FULL PAPER *Anatomy*

Immunohistochemical Studies on the Differential Maturation of Three Types of Olfactory Organs in the Rats

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ABSTRACT. Differential maturation of three types of olfactory organs, the olfactory epithelium (OE), the vomeronasal organ (VNO) and the septal olfactory organ of Masera (MO), was examined immunohistochemically in embryonic and newborn rats by the use of anti-protein gene product 9.5 (PGP 9.5) serum. These olfactory organs were derived in common from the olfactory placode as neuroepithelia. In the OE, PGP 9.5-immunopositive olfactory cells first appeared at 13 days of gestation. The OE maturated completely, and showed the same cytological features as in the adult at 20 days of gestation. The MO first appeared as a dense mass of PGP 9.5-immunopositive sensory cells on the most ventrocaudal part of the nasal septum at 15 days of gestation and was evidently isolated from the OE by the decrease of immunopositive cells in the intercalated epithelium between the OE and the MO at 20 days of gestation. However, even at 7 days after birth, the MO did not complete its development and contained sensory cells aggregating in the mass. The VNO was separated from the nasal cavity at 13 days of gestation as a tubular structure of a neuroepithelium including PGP 9.5-immunopositive sensory cells. These cells gradually increased in number in the sensory epithelium of the VNO and extended their dendritic processes to the free surface at 7 days after birth. These findings clarified the differential maturation of these olfactory organs. That is, the OE completes its development before birth, while the MO and VNO after birth.

KEY WORDS: development, olfactory epithelium, protein gene product 9.5, septal olfactory organ of Masera, vomeronasal organ.

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In mammals can occur three different types of olfactory organs, the olfactory epithelium (OE), the vomeronasal organ (VNO) and the septal olfactory organ of Masera (MO) [12, 24, 29, 32]. These olfactory organs were reported to be derived in common from the embryonic olfactory placode as neuroepithelia during their ontogenetic development to take part in different kinds of olfaction [6, 9, 10–13, 26, 34, 36]. The OE responds to general and volatile odorants and takes charge of the usual olfactory function, while the VNO is thought to be specialized for the perception of pheromonal and non-volatile substances and involved in reproductive and/or social behaviors [1, 3, 4, 7, 13, 19, 22, 28, 40, 41]. On the other hand, the MO appears as a small part of the primitive OE in the later embryonic stage and is separated from the OE by the non-sensory (respiratory) epithelium during the course of embryonic development, and finally becomes an isolated small patch of sensory epithelium located on the ventrocaudal part of the nasal septum in the adult [11]. As for its function, Kratzing [20] suggested that the MO could act to monitor the airflow in a quiet respiration, while Marshall and Maruniak [23] revealed electrophysiologically that the MO shows higher chemical sensitivity to some odorants such as pentyl-acetate and butanol than the OE.

These olfactory organs seem to undergo several modifications in their structure and function during phylogenic development and differentiate into three organs to take charge of respective functions, although the process of their differentiation is not fully clarified. In this context, it is expected that detailed examinations on the development and maturation of these olfactory organs may be useful to demonstrate their functional significance as individual organs.

Recently, protein gene product 9.5 (PGP 9.5) has attracted considerable attention as a neuron-specific marker [15, 33, 36]. PGP 9.5 is known to be a ubiquitin carboxyl-terminal hydrolase and is found predominantly in the cytoplasm of neurons and neuroendocrine cells [15, 17, 25, 38]. In the olfactory organs, PGP 9.5 is expressed in receptor cells of the OE, VNO and MO [11, 14, 16, 31, 33, 36]. In the present study, therefore, chronological development of three types of olfactory organs, the OE, VNO and MO, was examined immunohistochemically in rats by the use of anti-PGP 9.5 serum to compare the differential maturation of receptor cells of these olfactory organs during development.

MATERIALS AND METHODS

Wistar rat fetuses were obtained at 12, 13, 14, 15, 16, 18 and 20 days of gestation. Days of gestation were counted from the ejaculation following the intromission in the course of pairing, which was regarded as day 0 of gestation. Postnatal animals were also obtained on the day of birth and at 3 and 7 days after birth. Five animals were used for each group of age. Dams were anesthetized with ether and underwent a caesarean operation to take fetuses. After decapitation, the heads of fetuses were immersed in Bouin's solution and embedded in paraffin by routine procedures. Newborn

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rats were decapitated under ether anesthesia. Their heads were immersed in the same fixative, decalcified in a mixture of 10% formic acid and 10% formalin, and then embedded in paraffin in the same way as fetuses. Paraffin sections were cut serially at 4 μm in the frontal plane, deparaffinized with xylene, and processed for immunostaining by the avidin-biotin complex (ABC) method using a rabbit anti-PGP 9.5 serum (UltraClone, Wight, U.K.). The sections were incubated overnight at 4°C with the antiserum diluted to 1:2000. The immunoreactions were visualized with the Vectastain ABC kit (Vector, California, U.S.A.).

The characterization of the antiserum has been described elsewhere [38]. Control immunohistochemical stainings were performed by the use of normal rabbit serum or PBS to replace the biotinylated IgG or ABC. No specific reaction products were observed in the control stainings.

RESULTS

The olfactory epithelium: At 12 days of gestation, the embryonic nasal pit was lined with a neuroepithelium consisting mainly of undifferentiated stem cells. None of these cells was immunopositive to PGP 9.5 at this stage.

At 13 days of gestation, PGP 9.5-immunopositive receptor (olfactory) cells were scattered in the presumptive OE lining the nasal septum (Fig. 1). These cells showed weak immunoreactivity and possessed a round nucleus and scanty cytoplasm.

At 14 days of gestation, the presumptive OE increased its thickness gradually in the dorsal region of the nasal cavity and contained many olfactory cells showing moderate immunoreactivity in the middle to basal region of this epithelium (Fig. 2). Some olfactory cells distally extended their cytoplasm, dendritic process, which did not reach the free surface of this epithelium (Fig. 2). On the other hand, the presumptive OE gradually reduced its thickness and its immunopositive olfactory cells toward the ventral part of the nasal septum and was transformed into the presumptive non-sensory (respiratory) epithelium.

At 15 to 16 days of gestation, the presumptive OE differentiated into the OE. Immunopositive olfactory cells were conspicuously increased in number and accumulated densely in the middle to basal region of the epithelium (Fig. 3). In the upper one-third of the OE, elongated nuclei of probable supporting cells were arranged in a single layer. Almost all dendritic processes of the immunopositive olfactory cells reached the free surface of the OE, but did not form olfactory vesicles at their distal ends. In the ventral part of the nasal septum, the presumptive non-sensory (respiratory) epithelium showed almost the same cell configuration and cytological features as observed at previous stages except that a small number of immunopositive cells aggregated in a small area of the most ventral part of the nasal septum and might be destined to become the MO in the future (Fig. 3).

At 18 days of gestation, the general appearance of the OE was almost the same as that in the adult. A single layer of nuclei of supporting cells was clearly distinguished from the lower region of the epithelium where immunopositive olfactory cells were arranged in three to five irregular laws (Fig. 4). Dendritic processes of olfactory cells protruded into the lumen of nasal cavity to form olfactory vesicles at their distal ends.

From 20 days of gestation onward, the general features of the OE were almost undistinguishable from those in the adult (Figs. 5, 6).

The septal olfactory organ of Masera: At 15 days of gestation, the presumptive MO first appeared as the aggregation of PGP 9.5-immunopositive receptor (sensory) cells in the epithelium lining the most ventral part of the nasal septum (Fig. 3). The MO was separated from the OE by the intercalated epithelium with lower height and a small number of PGP 9.5-immunopositive cells. Immunopositive sensory cells of the MO were distributed throughout the thickness of the epithelium and possessed scanty cytoplasm (Fig. 3). Dendritic processes of the immunopositive sensory cells were not long enough to reach the free surface of the MO.

At 16 days of gestation, the general appearance of the MO was almost the same as that at 15 days of gestation.

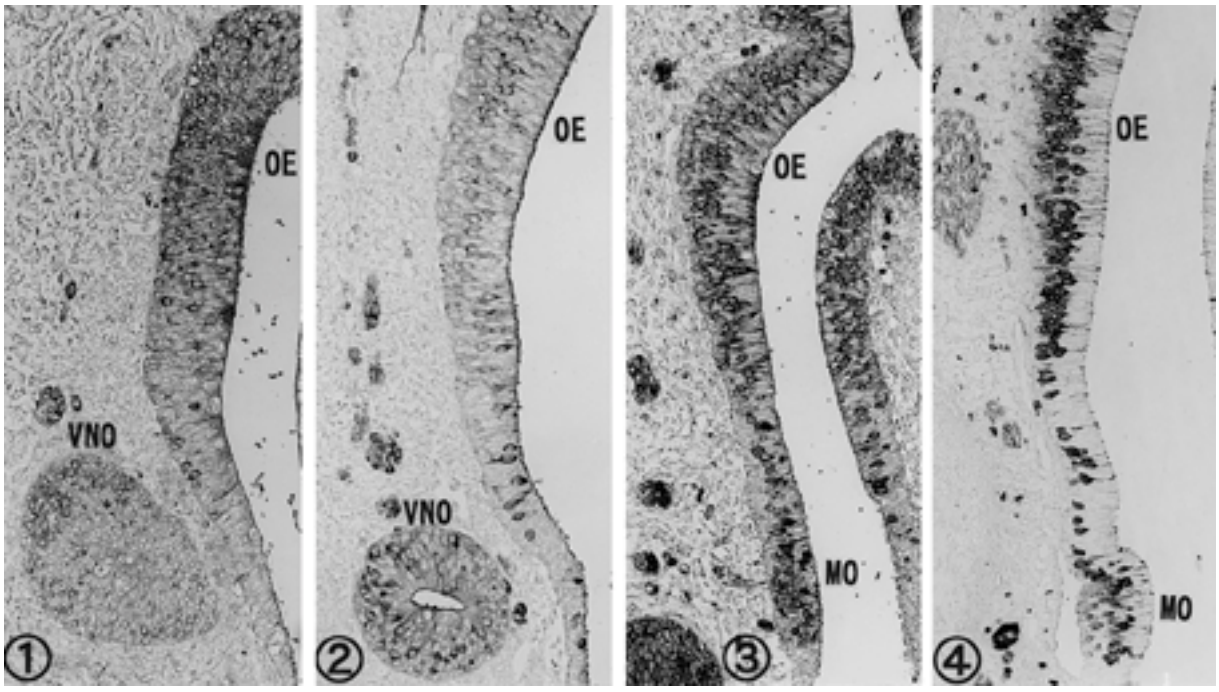
At 18 days of gestation, the MO increased its thickness and contained many immunopositive sensory cells although these cells decreased in the intercalated epithelium (Fig. 4). Numerous immunopositive sensory cells reached the free surface of the MO by sending dendritic processes upward (Fig. 4).

At 20 days of gestation, the immunopositive sensory cells increased in number in the MO and densely aggregated in the mass (Fig. 5). Stratification of sensory and supporting cells was still ambiguous in the MO at this stage. Dendritic processes of sensory cells were shorter than those in the adult but protruded beyond the free surface of the MO to form structures like olfactory vesicles (Fig. 7). The intercalated epithelium reduced the number of immunopositive cells and became similar to the non-sensory (respiratory) epithelium in its general appearance (Fig. 5).

At 7 days after birth, the MO gained almost the same histological features as those in the adult. However, several sensory cells still aggregated in the mass, as observed in the previous stage (Fig. 8). The presumptive non-sensory (respiratory) epithelium occupied a wide area between the MO and the OE and occasionally possessed a few immunoreactive cells in the boundary between this epithelium and the MO and this epithelium and the OE.

The vomeronasal organ: At 12 days of gestation, a recess was formed on the medial wall of the nasal pit and lined with a neuroepithelium consisting of PGP 9.5-immunonegative stem cells as in the presumptive OE.

At 13 days of gestation, the recess was separated from the nasal cavity to form the VNO (Fig. 1). The VNO was a tubular structure with a narrow lumen lined with the epithelium and closed at both the rostral and caudal ends at this stage. The weakly immunopositive receptor (sensory) cells were scattered in the dorsomedial part of the VNO.



Figs. 1–4. Frontal sections of the left side of the nasal septum. Medial is left and ventral is bottom in each figure. × 150. Fig. 1.: at 13 days of gestation. PGP 9.5-immunopositive olfactory cells are scattered in the presumptive OE and possess a round nucleus and scanty cytoplasm. Fig. 2.: at 14 days of gestation. Some olfactory cells distally extend dendritic process. Fig. 3.: at 15 days of gestation. Immunopositive olfactory cells are increased in number and accumulate densely in the middle to basal region of the epithelium. The MO first appears as the aggregation of PGP 9.5-immunopositive sensory cells in the epithelium lining the most ventral part of the nasal septum. Fig. 4.: at 18 days of gestation. Dendritic processes of olfactory cells form olfactory vesicles at their distal ends.

At 14 days of gestation, the epithelium increased in both its thickness and the number of immunopositive cells. These cells were elliptical in shape and gained abundant cytoplasm moderately immunopositive to PGP 9.5 (Fig. 2).

At 15 days of gestation, the lumen of the VNO became crescent in shape, and lined medially with the thickened epithelium but laterally with the thinner epithelium (Fig. 9). The former epithelium seemed to be destined to the vomeronasal sensory epithelium, while the latter one to the vomeronasal respiratory epithelium. Immunopositive cells were observed in both the medial and lateral epithelia, but rarely sent their dendritic processes to the free surface.

At 16 days of gestation, the sensory cells of the vomeronasal sensory epithelium increased in number and immunoreactivity to PGP 9.5.

At 18 days of gestation, oval nuclei of supporting cells were arranged in a single layer in the upper region of the vomeronasal sensory epithelium and round nuclei of sensory cells were accumulated densely in the middle to basal region (Fig. 10). These nuclear arrangements were the same as those in the adult. Long dendritic processes of sensory cells were less intensely immunopositive than the somata. In the vomeronasal respiratory epithelium, PGP 9.5-immunopositive cells were also observed, but they were devoid of dendrites and round in shape (Fig. 10).

From 20 days of gestation onward, numerous sensory cells were intensely immunopositive in both the dendrites and somata, and reached the free surface of the vomeronasal sensory epithelium by sending long dendrites. On the other hand, immunopositive cells were decreased in number in the vomeronasal respiratory epithelium. The brush border was not so prominent on the free surface of the vomeronasal sensory epithelium at 20 days of gestation, but developed well at 7 days after birth (Fig. 11). The VNO began to communicate with the nasal cavity by 7 days after birth. At 7 days after birth, virtually all dendrites of sensory cells protruded slightly beyond the free surface as those in the adult (Fig. 11).

DISCUSSION

PGP 9.5 is known to play an essential role in ubiquitin regulation and be expressed in both mature and immature olfactory receptor cells [16, 25, 31, 39]. In rats, all receptor cells are intensely immunopositive to PGP 9.5 in the OE, the sensory epithelium of the VNO and the MO [11, 16, 18, 33]. Therefore, PGP 9.5 may be a reliable marker for olfactory receptor cells in rats. In the present study, the OE, VNO and MO were compared with one another in their development and maturation to discuss the functional significance of these olfactory organs.

In the OE, PGP 9.5-immunopositive receptor (olfactory)

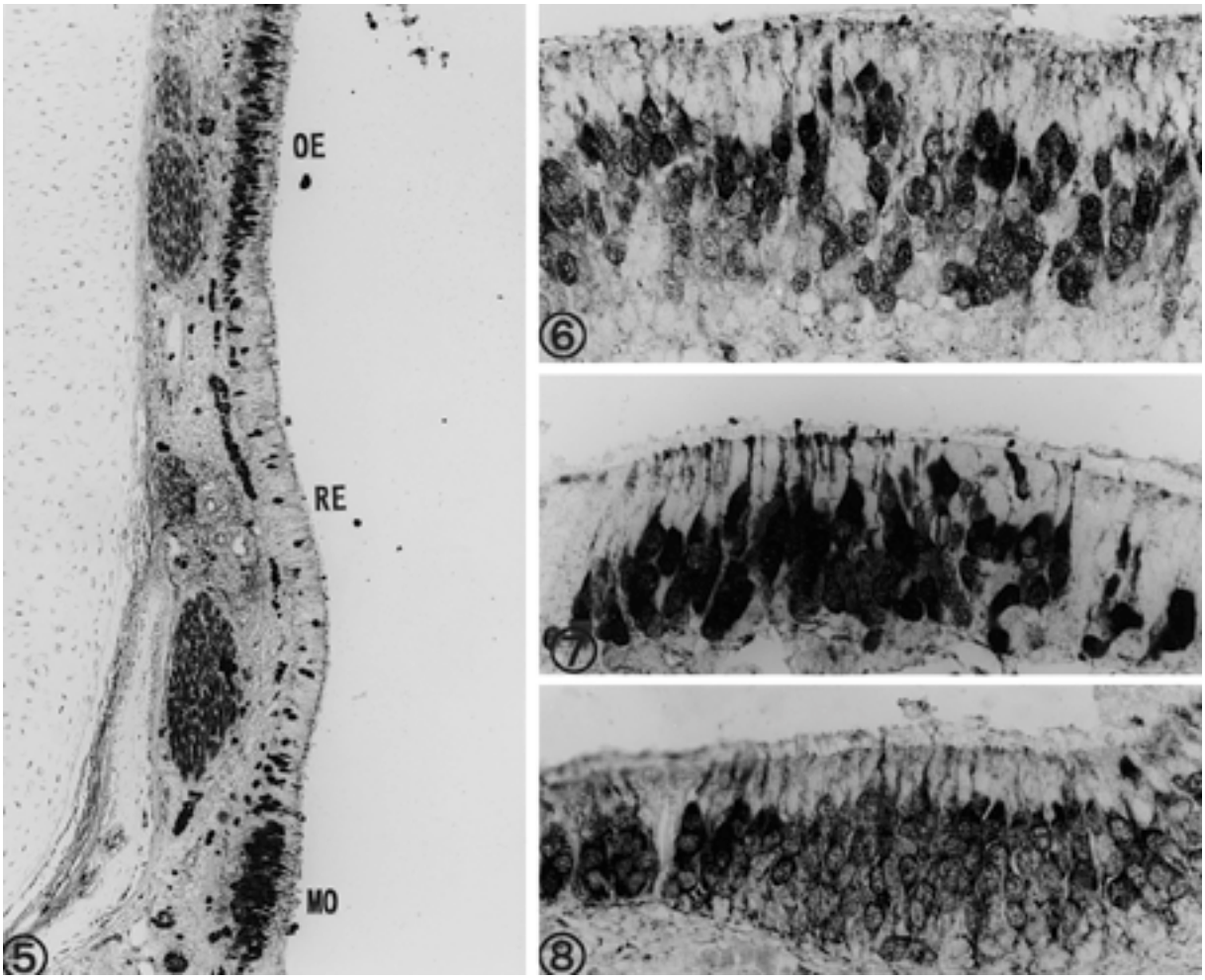


Fig. 5. Frontal sections of the left side of the nasal septum at 20 days of gestation. PGP 9.5-immunopositive sensory cells increase in number in the MO. The intercalated epithelium reduces the number of immunopositive cells and becomes similar to the respiratory epithelium (RE). × 180.

Fig. 6. Olfactory epithelium at 20 days of gestation. The general features of the OE were almost undistinguishable from those in the adult. × 400.

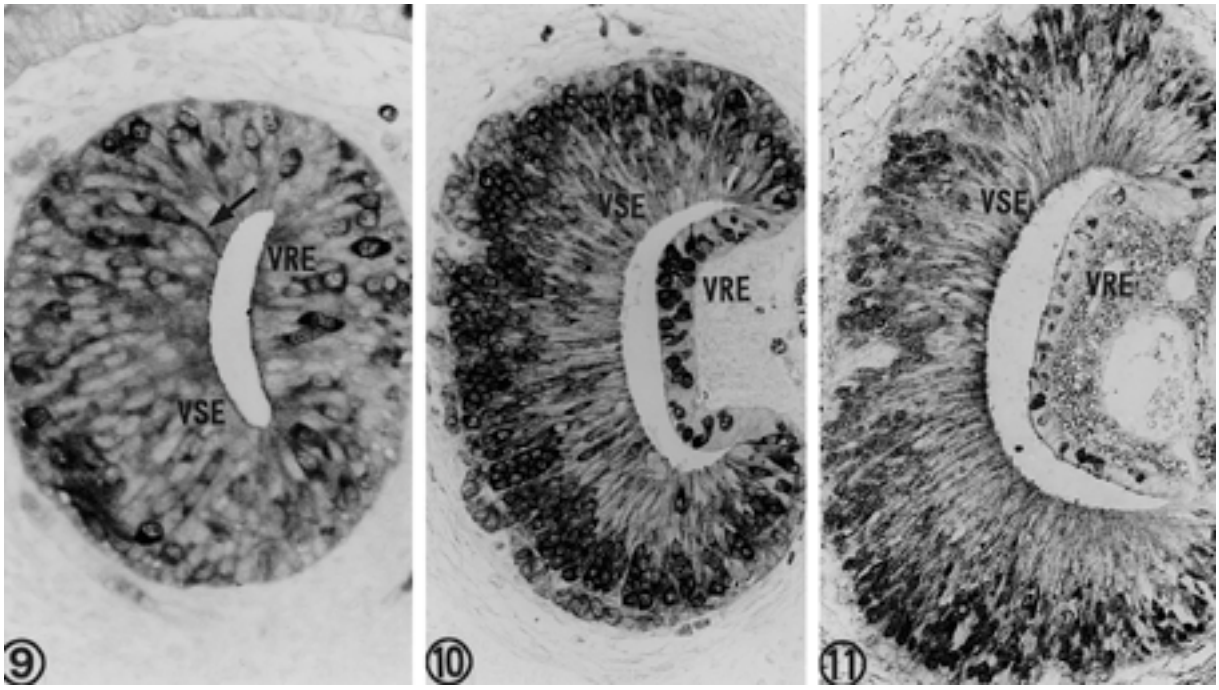
Fig. 7. Septal organ of Masera at 20 days of gestation. Short dendritic processes of sensory cells protrude beyond the free surface of the MO to form structures like olfactory vesicles. × 400.

Fig. 8. Septal organ of Masera at 7 days after birth. Several sensory cells aggregate in the mass, as observed in the previous stage. × 400.

cells first appeared at 13 days of gestation. At 15 to 16 days of gestation, the configuration of olfactory and supporting cells became similar to that in the adult, and nuclei of supporting cells were situated in the upper region of the OE, whereas those of olfactory cells in the middle to basal region. At 18 days of gestation, dendritic processes of olfactory cells were elongated and protruded into the lumen of the nasal cavity to form olfactory vesicles at their distal ends. At 20 days of gestation, the OE came to possess the same cytological features as in the adult, and seemed to differentiate and maturate completely at this stage. These findings agree well with those reported previously in rats, and may lead to reconfirm the hypothesis that the OE is already active in function at birth [11, 21, 27, 37].

As for the MO, it first appeared as densely packed PGP 9.5-immunopositive receptor (sensory) cells on the most ventral part of the nasal septum at 15 days of gestation. Thereafter, the MO became apparently distinguished from the OE by the decrease of PGP 9.5-immunopositive cells in the intercalated epithelium between the OE and the MO, to be isolated from the OE, and took an appearance of the distinct olfactory organ at 20 days of gestation. However, the MO does not seem to complete its development even at 7 days after birth, because several sensory cells still aggregated in the mass as observed in the previous stage.

On the other hand, Giannetti *et al.* [11] examined the development of the MO immunohistochemically by the use of the growth-associated protein B-50/GAP-43 as a neu-



Figs. 9–11. Coronal sections of vomeronasal organ. Fig. 9.: at 15 days of gestation. PGP 9.5-immunopositive cells rarely send their dendritic processes to the free surface (arrow). × 320. Fig. 10.: 18 days of gestation. Long dendritic processes of sensory cells are less intensely immunopositive than the somata. × 250. Fig. 11. at 7 days after birth. All dendrites of sensory cells protrude slightly beyond the free surface. × 250. (VSE: vomeronasal sensory epithelium, VRE: vomeronasal respiratory epithelium)

ronal marker in rats, and reported that the MO first appeared at the same stage as in the present study and went on developing even after birth. Breipohl *et al.* [2] observed the postnatal development of this organ by light and electron microscopy, and reported its ongoing development and the increase in number of olfactory cilia during the suckling period. In these two reports, the authors equally speculated that the MO begins to function after weaning.

The present findings on the development of the MO agree well with those reported previously in rats and may support the previous interpretation that the MO is slower than the OE in its development and the commencement of its function.

The VNO was separated from the nasal cavity as a tubular structure of a neuroepithelium at 13 days of gestation in the present study. PGP 9.5-immunopositive receptor (sensory) cells appeared simultaneously in this epithelium. By 18 days of gestation, the medial sensory epithelium became distinguishable from the lateral respiratory epithelium by the difference in their thickness and nuclear arrangement. At 7 days after birth, the free surface of the sensory epithelium was provided with the histological features like those in the adult, and showed the well-developed brush border and the protrusion of dendrites in the sensory cells.

There have been several reports on the development of the VNO [16, 34–36]. In rats, the VNO communicates with the nasal cavity at its rostral end postnatally, and its sensory cells do not maturate structurally nor express olfactory marker protein until after birth [5, 8, 10]. We previously examined the development of the VNO in the golden hamster by light and electron microscopy and reported that the sensory cells of the VNO are rather slowly maturated and some of them are devoid of microvilli on their free surface even at 10 days after birth when the weaning begins [34, 35]. On the other hand, Tarozzo *et al.* [36] reported that the VNO achieves significant morphological and biochemical maturation before birth in mice because of its expression of olfactory marker protein, but they speculated that the VNO is not necessarily active in function. These previous reports and the present findings strongly suggest that the VNO continues to develop and begin to function postnatally as same as the MO.

The functional assignment of these three olfactory organs is still unclear. Especially, the function of the MO is not established until now. Since the MO arises from the OE after the differentiation of the VNO from the nasal pit in the present study, the MO seems to participate in olfaction by supplementing the function of the OE, rather than the VNO. This supposition may be strengthened by our previous observation that the MO possesses two different types of sensory cells, the major type is covered with cilia, similar to the olfactory cells of the OE, and the other minor type with microvilli, similar to the sensory cells of the VNO [32]. This supposition may also be strengthened by our previous lectin-histochemical study demonstrating that lectin-binding patterns of the OE and MO are similar with each other to

be different from those of the VNO [30].

In summary, the present study indicates that PGP 9.5 is expressed in receptor cells of the OE, MO and VNO at an early stage of their development. This finding may suggest that PGP 9.5 plays a common fundamental role in the development of these olfactory organs. In addition, the present study makes it clear that each of these organs have its own distinct process of development and maturation. The OE completes its development before birth, while the MO and VNO after birth.

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