number of pale vesicles with mucous substance in their apical parts.

At 36 days after hatch, the olfactory cells were similar in fine structure to adult olfactory cells (Fig. 15). The secretory granules increased in number in the supporting cells. The non-sensory ciliated cells (Fig. 15, arrow) were still detectable but decreased in number. No melanin granules were observed at this time.

At 60 days after hatch, the OE almost completed its ultrastructural development and showed the similar features in fine structure to those in the adult. No non-sensory ciliated cells were detectable (Fig. 17).

Ultrastructural development of the VNO: At 4 days after hatch, the VNO was formed as a ventromedial invagination of the OE. The VNO contained a few number of melanin granules, but no yolkly materials. The VNO consisted of immature epithelial cells, cuboidal to low columnar in shape. These cells contained many free ribosomes and a small number of mitochondria (Fig. 10).

At 8 days after hatch, the VNO became taller than the previous stages (Fig. 12). A small number of melanin granules was observed throughout the epithelium. The sensory (Fig. 12, SE) and supporting cells (Fig. 12, SU) became distinguishable from each other. The former was covered with microvilli, and had round nuclei with prominent nucleoli, well-developed rER, neurotubules and mitochondria in the apical cytoplasm. The latter was covered with cilia and had elongated oval nuclei with peripheral accumulation of heterochromatin. They contained rER and mitochondria in the cytoplasm.

At 24 days after hatch, the free surface of the VNO was mostly covered with cilia of the supporting cells (Fig. 14).

At 36 days after hatch, cells in the VNO became much elongated (Fig. 16). The majority of apical ends of sensory cells did not reach to the apical surface yet. Many dendritic processes with centrioles (Fig. 16, arrows) were observed in the epithelium to extend toward the free surface.

At 60 days after hatch, the ultrastructural development of the VNO was not still completed (Fig. 18). Some centrioles were detected in the extending dendrites of sensory cells (Fig. 18, arrows). The number of microvilli of the sensory cells was much smaller than that in the adult.

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Fig. 6. Histogenesis of the olfactory epithelium and vomeronasal organ during development. A: 0 day, B: 4 days, C: 10 days, D: 24 days, E: 44 days, F: 60 days after hatch. Arrowhead: Jacobson's glands, Arrows: Bowman's glands. Hematoxylin-eosin. A-C: ×240, D-F: ×88.

Fig. 7. Apical part of the olfactory placode at hatch. It contains many melanin granules, and is covered with cilia and microvilli, but does not equip with olfactory vesicles. ×10,200.

Fig. 8. Basal part of the olfactory placode at hatch, containing many yolkly materials (arrows) and lipid droplets (arrowheads). ×2,300.
DISCUSSION

Structure of the OE and VNO in the adult: In the present study, several differences were found between the OE and the VNO. By light microscopy, the OE was positive to PAS, but the VNO was negative to PAS. By electron microscopy, the olfactory cells of the OE were equipped with cilia, but the supporting cells of the OE with microvilli. On the contrary, the sensory cells of the VNO were equipped with microvilli, but the supporting cells of the VNO with cilia. These differences in the PAS-staining and in the surface structure of the OE and VNO suggest that these two epithelia may be sensitive to different sort of odorants. Accumulating data have shown that the VNO is involved in...
the detection of pheromones in mammals [22]. The VNO in amphibians may detect pheromones as that in mammals.

By electron microscopy, the supporting cells of the OE contained a large number of mucous granules in their apical cytoplasm, but those of the VNO did not contain mucous granules at all. Therefore, the mucous substance by electron microscopy seems to correspond to the PAS-positive material by light microscopy, and suggests the existence of neutral mucopolysaccharides in the OE. In the lizard, however, the OE have been reported to be intensely positive to alcian blue to suggest the existence of acid mucopolysaccharides [16]. This may reflect the species differences in the properties of the OE.

Although the OE is reported to exist in all vertebrates from fish to mammals [1, 7, 8, 10, 11, 19], the VNO first appears in amphibians phylogenetically [2, 4, 5, 13]. In fish lacking the VNO, some of the receptor cells of the OE are reported to be covered with cilia, others with microvilli [3]. In amphibians, however, the receptor cells of the OE are covered with cilia and those of the VNO with microvilli. Therefore, the primitive single epithelium may be divided into two kinds of epithelia, the OE and VNO, as the phylogenetical development proceeds.

**Development of the OE and VNO:** As shown in Table 1, tadpoles respirated with the external gill for the first two or three weeks after hatch, and thereafter with the lung. The OE was already formed at hatch, and showed the similar structure as in the adult at two or three weeks after hatch. Therefore, the OE may have started their function at this time already and may function in both the “gill-stage” and the “lung-stage” to detect odorants both in the water and in the air.

On the other hand, the VNO appeared at 4 days after hatch, matured gradually, but did not complete its morphogenesis even at 60 days after hatch, the end of metamorphosis. Since the “gill-stage” finished earlier than the ultrastructural maturation of the cells in the VNO, the VNO may function only in the “lung-stage”. These findings may further suggest that the VNO functions not in larvae but in frogs, and detects odorants in the air.

In the present study, the cells of the OP contained numerous number of melanin granules, as those in Xenopus laevis [12]. At 4 days after hatch, the VNO appeared as a concave of the epithelium, with smaller number of melanin granules than in the OE. Because the VNO did not originate directly from the OP but from the OE, the number of the melanin granules in the VNO may be smaller than in the OE.

Furthermore, we revealed the transient appearance of non-sensory ciliated cells from 0 to 36 days after hatch in the OE. These cells decreased in number at 36 days after hatch, and disappeared completely at 60 days after hatch. These findings suggest that they died during the developmental process of the OE, or transformed to another type of cells. Pellier and Astic reported the appearance of dying cells during the developmental process of the VNO in rats by the use of nick end labeling method of fragmented DNA [21]. In the present study, however, we seldom observed apoptotic or necrotic cells in the OE during 16–60 days after hatch. On the other hand, Kratzing reported the transit appearance of cilia in the sensory cells of the VNO in the suckling rats [15]. Since cilia are absent in both the sensory and supporting cells of the VNO in the adult rats, non-sensory ciliated cells in the present study may be transformed into
non-ciliated supporting cells. Further investigations are necessary to make clear the fate of these cells.

In conclusion, the OE matures earlier than the VNO and may take part in olfaction earlier in ontogeny than the VNO. The OE may function in both aquatic and terrestrial lives, but the VNO only in the terrestrial life.

REFERENCES


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