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Differential Expression of Neurofilament 200-Like Immunoreactivity in the Main Olfactory and Vomeronasal Systems of the Japanese Newt, *Cynops pyrrhogaster*

Satoshi SOETA[1], Yayoi IZU[1], Toru R. SAITO[2], Shuji YAMANO[1] and Kazuyuki TANIGUCHI[3]

[1]Departments of Veterinary Anatomy and [2]Laboratory Animal Science, Nippon Veterinary and Animal Science University, 1–7–1 Kyonan-cho, Musashino-shi, Tokyo 180–8602 and [3]Laboratory of Veterinary Anatomy, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka 020–8550, Japan

ABSTRACT. Expression of neurofilament 200 (NF200)-like immunoreactivity was examined in the main olfactory system and the vomeronasal system of the Japanese newt, *Cynops pyrrhogaster*, using anti-porcine NF200 monoclonal antibody (clone N52) to investigate the differences in phenotypical characteristics between these systems. The entire nasal cavity was a flattened single chamber consisting of the main nasal chamber (MNC) and the lateral nasal sinus (LNS) communicating with each other. The olfactory epithelium (OE) was present in the MNC, and the vomeronasal epithelium (VNE) was in the LNS. The OE possessed only a small number of NF200-like immunoreactive receptor neurons. The olfactory nerve and the olfactory nerve layer of the main olfactory bulb also contained a small number of NF200-like immunoreactive axons. In contrast, the VNE possessed many NF200-like immunoreactive receptor neurons. The vomeronasal nerve and the vomeronasal nerve layer of the accessory olfactory bulb contained many NF200-like immunoreactive axons. These findings in the Japanese newt indicate that NF200-like immunoreactive receptor neurons constitute a major subpopulation in the VNE and a minor subpopulation in the OE. In addition, NF200-like immunoreactivity seems to be a useful marker to distinguish the vomeronasal system from the other nervous systems including the main olfactory system in the Japanese newt. The localization of a few NF200-like immunoreactive receptor neurons in the OE might indicate that pheromone-sensitive receptor neurons are intermingled in the OE of the Japanese newt.
KEY WORDS: *Cynops pyrrhogaster*, immunohistochemistry, Japanese newt, NF200, olfactory systems.

In vertebrates, olfactory organs can possess two types of sensory epithelia, i.e., olfactory epithelium (OE) and vomeronasal epithelium (VNE). The olfactory receptor neurons in the OE project centrally through the olfactory nerve (ON) to the main olfactory bulb (MOB). On the other hand, the vomeronasal receptor neurons in the VNE project centrally through the vomeronasal nerve (VN) to the accessory olfactory bulb (AOB) [1, 18, 23, 36, 52].

Phylogenetically, the OE exists in all vertebrates from fish to mammals, while the VNE is thought to be absent in fish and first appears in urodele amphibians. The main olfactory system detects general odorants, while the vomeronasal system is involved in the perception of pheromonal molecules [1, 18, 23, 36, 52]. However, the physiological distinction between the OE and the VNE still remains unclear. The VNE can also respond to some kinds of general odorants [40], while reproductive behaviors and hormonal changes are also induced by pheromones in pig and sheep with destroyed VNE but intact OE [11, 15].

In the Japanese newt, *Cynops pyrrhogaster*, the OE possesses both ciliated and microvillous receptor neurons as the primitive OE in fish [4, 28], and the VNE possesses only microvillous receptor neurons as that in the other tetrapod animals [2, 22, 28, 32]. These morphological findings in the Japanese newt suggest that the main olfactory system retains phylogenetically primitive characteristics, while the vomeronasal system acquires characteristics common to the tetrapod animals. These characteristics observed in the Japanese newt olfactory systems may make the Japanese newt a good model to examine the evolution of the main olfactory and vomeronasal systems in tetrapod animals.

Neurofilament (NF), one of neuron-specific intermediate filaments, is composed of three subunits with molecular masses of approximately 200 (NF200), 150 (NF150) and 70 kDa (NF70), and these subunits are assembled to constitute a filament about 10 nm in diameter [45]. NF is widely expressed in the central and peripheral nervous systems. However, recent studies have demonstrated that assembled NFs are absent in the olfactory receptor neurons in salamanders, rats and humans [6, 8, 29, 47]. The absence of assembled NFs in the olfactory receptor neurons is thought to relate to the continual neurogenesis of the olfactory receptor neurons, since no assembled NF was detected in immature neurons [9, 10]. In contrast, both the ON and VN contain assembled NFs and the VN shows difference in number of assembled NFs between the proximal and distal ends in bullfrogs [7]. These findings indicate that the expression of NF in the olfactory systems varies among species and may correspond to functional difference between the olfactory and vomeronasal systems.

In our preliminary screening of several antibodies for NF subunits to reveal their immunohistochemical titers, we found that an anti-porcine NF200 monoclonal antibody showed intense immunoreactivity for a subpopulation of the olfactory and vomeronasal receptor neurons in the Japanese newt. In the present study, therefore, we used this antibody for morphological studies to examine in detail the difference in the subpopulation of receptor neurons and their projection

patterns leading to their functional differentiation in the main olfactory and vomeronasal systems from a phylogenetical point of view.

MATERIALS AND METHODS

Ten Japanese newts (5 males and 5 females) obtained from (Hamamatsu Seibutsukyozai, Hamamatsu, Japan) were anesthetized by a 0.3% solution of tricaine methanesulfonate and euthanized by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer. The upper jaw together with the head was removed, immersed in the same fixative, decalcified in 10% EDTA for 1 week and embedded in paraffin. Paraffin sections were double-stained with hematoxylin-eosin (HE) or processed for immunohistochemistry. The immunohistochemistry using a monoclonal antibody (clone N52, Sigma Chemical, St. Louis, MO., U.S.A.) raised against porcine neurofilament 200 (NF200) was performed by streptavidin-biotin-peroxidase complex (sABC) method. Briefly, after blocking of endogenous peroxidase with 0.3% H_2O_2 in methanol, the deparaffinized sections were treated with normal rabbit serum (DAKO A/S, Glostrup, Denmark). Then, the sections were incubated with monoclonal mouse anti-porcine NF200 (1:100) antibody at 4°C overnight. After treatment with rabbit anti-mouse immunoglobulin (DAKO A/S), the sections were incubated with sABC (DAKO A/S). To visualize the products of antigen-antibody reaction, the sections were treated with 0.02% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2. Finally, the sections were counterstained with hematoxylin.

RESULTS

In the Japanese newt, the entire nasal cavity was a bilaterally symmetric, flattened chamber. It consisted of the main nasal chamber (MNC) and the lateral nasal sinus (LNS) communicating with each other (Fig. 1A). The LNS was a small diverticulum situated laterally to the large MNC (Fig. 1A). The epithelium of the MNC was organized in a pattern of grooves and ridges, and the OE represented grooves and the respiratory epithelium ridges (Fig. 1B). In contrast, the LNS was lined solely with the VNE and did not show such a pattern (Fig. 1C). In both the OE and VNE, elongated nuclei of the supporting cells were arranged in the superficial third of the epithelium, and ovoid nuclei of the receptor neurons were packed in the deep two thirds of the epithelium. In addition, small and amorphous basal cells with ovoid nuclei were situated in a row at the bottom of the epithelium (Fig. 1B and C). In the forebrain, the MOB was situated at the rostral portion of the telencephalon [16, 19, 24–26]. The MOB was divided into 4 layers; the olfactory nerve layer, glomerular layer, mitral / tufted cell layer and granule cell layer (Fig. 1D). The AOB was situated dorsocaudally to the MOB and consisted of the vomeronasal nerve layer, glomerular layer, mitral / tufted cell layer and granule cell layer [19] (Fig. 1E).

By immunohistochemistry, a small number of NF200-like immunoreactive receptor neurons were detected in the OE (Fig. 2A and B). The immunoreactivity was observed in the perikarya, thin dendrites extending to the free surface of the epithelium and axonic extensions at the basal portion of the receptor neurons (Fig. 2B). There was no difference in the distribution of NF200-like immunoreactive receptor neurons among the grooves representing the OE. In the VNE, numerous NF200-like immunoreactive receptor neurons were densely distributed, although a very small number of receptor neurons were NF200-like immunonegative (Fig. 2C). The immunoreactivity was observed in the perikarya, dendrites and axons of the receptor neurons as in the NF200-like immunoreactive receptor neurons of the OE (Fig. 2D). NF200-like immunoreactive receptor neurons gradually decreased in number from the caudal one third of the LNS toward the choana and were not detected near the choana. No immunoreactivity for NS200 was observed in either supporting cells or basal cells in both the OE and the VNE.

The VN was identified as the nerve bundle containing many NF200-like immunoreactive axons in the serial sections of the upper jaw and the head of the Japanese newt. In the submucosa of LNS, the VN originated from NF200-like immunoreactive receptor neurons in the VNE (Fig. 2E) to run mediocaudally in the submucosa of the MNC toward the nasal septum (Fig. 2F). Other nerve bundles in the submucosa of the MNC possessed a few NF200-like immunoreactive axons and constituted the ON. In the nasal septum, the VN ran dorsocaudally along with the ON containing a few NF200-like immunoreactive axons (Fig. 2G and H).

The VN ran dorsocaudally to enter the cranial cavity and formed a common bundle with the ON situated dorsally to the VN (Fig. 3A). In the forebrain, the VN running on the surface of the MOB still contained many NF200-like immunoreactive axons, while the MOB contained only a few NF200-like immunoreactive axons in the olfactory nerve layer (Fig. 3B and C). The AOB contained many NF200-like immunoreactive axons in the vomeronasal nerve layer and a few NF200-like immunoreactive axons in the glomerular layer (Fig. 3D). No NF200-like immunoreactivity was observed in the other layers of the AOB.

DISCUSSION

In the present study, the organizations of the nasal chamber, the MOB and AOB of the Japanese newt were almost consistent with those of several urodele species [5, 12–14, 16, 17, 19, 24–26, 35, 39, 41, 50]. As previously described by Jones *et al.* [28] and Saito *et al.* [39], the OE of the Japanese newt was divided into several grooves by ridges of the respiratory epithelium. The pattern of the grooves and ridges is also demonstrated in the epithelia of the MNC of aquatic salamanders, such as *Ambystoma, Necturus* and *Silen* [16, 17, 41]. In *Plethodon*, a terrestrial urodele, however, the OE lines whole of the dorsal and ventral walls of the MNC, and the respiratory epithelium is limited to the medial wall of the MNC and the boundary regions between
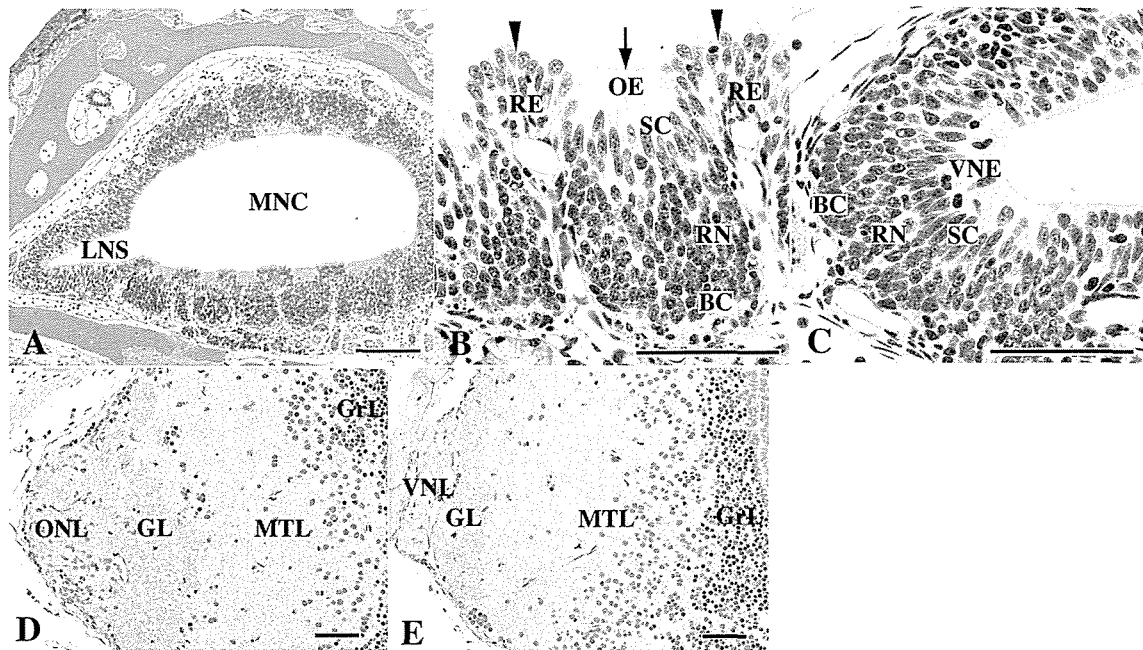
Fig. 1.   Transverse sections of the right entire nasal cavity, the main (MOB) and accessory olfactory bulbs (AOB) of the Japanese newt. A : The entire nasal cavity consists of the main nasal chamber (MNC) and the lateral nasal sinus (LNS). Bar=200 μm. B : The epithelium of the MNC is organized in a pattern of grooves (arrow) representing the olfactory epithelium (OE) and ridges (arrowheads) representing respiratory epithelium (RE). Elongated nuclei of the supporting cells (SC) are arranged in the superficial third of the epithelium, and ovoid nuclei of the receptor neurons (RN) are packed in the deep two thirds of the epithelium. Small and amorphous basal cells (BC) with ovoid nuclei are situated in a row at the bottom of the epithelium. Bar=100 μm. C: The LNS is lined solely with the vomeronasal epithelium (VNE) and devoid of a pattern of grooves and ridges. The RN, SC and BC are arranged as in the OE. Bar=100 μm. D: The MOB is divided into 4 layers; olfactory nerve layer (ONL), glomerular layer (GL), mitral/tufted cell layer (MTL) and granule cell layer (GrL). Bar=100 μm. E: The AOB consists of vomeronasal nerve layer (VNL), GL, MTL and GrL. Bar=100 μm. H.E.

the OE and VNE [12–14]. Therefore, the pattern of grooves and ridges in the epithelia of the MNC may be a specific feature of aquatic and semiaquatic urodeles, such as the Japanese newt.

The differences in phenotypical properties between the main olfactory and vomeronasal systems were demonstrated by lectin-histochemistry in several vertebrates [20, 27, 30, 31, 34, 38, 43, 46]. In urodeles, Franceschini and Ciani [19] demonstrated that *Dolichos biflorus* agglutinin (DBA) and succinyl wheat germ agglutinin (s-WGA) bound predominantly to the MOB, but not to the AOB. The receptor neurons in the OE showed more intense reactivity than those in the VNE by histochemistry using several lectins [39]. However, there was no report showing phenotypical differences between the main olfactory and vomeronasal systems by immunohistochemical examinations.

In the present study, we demonstrated that NF200-like immunoreactive receptor neurons were predominantly distributed in the vomeronasal system, but sparsely in the main olfactory system of the Japanese newt. The VN containing many NF200-like immunoreactive axons could be traced from the submucosa of the VNE to the vomeronasal nerve layer in the AOB. The course of the VN of the Japanese newt was almost consistent with that of the salamanders

[42]. In contrast, NF200-like immunoreactivity was rarely observed in the olfactory receptor neurons and absent in the other areas of the central nervous system (data not shown). These findings in the Japanese newt indicate that NF200-like immunoreactive receptor neurons constitute a major subpopulation in the VNE and a minor subpopulation in the OE. In addition, the epitope recognized by the present monoclonal antibody for NF200 (clone N52) seems to be a useful marker to distinguish the vomeronasal receptor neurons from the other nervous tissues including the main olfactory system in the Japanese newt. Since the clone N52 recognizes an epitope present in the tail domain (H subunit) of NF200 in mammals, it may also be true in the Japanese newt. In urodeles, however, NF200 is widely expressed in the central and peripheral nervous systems as in the other vertebrates [3, 8, 37]. Lewis and Nixon identified four NF200 variants generated by phosphorylation of a single polypeptide in rodents [33]. These NF200 variants show distinct reaction patterns with several monoclonal antibodies against NF200 and are expressed in different regions of the nervous tissues. It is speculated that N52 monoclonal antibody recognizes an epitope of a NF200 variant predominantly expressed in the olfactory systems of the Japanese newt. This immunohistochemical specificity may lead to
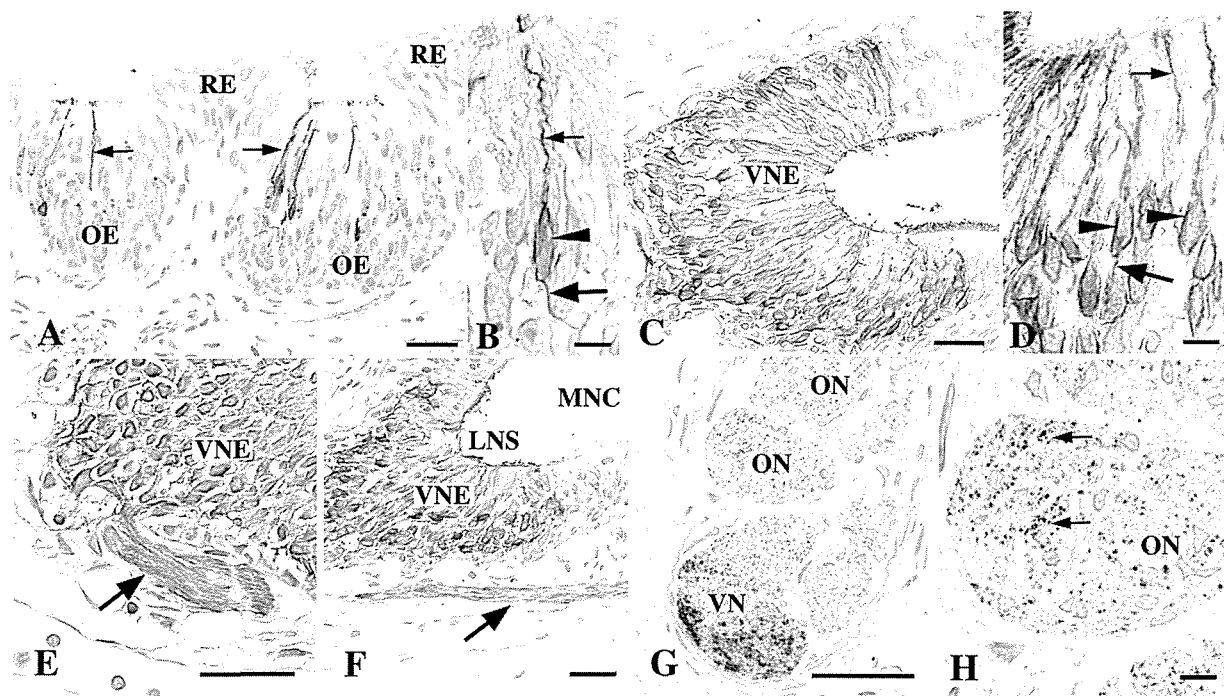
Fig. 2.   NF200 immunohistochemistry in the olfactory epithelium (OE), vomeronasal epithelium (VNE), olfactory nerve (ON) and vomer-
onasal nerve (VN) of the Japanese newt. A: A small number of NF200-like immunoreactive receptor neurons (small arrows) are detected
in the OE. Bar=50 μm. B: NF200-like immunoreactivity is detected in the perikarya (arrowhead), dendrites (small arrow) and axons
(large arrow) of the receptor neurons. Bar=10 μm. C: Numerous NF200-like immunoreactive receptor neurons are densely distributed in
the VNE. Bar=50 μm. D: NF200-like immunoreactivity is detected in the perikarya (arrowheads), dendrites (small arrow) and axons
(large arrow) of the receptor neurons. Bar=10 μm. E: NF200-like immunoreactive VN (large arrow) originates from NF200-like immu-
noreactive receptor neurons in the VNE. Bar=50 μm. F: The VN (arrow) runs mediocaudally in the submucosa of the lateral nasal sinus
(LNS) and main nasal chamber (MNC) toward the nasal septum. Bar=50 μm. G: The VN contains many NF200-like immunoreactive
axons in the nasal septum. Bar=50 μm. H: The ON contains only a few NF200-like immunoreactive axons (arrows) in the nasal septum.
Bar=10 μm.

the limited immunoreaction in the olfactory systems of the
Japanese newt observed in the present study.

In the OE, a few NF200-like immunoreactive receptor
neurons were detected among many NF200-like immunone-
gative receptor neurons. A few NF200-like immunoreactive
nerve fibers detected in the ON and the olfactory nerve layer
of the MOB were thought to be axons of the NF200-like
immunoreactive receptor neurons in the OE. NF200-like
immunoreactive receptor neurons may constitute a subpop-
ulation functionally different from the other subpopulations
in the OE. Therefore, the OE may possess phenotypically
similar receptor neurons to vomeronasal receptor neurons.
Previous ultrastructural examinations revealed that the OE
of the Japanese newt possessed microvillous and ciliated
receptor neurons as in fish [4, 28], while in the other tetra-
pods, the OE possessed only ciliated receptor neurons [2,
22, 32]. In contrast, the VNE in the Japanese newt pos-
sessed only microvillous receptor neurons as in the other tet-
rapods [28]. These findings indicated that the microvillous
receptor neurons in the OE resembled the vomeronasal
receptor neurons in the Japanese newt. In addition, Toyoda
and Kikuyama [49] examined the sensitivity of the olfactory

receptor neurons to sodefrin, a female-attracting phero-
mone, and demonstrated that the response to sodefrin was
detected not only in the VNE, but also in the OE. In the
goldfish lacking the VNE anatomically, some populations
of the receptor neurons in the OE were pheromone-sensitive
and projected to the MOB [21, 44]. These findings lead to a
possibility that the pheromone-sensitive receptor neurons
are intermingled with the ordinary olfactory receptor neu-
rons in the OE and projected to the MOB in the Japanese
newt as in the goldfish. This supposition might be sup-
ported by the results in the present study that a few receptor
neurons in the OE showed NF200-like immunoreactivity
similar to the vomeronasal receptor neurons. Additional
examinations are necessary to determine whether the
NF200-like immunoreactive and / or microvillous receptor
neurons could detect pheromonal molecules such as sode-
frin in the OE of the Japanese newt.

As for the main olfactory and vomeronasal systems, the
present study revealed NF200-like immunoreactive and
immunonegative neuronal subpopulations in the Japanese
newt. Although the main olfactory and vomeronasal sys-
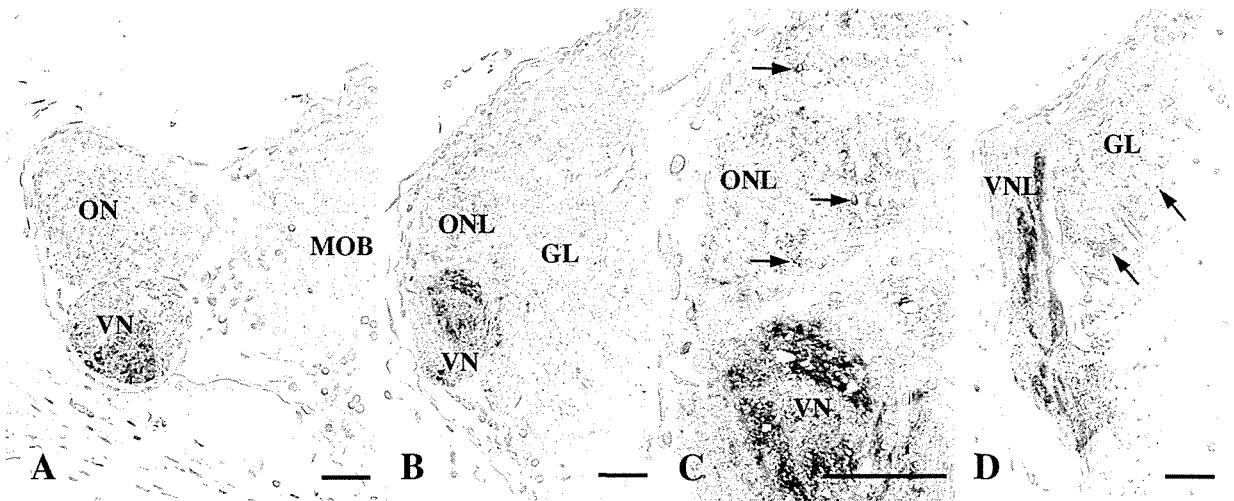tems are composed of distinct neuronal subpopulations sub-

Fig. 3. NF200-like immunohistochemistry in the olfactory nerve (ON), vomeronasal nerve (VN), main olfactory bulb (MOB) and accessory olfactory bulb (AOB) of the Japanese newt. A: The VN forms a common bundle with the ON situated dorsally to the VN. The VN contains many NF200-like immunoreactive axons, while the ON contains only a few NF200-like immunoreactive axons. Bar=50 μm. B: The VN running on the surface of the MOB still contained many NF200-like immunoreactive axons. Bar=50 μm. C: The MOB contained only a few NF200-like immunoreactive axons (arrows) in the olfactory nerve layer (ONL). Bar=50 μm. D: The AOB contains many NF200-like immunoreactive axons in the vomeronasal nerve layer (VNL) and a few NF200-like immunoreactive axons (arrows) in the glomerular layer (GL). Bar=50 μm.

divided clearly by histochemistry in rodents [48, 51], the present study first demonstrated that the histochemical subdivision in the main olfactory and vomeronasal systems have already appeared in urodeles in phylogeny. In the Japanese newt, however, there was no difference in the distribution of NF200-like immunoreactive receptor neurons among the grooves representing the OE. In addition, NF200-like immunoreactive and immunonegative neuronal subpopulations showed no clearly segregated distribution pattern in the VNE. Therefore, functionally different neuronal subpopulations may be intermingled in the olfactory systems of the Japanese newt unlike those of rodents.

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