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Timing of Neuronal Intermediate Filament Proteins Expression in the Mouse Vomeronasal Organ During Pre- and Postnatal Development. An Immunohistochemical Study

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Abstract

Several types of intermediate filament proteins are expressed in developing and mature neurons; they cooperate with other cytoskeletal components to sustain neuronal function from early neurogenesis onward. In this work the timing of expression of nestin, peripherin, internexin, and the neuronal intermediate filament triplet [polypeptide subunits of low (NF-L), medium (NF-M), and high (NF-H) molecular weight] was investigated in the developing fetal and postnatal mouse vomeronasal organ (VNO) by means of immunohistochemistry. The results show that the sequence of expression of intermediate filament proteins is internexin, nestin, and NF-M in the developing vomeronasal sensory epithelium; internexin, peripherin, and NF-M in the developing vomeronasal nerve; and nestin, internexin and peripherin, NF-L, and NF-M in the nerve supply to accessory structures of the VNO. At sexual maturity (2 months) NF-M is only expressed in vomeronasal neurons and NF-M, NF-L and peripherin are expressed in extrinsic nerves supplying VNO structures. The differential distribution of intermediate filament proteins in the vomeronasal sensory epithelium and nerve is discussed in terms of the cell types present therein. It is concluded that several intermediate filament proteins are sequentially expressed during intrauterine development of the VNO neural structures in a different pattern according to the different components of the VNO.

Key words: nestin, peripherin, internexin, NF-L, NF-M, NF-H

Introduction

The vomeronasal organ (VNO) is the chemoreceptive organ of the accessory olfactory system detecting pheromones released in the environment by conspecifics. The VNO affects sexual and reproductive behavior (Døving and Trotier, 1998). In the mouse, the VNO is placed in tubular bones on either side of the anterior–inferior nasal septum; the VNO comprises a medial sensory epithelium and a lateral nonreceptor epithelium delimiting the vomeronasal lumen, which in turn is in connection with the nasal cavity; other structures of the VNO are the laterally placed cavernous tissue (the so-called vomeronasal pump), probably involved in the access/removal of pheromones from the lumen (Meredith *et al.*, 1980; Meredith, 1994), and the vomeronasal glands, secreting putative pheromone carriers (vomeroglandin) into the lumen (Matsushita *et al.*, 2000). Axons leaving the sensory epithelium form the vomeronasal nerve, which eventually reaches the accessory olfactory bulb. The mouse VNO develops from the olfactory placode starting from embryonic (E) day 11 (Cuschieri and Bannister, 1975). At birth, all the basic structural constituents of the VNO are present; however, the VNO only reaches its final size and structure upon puberty (Wilson and Raisman, 1980; Coppola *et al.*, 1993).

In the cytoplasm of Metazoan cells three classes of fibrous cytoskeletal polymers are found: F-actin, microtubules, and intermediate filaments. Intermediate filaments are in turn classified in to six classes (I–VI) according to genomic structure and nucleotide sequence homology (Coulombe *et al.*, 2001). Mature mammalian neurons contain intermediate filaments [neuronal intermediate filaments (NIF)] involved in the maintaining of neuronal shape and internal dynamics; albeit other cytoskeletal proteins, such as microtubules

and microfilaments, collaborate to the above functions, NIF are unique to neurons and can serve as a marker of mature neurons (Lee and Cleveland, 1996). NIF belong to the Class IV intermediate filament proteins and are composed of three polypeptide subunits of low (NF-L), medium (NF-M), and high (NF-H) molecular weight. In vertebrates, the three subunits are generally expressed in sequence during development: NF-L and NF-M appear during neurite differentiation, and NF-H is found as the cytoskeleton is stabilized (Willard and Simon, 1983; Carden *et al.*, 1987; Szaro *et al.,* 1989). α-Internexin, an other Class IV intermediate protein (Ching and Liem, 1991), is mainly expressed by immature neurons preceding the expression of NIF proteins (Kaplan *et al.*, 1990). Moreover, during early neurogenesis different other intermediate filament proteins are expressed: peripherin (Portier *et al.*, 1983), which belongs to Class III intermediate protein, and nestin (Lendahl *et al.*, 1990), which belongs to Class VI intermediate protein. Peripherin is mainly expressed in differentiating neurons, and its presence correlates with the phase of axon outgrowth (Troy *et al.*, 1990). Nestin and peripherin are therefore markers of neuron progenitors.

Despite extensive investigation, the precise function of these proteins in neurons is still not fully understood (for review, see Lariviere and Julien, 2004). In mature neurons, NIF control axonal caliber and thus conduction velocity (Hoffman *et al.*, 1987) as confirmed in studies of transgenic mice lacking axonal neurofilaments (Eyer and Peterson, 1994). In developing neurons, intermediate filament proteins are probably involved in the dynamics of the cytoskeleton during neuronal differentiation, axon outgrowth, and guidance (Grant and Pant, 2000), albeit some neuronal subsets appear to be affected by the lack of a particular intermediate filament type by a gene-targeting approach. Also, NIF are required for proper dendritic arborization of large motor neurons. Independently from possible function(s), the expression of the above quoted intermediate filament proteins has been widely used as marker to define maturation steps of neurons; in the main olfactory system (Schwob *et al.*, 1986; Bruch and Carr, 1991; Gorham *et al.*, 1991; Drapkin and Silverman, 1999), inclusive of the accessory olfactory bulb (Chien *et al.*, 1998), the expression of NIF proteins in the adult and developing neurons revealed a more immature pattern than elsewhere in the central nervous system. This is probably related to the continuous turnover and replacement olfactory neurons undergo throughout life. However, the appearance and distribution of neuron-specific intermediate filament proteins in neurons of the accessory olfactory system were not investigated but for nestin (Osada *et al.*, 1995; Matsuoka *et al.*, 2002). In the present work we present immunohistochemical results showing the timing of expression of several neurofilament proteins in the mouse VNO from early intrauterine development to sexual maturity to get insights into the maturation steps of neural structures therein.

Materials and methods

Animals

A total of 50 embryos and postnatal mice of the Swiss strain were used. Timed pregnant mice were anesthetized with ketamine (75 mg/kg) and xylazine (20 mg/kg) and perfused through the aorta with 4% paraformaldehyde. Embryonic mice were removed by cesarean section and their crown–rump length was determined. Embryos were collected at E13, E14, E15, E16, E17, E18, E19, and E20 and decapitated. Heads were further fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for several hours at 4°C. Postnatal mice were anesthetized and perfused as above at the end of postnatal (P) day 1 (E21 = P1), P8, P15, P21, and P60 and decapitated. Neonates had free access to suckling, and they were left with the dam until about 1 h before killing. Where needed, heads was decalcified with 3.72% ethylenediaminetetraacetic acid in 0.1 M phosphate buffer for several days. Finally, heads were embedded in paraffin and sectioned. All the procedures comply with the Italian law on animal experiments and were performed according to protocols approved by the Italian Ministry of Health.

Immunohistochemistry

At least three heads were used for immunohistochemistry at each embryonic or postnatal age. Coronal paraffin sections were collected on polylysine-coated microscope slides. For immunohistochemistry, the Labeled Polymer method (Van der Loos *et al.*, 1996; Zancanaro *et al.*, 2002) or the avidin–biotin complex (ABC) method was used. Briefly, deparaffinized sections were rehydrated, and endogenous peroxidase activity was inhibited by 10 min incubation with the Dako peroxidase-blocking agent (Dako, Milan, Italy). After washing in 0.05 M Tris–HCl buffer (pH 7.6), the sections were incubated with the primary antibody for 30 min at room temperature. The source and final dilution of antibodies is indicated in Table 1.

Immunoreaction was revealed by incubating sections with the proper biotinylated suine anti-rabbit IgG (Dako) or goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (Envision+, Dako). Sections processed according to the ABC method were then incubated for 30 min with avidin–biotinylated horseradish peroxidase (Vectastain Elite, Vector Laboratories, Burlington CA). For nestin immunolabeling, an immunodetection kit (Vector M.O.M., Vector Laboratories) that is specifically designed to localize mouse primary antibody on mouse tissues was used. Finally, all sections were reacted with 0.05 M 3,3-diaminobenzidine tetrahydrochloride for 3–5 min. Sections were then mounted and observed in an Olympus BX51 photomicroscope equipped with a KY-F58 CCD camera (Vietol Company of Japan, Tokyo, Japan). Electronic images were analyzed and stored using the software Image ProPlus (Media

Table 1 List of antibodies used in immunohistochemical staining

Antibody	Dilution	Source	Code
Nestin (monoclonal)	1:1000	BD PharMingen (San Diego, CA)	556309
Peripherin (polyclonal)	1:1400	Chemicon International (Temecula, CA)	AB1530
α-Internexin (polyclonal)	1:1400	Chemicon International	AB1515
Neurofilament 68 kD NF-L (polyclonal)	1:1800	Chemicon International	AB1983
Neurofilament 150 kD NF-M (polyclonal)	1:1000	Chemicon International	AB1981
Neurofilament 200 kD NF-H (polyclonal)	1:1000	Chemicon International	AB1989

Cybernetics, Silver Spring, MD). All illustrations were composed in Adobe Photoshop software (version 6.0, Adobe Systems, Mountain View, CA), adjusting only brightness and contrast.

Results

The results of immunohistochemical investigation of the mouse embryonic and adult VNO are summarized in Tables 2 and 3. Figures 1–6 illustrate representative immunohistochemical findings in the prenatal and postnatal VNO.

Nestin (Figures 1A,B,D,E,G–M and 5A–C,E; Table 2) is expressed in the sensory epithelium at E14 until P8, showing maximum intensity at E18–E20. At E14, labeling is found in the vomeronasal nerve (Figure 1A) as well; at this stage, continuity of epithelial, nestin-positive cells and labeled prolongements in the mesenchyma is present (Figure 5B), which is not found in later development. In embryos, labeling is in cells with their body located in the basal region of the epithelium and apical prolongements extending to the epithelial surface (Figure 5A); in postnatal mice, labeling is restricted to the basal region of the epithelium (Figures 1M and 5C). Nestin-positive cells often show intense labeling in the nucleus (Figure 5E).

Nestin immunoreactivity is found at E14–E20 in nerve fibers around the developing VNO cavernous tissue and blood vessels of the sensory epithelium and from E16 to term in fibers around vomeronasal glands; no immunoreactivity is present in these locations in postnatal mice. In the olfactory epithelium (Figure 1A,C,F; Table 2), the timing of appearance of nestin immunoreactivity is similar as in the vomeronasal sensory epithelium. Labeling is present in basal prolongements of cells reaching the basal lamina or ending on the body of basal cells (Figure 5D).

Peripherin immunoreactivity (Figures 2A–I and 5F–H; Table 2) is occasionally present in cells of the vomeronasal epithelium sending projections to the mesenchyma at E14 (Figure 5F,G) and is not found thereafter; it is expressed in the vomeronasal nerve from E14 onwards and in cells migrating along with fibers (Figure 5H); the labeling intensity of the nerve increases in the last days of intrauterine life, and peripherin is absent therein during the postnatal development of the VNO. Peripherin is also present in fibers around the developing VNO cavernous tissue, blood vessels of the sensory epithelium, and the vomeronasal glands from E16 onwards with maximum intensity during the last days before delivery; in the postnatal life, some peripherin immunoreactivity is still found around the blood vessels of the VNO cavernous tissue and glands up to 2 months of age. Peripherin immunoreactivity is consistently absent in the olfactory epithelium (not shown) during both the intra- and extrauterine life.

In the mouse embryo, internexin (Figures 3A–L and 6A–E; Table 2) is expressed in the developing sensory epithelium from E13 onwards; labeling is found in the cell body and, more intense, in the apical prolongements of cells in almost the entire epithelial thickness (Figure 6B–D). Occasionally, intense labeling is present in basal prolongements apparently leaving the epithelium (Figure 6A) or in globose cells at the lateral side of the epithelium (Figure 6E) Immunoreactivity is mild or absent therein by the end of pregnancy. In the vomeronasal nerve, positive fibers (Figure 3A,B,D,F; Table 2) are found from E13 to the end of pregnancy, together with positive, nerve-associated cells. Internexin immunoreactive fibers are also found around the developing VNO cavernous tissue and glands from E16 to the end of pregnancy, with maximum intensity at E19; some immunoreactivity is also found in the olfactory epithelium (not shown) at E13–E19. No internexin immunoreactivity is found in the postnatal VNO.

Immunoreactivity for NF-L (Figure 4A,C; Table 3) appears in fibers around the vomeronasal cavernous tissue, among developing VNO glands and around blood vessels of the sensory epithelium at E15, E16, and E18 to persist therein till the end of pregnancy; in the same VNO structures, NF-M immunoreactivity (Figure 4B,D; Table 3), shows a similar pattern, apart for a slightly delayed appearance. In the sensory epithelium and nerve fibers therefrom NF-M immunoreactivity is only found, starting at E18 (Figure 4B,D; Table 3); in the sensory epithelium labeling is in the apical prolongements of epithelial cells (Figure 6G,H). During postnatal development, immunoreactivity for NF-L and NF-M is still present in the VNO vasculature and glands at 2 months of age (Figure 4E–H; Table 3). In the sensory epithelium

Table 2 Nestin, peripherin, and internexin immunostaining in the embryonic mouse nose

	Embryonic age (day)																							
	13			14			15			16			17			18			19			20		
Antibodies	N	P	I	N	P	I	N	P	I	N	P	I	N	P	I	N	P	I	N	P	I	N	P	I
Vomeronasal nerve	–	–	●	●	•	●	–	●	●	–	●	●	–	●	●	–	●	●	–	●	•	–	●	•
Sensory epithelium	–	–	•	●	–	•	●	–	•	●	–	•	●	–	•	●	–	•	●	–	•	●	–	–
Vomeronasal fibers in the lamina propria of the sensory epithelium	–	–	–	–	–	–	–	–	●	–	●	●	–	●	●	–	●	●	–	●	●	–	●	–
Vomeronasal cavernous tissue	–	–	–	●	–	–	●	–	–	●	•	•	●	•	•	●	●	●	●	●	●	●	●	●
Blood vessel of the sensory epithelium	–	–	–	●	–	–	●	–	–	●	–	–	●	–	–	●	●	●	●	●	●	●	●	●
Vomeronasal glands	np	np	np	np	np	np	np	np	np	●	•	●	●	•	●	●	●	●	●	●	●	●	●	●
Olfactory epithelium	–	–	•	●	–	•	●	–	•[a]	●	–	•[a]	●	–	•[a]	●	–	•[a]	●	–	•[a]	●	–	–

Antibodies: N, nestin; P, peripherin; I, internexin. Immunostaining intensity: –, negative; •, weakly positive; ●, positive; ●, strongly positive; np, glands not present.
[a]Apical part.

Table 3 NF-L and NF-M immunostaining in the embryonic and postnatal mouse nose

	Embryonic age (day)																Postnatal age (day)									
	13		14		15		16		17		18		19		20		1		8		15		21		60	
Neurofilament type	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M	L	M
Vomeronasal nerve	–	–	–	–	–	–	–	–	–	–	–	●	–	●	–	●	–	●	–	●	–	●	–	●	–	●
Sensory epithelium	–	–	–	–	–	–	–	–	–	–	–	●	–	●	–	●	–	●	–	●	–	●	–	●	–	●
Vomeronasal fibers in the lamina propria of the sensory epithelium	–	–	–	–	–	–	–	–	–	–	–	●	–	●	–	●	–	●	–	●	–	●	–	●	–	●
Vomeronasal cavernous tissue	–	–	–	–	•	–	•	–	•	•	●	●	●	●	●	●	●	●	•	•	–	–	–	–	•	•
Blood vessel of the sensory epithelium	–	–	–	–	–	–	–	–	–	–	●	●	●	●	•	•	•	•	–	–	–	–	–	–	–	–
Vomeronasal glands	np	np	np	np	np	np	•	•	•	•	●	●	●	●	●	●	•	•	•	•	–	–	–	–	•	•
Olfactory epithelium	–	–	–	–	–	–	–	–	–	–	–	●	–	●	–	●	–	●	–	●	–	●	–	●	–	–

Neurofilament type: L, light subunit; M, medium subunit. Immunostaining intensity: –, negative; •, weakly positive; ●, positive; np, glands not present.

(Figures 4F,I and 6I) and the vomeronasal nerve (Table 3), NF-M immunoreactivity only persists up to 2 months of age. In the olfactory sensory epithelium, NF-M is expressed at E18–P21 (Table 3); NF-L immunoreactivity was absent at all ages. NF-H immunoreactivity was never found in both pre- and postnatal mice.

Discussion

The VNO originates from the olfactory placode (Cuschieri and Bannister, 1975), and neuronal precursor cells are found in the VNO anlage as early as E12–E13; extrinsic innervation of VNO structures starts developing very early as well. The expression of several markers and factors has been investigated in the developing VNO (Johnson *et al.*, 1993; Nakajima *et al.*, 1998; Tarozzo *et al.*, 1998; Zancanaro *et al.*, 1999; Camoletto *et al.*, 2001; Zancanaro *et al.*, 2002), inclusive of neuronal guidance molecules (Knoll *et al.*, 2001; Cloutier *et al.*, 2002); however, data on expression and localization of intermediate filament proteins in the VNO were lacking. The results presented here show that several intermediate filament proteins are sequentially expressed during intrauterine development of the VNO neural structures, and their expression and/or timing of appearance differ among the vomeronasal sensory epithelium, the vomeronasal nerve, and the extrinsic innervation to the VNO.

With regard to the embryonal vomeronasal sensory structures, nestin was found in the developing sensory epithelium (in parallel to the olfactory epithelium), and it was transiently present in the neural structures therefrom at E14 (Table 2), whereas the opposite was found for peripherin

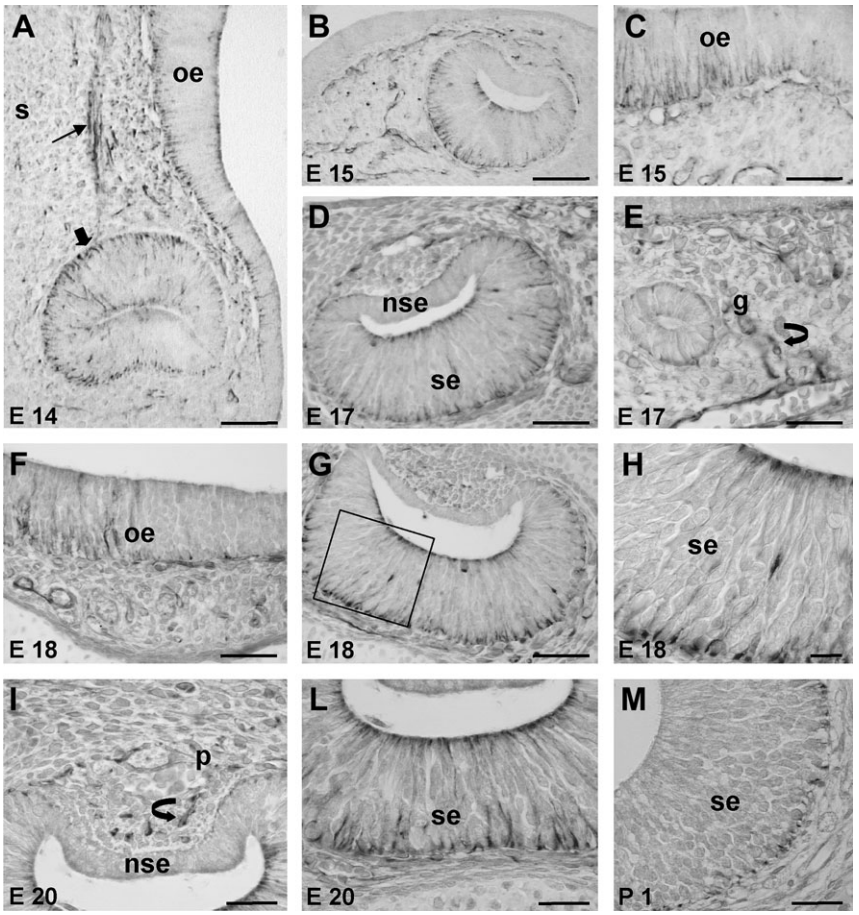

Figure 1 Nestin immunoreactivity in the mouse developing VNO. The embryonic (E) or postnatal (P) day is indicated at the bottom of each panel. s, nasal septum; oe, olfactory epithelium; se, vomeronasal sensory epithelium; g, vomeronasal glands; p, vomeronasal cavernous tissue; nse, nonsensory epithelium; thin arrow, vomeronasal nerve; thick arrow, early VNO; curved arrow, immunoreactive fibers. The framed area in panel **G** is enlarged in panel **H**. Scale bar: 30 μm **(A**, **B)**, 15 μm **(C**, **D**, **F**, G**)**, 10 μm **(E**, **I**, **L**, **M)**, and 5 μm (H).

(Table 2); instead, internexin was expressed in both the vomeronasal epithelium and nerve (Table 2). These data on the patterns of early intermediate filament protein expression are supported by previous findings showing that nestin is limited to the vomeronasal (Osada *et al.*, 1995; Matsuoka *et al.*, 2002) and olfactory (Doyle *et al.*, 2001) epithelia in the rat, whereas peripherin is predominantly expressed in olfactory axons of both mouse (Chien *et al.*, 1998) and rat (Escurat *et al.*, 1990; Gorham *et al.*, 1991); further, internexin immunoreactivity was found at low intensity in the olfactory epithelium (Chien *et al.*, 1998) and more obviously in the olfactory nerve (Kaplan *et al.*, 1990; Chien *et al.*, 1998) during development. NF-M was expressed in sensory vomeronasal structures (and the olfactory epithelium) at a later stage of development (E18) in comparison with nestin, peripherin, and internexin; this is in accordance with the sequential expression of intermediate filament proteins in vertebrate development (Willard and Simon, 1983; Carden *et al.*, 1987; Kaplan *et al.*, 1990). The presence of NF-M immunoreactivity in either apical prolongements of epithelial cells and vomeronasal fibers and the higher density of labeled cells in the central region of the vomeronasal sensory epithelium (Figures 1B,D,F,I and 6G–I) strongly suggest that these cells are neurons. However, the absence of NF-L and NF-H immunoreactivity in the sensory epithelium and nerve at any stage of prenatal and postnatal development indicates a special maturation pattern of the cytoskeleton in vomeronasal neurons. Consistently, previous findings showed that the NIF triplet proteins are absent in the olfactory epithelium and nerve of the mouse and weakly present in the olfactory bulb starting with NF-M at E14 (Trojanowski *et al.*, 1991; Chien *et al.*, 1998), and they are not expressed to a significant extent in the human (Ophir and Lancet, 1988; Trojanowski *et al.*, 1991) and bovine (Ophir and Lancet, 1988) adult olfactory neuron; however, immunoreactivity for the dephosphorylated form of NF-M was present in axons (Talamo *et al.*, 1989), and NF-H was found in rat olfactory neurons (Bruch and Carr, 1991; Trojanowski *et al.*, 1991). Such discrepancies could be explained by species and methodological differences.

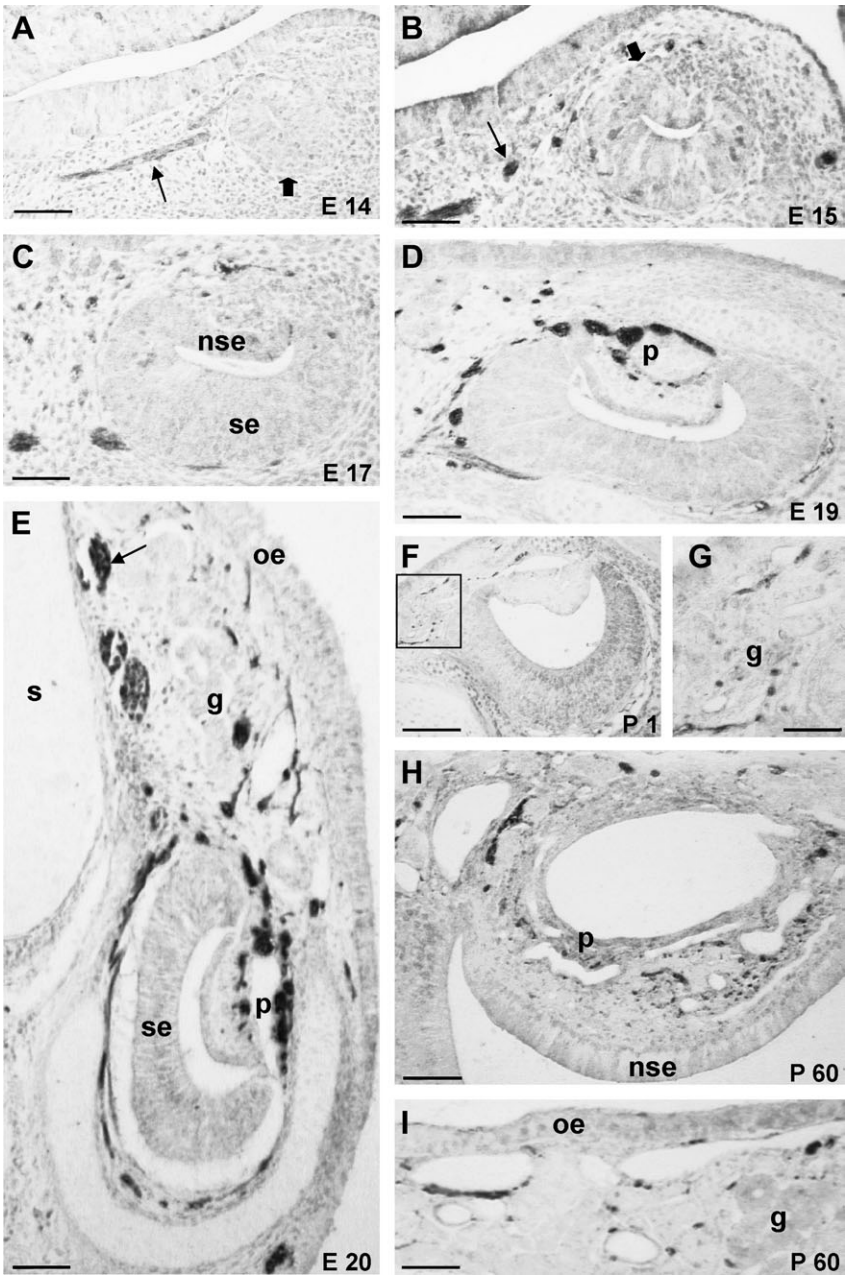

Figure 2 Peripherin immunoreactivity in the mouse developing VNO. The embryonic (E) or postnatal (P) day is indicated at the bottom of each panel. s, nasal septum; oe, olfactory epithelium; se, vomeronasal sensory epithelium; g, vomeronasal glands; p, vomeronasal cavernous tissue; nse, nonsensory epithelium; thin arrow, vomeronasal nerve. The framed area in panel **F** is enlarged in panel **G**. Scale bar: 60 μm **(A**, **B**, **D**, **H)**, 30 μm **(C**, **E**, F**)**, and 15 μm **(**G, **I)**.

The labeling pattern of intermediate filament proteins antibodies was obviously different in the vomeronasal sensory epithelium; therefore, the question arises as to which cell type is expressing each of the investigated intermediate filament proteins. In embryos, nestin (Figures 1A–D,G,H,L and 5A,B,E) was mainly expressed in the basal (and, to a lesser extent, apical) region; this pattern is consistent with the finding by Osada *et al.* (1995) and Matsuoka *et al.* (2002). Nestin-positive cell prolongements contacting the basal lamina were abundant, thereby suggesting that they originate from supporting cells whose cell body is apically located already during development (Garrosa *et al.*, 1998). This is consistent with findings in the olfactory epithelium of the same mice (Figure 5D) and of adult rats (Doyle *et al.*, 2001) showing superimposable nestin-labeling patterns. However, we found that nestin-positive cell bodies sending labeled apical prolongements are present as well in the basal region of the embryonal sensory epithelium (Figure 5E) and, at the very early stage of E14, nestin-positive cell prolongements, presumably axons, were found leaving the epithelium (Figure 5B). These

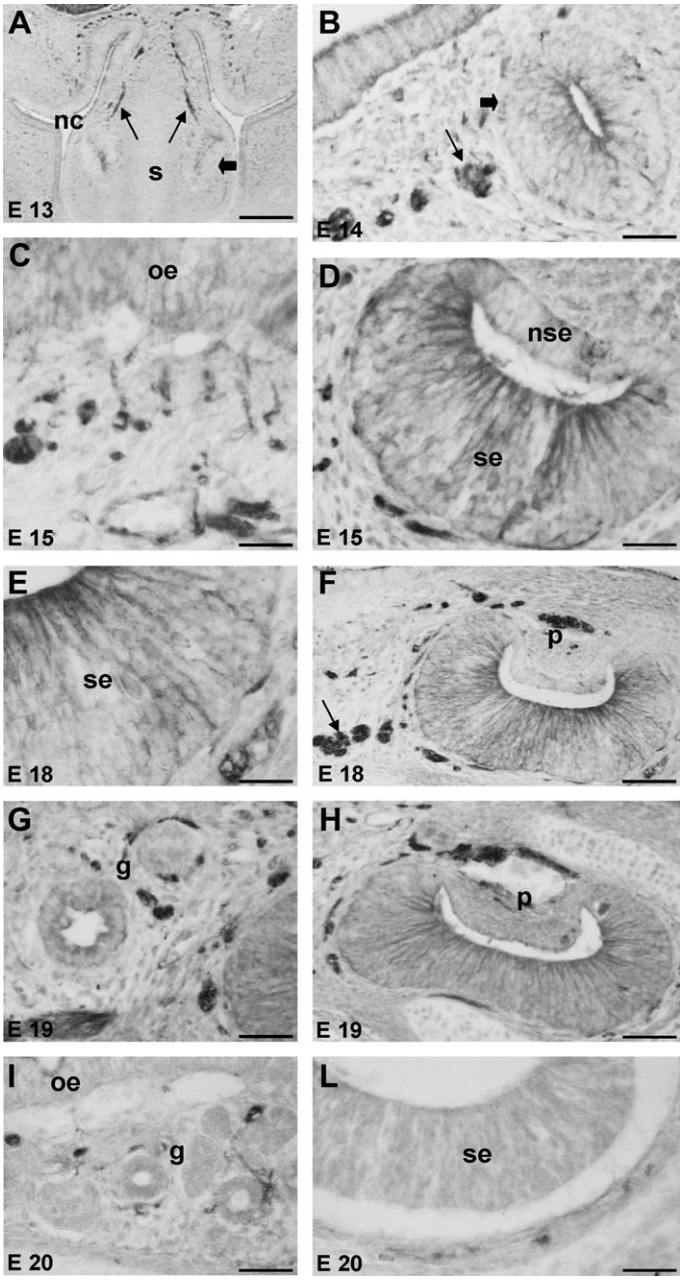

Figure 3 Internexin immunoreactivity in the mouse developing VNO. The embryonic (E) day is indicated at the bottom of each panel. s, nasal septum; nc, early nasal cavity; oe, olfactory epithelium; se, vomeronasal sensory epithelium; nse, nonsensory epithelium; g, vomeronasal glands; p, vomeronasal cavernous tissue; thin arrow, vomeronasal nerve; thick arrow, anlage of the VNO. Scale bar: 100 μm **(A)**, 30 μm **(F, H)**, 15 μm **(B–D, G, I, L)**, and 10 μm **(E)**.

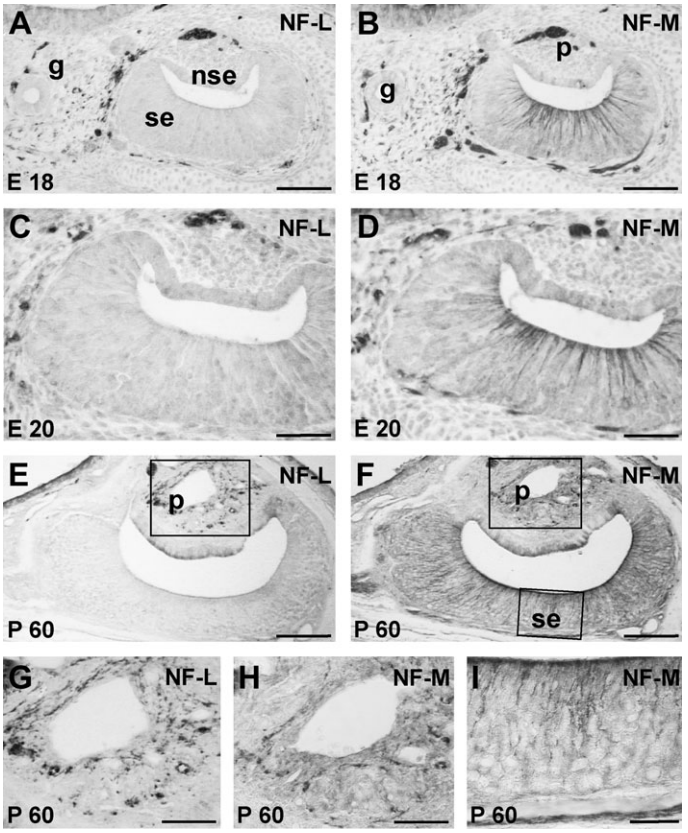

Figure 4 NF-L and NF-M immunoreactivity in the mouse developing VNO. The embryonic (E) or postnatal (P) day and the filament type (NF-L or NF-M) is indicated at the bottom and the top of each panel, respectively. se, vomeronasal sensory epithelium; p, vomeronasal cavernous tissue; nse, nonsensory epithelium. The framed area in panel **E** is enlarged in panel **G**; the framed areas in panel **F** are enlarged in panels **H** and **I**. Scale bar: 60 μm (E, F), 30 μm **(A–C, D**, G, H**)**, 10 μm **(I)**.

findings point to a neuronal nature of these cells. Consistently, Matsuoka *et al.* (2002) found nestin immunoreactivity in regenerating vomeronasal axons after vomeronasal nerve transection (they did not investigate embryonal stages earlier than E15). It is suggested that nestin identifies both neuronal and nonneuronal cells in the embryonal sensory epithelium; postnatally, nestin immunoreactivity just above the basal lamina (Figure 5C) suggests transient expression of this protein in basal cells that could be progenitors of cells differentiating into neurons and migrating vertically in the mouse vomeronasal sensory epithelium (Giacobini *et al.*, 2000). Accordingly, Osada *et al.* (1995) found in the rat at P22 that nestin immunoreactivity was "restricted to a narrow layer along the basement membrane," whereas immunoreactivity for the neuronal tubulin (β-III) was obvious in both the epithelium and the vomeronasal nerve. Taken together, the current findings confirm and extend the data by Osada *et al.* (1995) and Matsuoka *et al.* (2002). However, further work is necessary to define the nestin-positive cell types in the vomeronasal sensory epithelium since nestin is expressed in neuronal and nonneuronal cells, for example, the radial glia (Liu *et al.*, 2002) and Schwann cells (Friedman *et al.*, 1990).

The presence of nestin immunolabeling in nuclei (Figure 5E) suggests that nestin plays some hitherto unrecognized role in the maturation of cells of the vomeronasal sensory

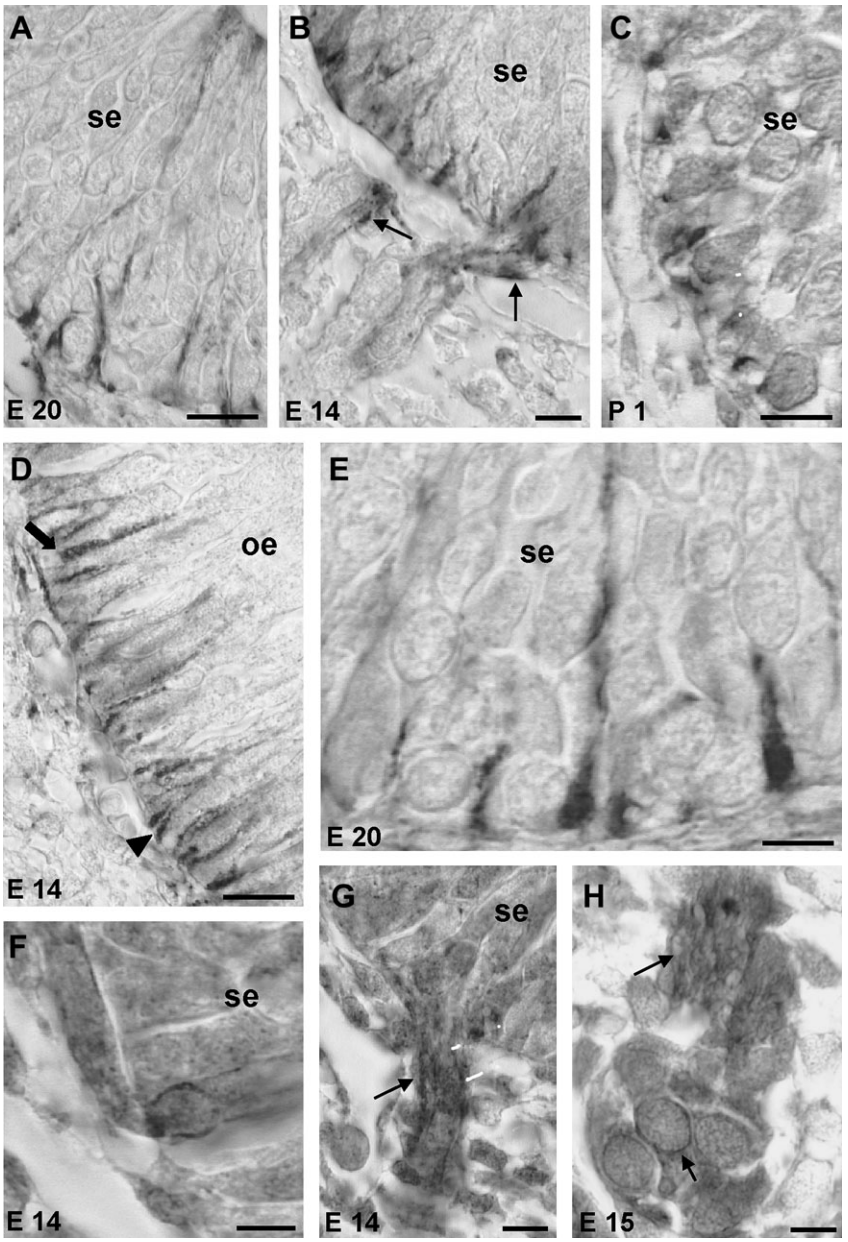

Figure 5 High magnification of immunoreactive structures in the mouse developing VNO. The embryonic (E) or postnatal (P) day is indicated at the bottom of each panel. **(A–E)**, nestin immunoreactivity, **F** and **G**, peripherin immunoreactivity. se, vomeronasal sensory epithelium; oe, olfactory epithelium; thin arrow, fibers in the vomeronasal nerve; thick arrow and arrowhead, basal prolongement of putative sustentacular cells ending on the basal cell body or the basal lamina, respectively. short thin arrow, cell associated to the vomeronasal nerve. Scale bar: 10 μm (D), 5 μm (A–C, E, G, H), and 2.5 μm (F).

epithelium; this is in accordance with the recent finding of nestin in the nucleus of human neuroblastoma cells where it could regulate the expression of genes associated with malignancy (Thomas *et al.*, 2004).

The labeling pattern with the α-internexin antibody was quite different from that of nestin; albeit staining was generally lighter, it was clearly present in the cell body of cells placed in the entire thickness of the epithelium except for its very apical and basal portions (Figure 6B–D) as well as in the vomeronasal nerve all along the intrauterine development; these findings strongly suggest that α-internexin is a marker of chemosensory developing neurons in the vomeronasal epithelium. The expression of α-internexin in the apical prolongement (equivalent to dendrite) of these cells confirms the peculiarity of chemoreceptor neurons because α-internexin is regularly found in axons only in the central nervous system (Kaplan *et al.*, 1990). Consistently, α-internexin has been shown in the dendritic processes of mitral and tufted cells in the external plexiform layer of the olfactory bulb (Chien *et al.*, 1998).

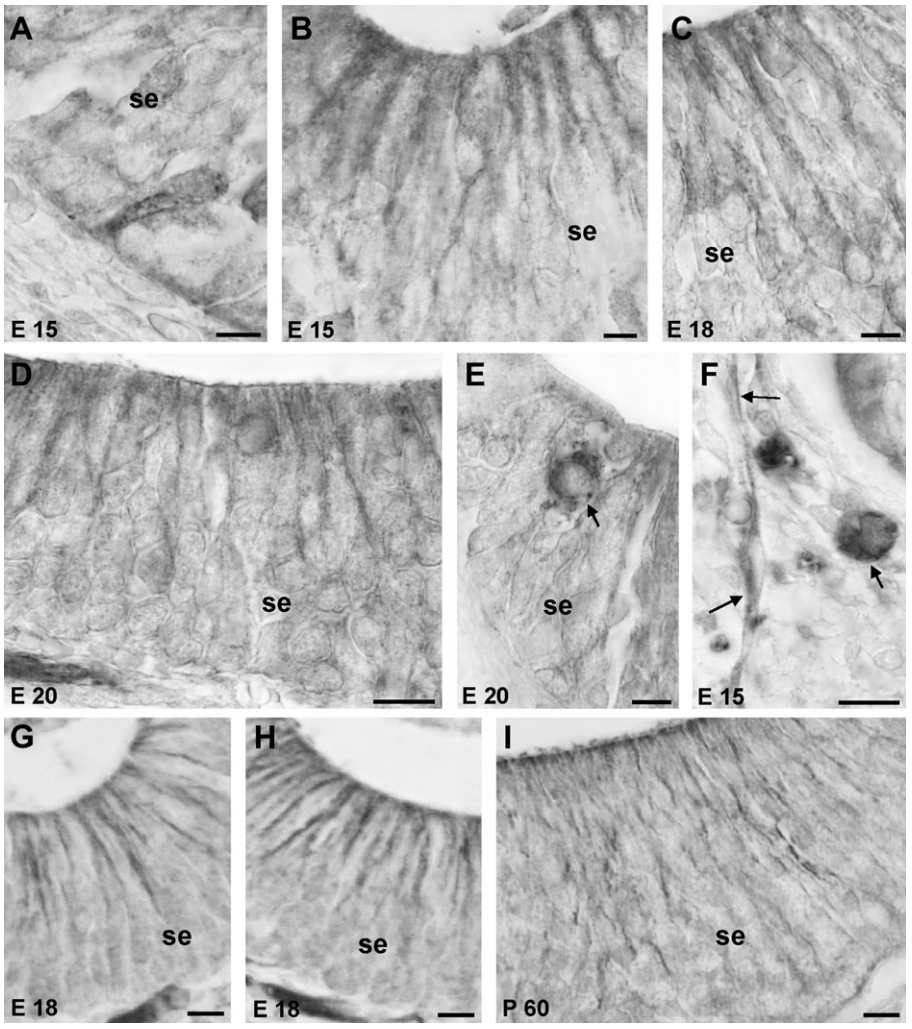

Figure 6 High magnification of immunoreactive structures in the mouse developing VNO. The embryonic (E) or postnatal (P) day is indicated at the bottom of each panel. **(A–F)**, internexin immunoreactivity, **(G–I)**, NF-M immunoreactivity. se, vomeronasal sensory epithelium; thin arrow, vomeronasal nerve fibers; short thin arrow, cell associated to the vomeronasal nerve. Scale bar: 10 μm (D, F) and 5 μm (A–C, E, G, H, I).

The presence of peripherin in the vomeronasal nerve is specially intriguing because of its timing. In the mouse, Troy *et al.* (1990) showed that peripherin-containing cells "produce long axons which either enter or exit the central nervous system" and that peripherin is present in the same regions during development and in the adult, indicating that it is a functional intermediate filament and not a remnant; moreover, on the basis of its molecular properties, peripherin could be useful in axons of small diameter or undergoing rapid remodeling of their cytoskeleton. However, Troy *et al.* (1990) found very few peripherin immunoreactive fibers in the olfactory mucosa both during development and in the adult. Chien *et al.* (1998) showed the expression of peripherin in the mouse olfactory nerve from E12 to the adult (where it coexists with α-internexin) and to some extent in the olfactory epithelium as well; since NIF immunoreactivity was absent in the olfactory nerve, Chien *et al.* (1998) concluded that intermediate filaments different from NIF could serve in maintaining axonal caliber in the olfactory nerve. More recently, Lariviere *et al.* (2002) showed that disrupting the peripherin gene in mice has no effect on the caliber of axons; however, the peripherin-null mice have a reduced number of unmyelinated sensory axons. Our data (Table 2, 3) show that peripherin immunoreactivity appeared slightly later in the embryonal vomeronasal nerve in comparison with α-internexin as did peripherin peak-labeling intensity (E19–E20), which was associated with the initial expression of NF-M. Peripherin is absent in the vomeronasal nerve postnatally (as is α-internexin); instead, NF-M is expressed therein up to adulthood (Table 3). There is no obvious explanation for the disappearance of peripherin in the postnatal life. It could be suggested that peripherin (and internexin) has a temporally defined and limited role in organizing the cytoskeleton of vomeronasal neurons insofar they are different from the olfactory ones. An intriguing complementary explanation is that peripherin-positive fibers

in the vomeronasal nerve serve as neuronal track for the migration of cells leaving the olfactory placode area to the forebrain from about E11 to the end of pregnancy (Wray *et al.*, 1994, Figures 5H and 6F). These cells were first identified as LHRH cells, but several other types of migrating cells were identified later on including GABA, NPY, and tyrosine hydroxylase–expressing cells (Wray, 2002).

In the last several years, two families of putative vomeronasal receptors genes have been identified whose expression is distributed differently in the vomeronasal epithelium of many mammalian species. The first gene family (Dulac and Axel, 1995) is expressed in more apically situated chemoreceptor neurons that coexpress the G protein $G_{i2\alpha}$; the second family (Herrada and Dulac, 1977; Matsunami and Buck, 1977; Ryba and Tirindelli, 1997) is expressed in more basally placed chemoreceptor neurons which coexpress the G protein $G_{0\alpha}$. The distribution of these two types of neurons could be reminiscent of that of α-internexin and nestin, respectively (see Figures 3 and 1); however, the fact that nestin immunoreactivity in cell bodies is limited to the epithelial region just above the basal lamina and that at high magnification α-internexin is found expressed in cells across almost the whole epithelial thickness would argue against a relationship between the differential distribution of these two intermediate filament proteins in the embryonal vomeronasal epithelium and the segregation of $G_{i2\alpha}$- and $G_{0\alpha}$-positive receptor neurons, the latter having only shown in the postnatal life to date (Jia and Halpern, 1996, Jia *et al.*, 1997).

Finally, data presented here show a different pattern of cytoskeletal development in mouse vomeronasal neural structures in comparison with extrinsic innervation to the VNO: peripherin and internexin expression was delayed in extrinsic nerve fibers (Table 2) with peripherin persisting to some extent in the postnatal life, whereas NIF triplet proteins expressed earlier therein with NF-L never found in vomeronasal neurons (Table 3). This could be related to difference in maturation pattern between special sense vomeronasal central nervous system (CNS) neurons and sensory or motor peripheral nervous system neurons; accordingly, several neuropeptides were found in the extrinsic nerves to the VNO during postnatal development which were not present in vomeronasal neurons (Zancanaro *et al.*, 1999).

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