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Maturation of Vomeronasal Receptor Neurons *In Vitro* by Coculture with Accessory Olfactory Bulb Neurons

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Abstract

To analyze the mechanisms of perception and processing of pheromonal signals *in vitro*, we previously developed a new culture system for vomeronasal receptor neurons (VRNs), referred to as the vomeronasal pocket (VN pocket). However, very few VRNs were found to express the olfactory marker protein (OMP) and to have protruding microvilli in VN pockets, indicating that these VRNs are immature and that VN pockets are not appropriate for pheromonal recognition. To induce VRN maturation in VN pockets, we here attempted to coculture VN pockets with a VRN target—accessory olfactory bulb (AOB) neurons. At 3 weeks of coculture with AOB neurons, the number of OMP-immunopositive VRNs increased. By electron microscopy, the development of microvilli in VRNs was found to occur coincidentally with OMP expression *in vitro*. These results indicate that VRN maturation is induced by coculture with AOB neurons. The OMP expression of VRNs was induced not only by AOB neurons but also by neurons of other parts of the central nervous system (CNS). Thus, VRN maturation requires only CNS neurons. Since the maturation of VRNs was not induced in one-well separate cultures, the nonspecific induction of OMP expression by CNS neurons suggests the involvement of a direct contact effect with CNS in VRN maturation.

Key words: development, electron microscopy, microvillous, olfactory marker protein, rat, vomeronasal system

Introduction

The vomeronasal system, which consists of the vomeronasal organ (VNO), the accessory olfactory bulb (AOB) and other central nervous system (CNS) structures receiving efferents from the AOB, plays a critical role in the perception and processing of pheromonal signals, which are conspecific chemical signals that provide information on the social and sexual states of other individuals within mammalian species (Wysocki, 1979; Halpern, 1987; Halpern and Martinez-Marcos, 2003; Dulac and Torello, 2003). The VNO is located at the base of the nasal septum, and the AOB forms part of the dorsocaudal region of the main olfactory bulb (MOB) in rodents. The vomeronasal epithelium (VNE), a chemosensory region in the VNO, is composed of vomeronasal receptor neurons (VRNs), supporting cells and basal cells. The axon terminals of VRNs form synaptic contacts with the dendrites of AOB neurons.

In the past decade, we have developed a primary culture system for the VNO and AOB to study the functional role of VRNs and AOB neurons in pheromonal signal perception and processing (Ichikawa and Osada, 1995; Ichikawa *et al*., 1995; Osada *et al*., 1999; Muramoto *et al*., 2003). This included a novel culture system that contained the structure we termed the vomeronasal pocket (VN pocket) (Osada *et al*., 1999). These VN pockets are spherical structures with central cavities and morphological characteristics similar to those of the VNE. Moreover, VRNs in a VN pocket undergo

continuous degeneration and regeneration as observed in the VNE *in vivo (*Graziadei and Monti-Graziadei, 1978). It has been postulated that a VN pocket is a useful tool for the investigation of pheromonal perception. However, by immunocytochemical studies, the VN pockets did not contain VRNs immunopositive for olfactory marker protein (OMP) (Osada *et al.*, 1999). OMP is a cytoplasmic protein found in mature olfactory receptor neurons and VRNs (Margolis, 1985). By electron microscopy, it was observed that VN pockets contained numerous VRNs, but the microvilli of these VRNs, where pheromone molecules are received, did not protrude into the cavity of the VN pockets, and other cellular organelles of the VRNs were not well developed (Osada *et al.*, 1999). These results suggest that VRNs in VN pockets are immature. VN pockets in which the microvilli of VRNs are not fully developed are of no use in the analysis of the perception and processing of pheromonal information.

In *in vivo* developmental studies, microvilli have not been observed on VRNs at the luminal surface of the VNO during the embryonic period, though a slight increase in microvillar number is observed during the postnatal days 1–7 (Garrosa and Coca, 1991; Ichikawa and Osada, 1995). The axon terminals of VRNs reach the AOB in the late prenatal stage (Ichikawa *et al.*, 1994; Jia *et al.*, 1997; Matsuoka *et al.*, 2002). The development of microvilli coincides with the initial wave of axonal termination in the AOB. Thus, it is presumed that the characteristics of VRNs in VN pockets correspond to those in the early developmental stage *in vivo*, and it is hypothesized that the maturational deficiency of VRNs in VN pockets is caused by the absence of target neurons. In a similar culture system, it has been reported that the *in vitro* maturation of olfactory receptor neurons (ORNs) is induced by coculture with central nervous system (CNS) cells (Pixley, 1992; Grill and Pixley, 1997). In the present study, we investigated whether the maturation of VRNs in VN pockets is induced by coculturing with AOB cells. We measured the induction of immunoreactivity of OMP on cocultured VN pockets and the frequency and morphology of microvilli of VRNs, observed by electron microscopy.

Materials and methods

Cell culture

VN pockets were prepared as previously described (Osada *et al.*, 1999). Briefly, VNOs were dissected from the heads of Wistar rats on embryonic day 15 (E15) and immersed in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL) containing 1 mg/ml collagenase/dispase (Boehringer Mannheim) at 37°C for 60 min to remove the surrounding tissues. After the inhibition of proteinase by DMEM/F12 containing 10% fetal bovine serum, VNOs were plated onto LF1 plastic cell culture discs (Sumitomo Bakelite Co., Japan) covered with a feeder cell layer (Osada *et al.*, 1999). The culture medium for VN pockets consisted of DMEM/F12 medium containing 50 ng/ml hydrocortisone, 161 ng/ml putrescine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 3 ng/ml fibroblast growth factor basic, 100 U/ml penicillin and 100 μg/ml streptomycin (all these reagents were purchased from Sigma-Aldrich).

Primary cultures of dissociated AOB cells were prepared following a modification of a previously reported procedure (Muramoto *et al.*, 2003). The AOB region, which was approximately one-quarter of the dorsal region of the entire OB, was determined the same as Muramoto *et al.* (2003) and excised from a rat on E20. After removing the surrounding meninges, the dissected AOB tissues were pooled and incubated at 37°C for 20 min in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.5 U/ml papain (Worthington), 0.02% L-cysteine HCl (Wako, Japan), 0.02% bovine serum albumin (Gibco BRL) and 0.5% glucose. After removing the supernatant of the enzyme solution, the tissues were washed twice in DMEM/F12 containing 5% newborn calf serum (Mitsubishi Kagaku, Japan) and 5% heat-inactivated house serum (General Scientific Laboratory, USA) in DMEM/F12, and then washed in the serum-free culture medium. Finally, the tissues were again transferred to another tube and suspended in the serum-free culture medium for AOB, which consisted of DMEM/F12 containing 1.2 mg/ml $NaHCO_3$, 50 U/ml penicillin G (Meiji Seika, Japan), 25 μg/ml streptomycin (Meiji Seika), 25 μM 2-mercaptoethanol (Sigma-aldrich), 100 μM L-(+)-ascorbic acid (Wako) and B27 serum-free additive (Gibco BRL). The resultant cell suspension was plated at a density of 5×10^5 cells/cm^2 on LF1 plastic cell culture discs or LabTek chamber slides (Nalge Nunc international, USA) coated with polyethyleneimine (Sigma).

After the AOB had been maintained *in vitro* for 2 days (2 DIV), a few VN pockets were transferred using a micropipette into wells and cocultured for 2–3 weeks. For coculture, a medium composed of equal amounts of culture medium for VN pocket and AOB was used.

In addition to the AOB, the MOB and cerebral cortex were prepared for coculture. MOB cells were excised from the anterior region of the MOB, and cerebral cortex cells were excised from the rostrolateral region of the cerebral cortex. The culture method was the same as that for the AOB. The astrocyte layer for coculture was prepared following general methods (Conn, 1990).

For histological comparisons, to determine if physical damage occurred when VN pockets are transferred to the AOB culture, single-cultured VN pockets were transferred to new feeder layers and then cultured in the same manner as the cocultured VN pockets. After 3 weeks in culture, the diameter of each VN pocket was measured using the NIH image software, and the number of cells in a VN pocket was determined using a hematocytometer after the treatment of each VN pocket with trypsin/EDTA (Gibco BRL) and physical dissociation.

To avoid cell migration and axon targeting, but to allow soluble influences, cocultures were prepared that prevented

cell contact. Feeder cells for VN pockets and AOB cells (after 2 DIV) were separately plated on half-size cut LF1 plastic cell culture disks and placed on either side of a 2% agarose gel divider in the same culture wells. Then VN pockets were transferred onto each side and cocultured for 3 weeks. Furthermore, in order to confirm the neurotrophic effects from AOB cells, the conditioned medium were adjusted from separately cultured AOB cells, and added to single-cultured VN pockets every 3 days for 3 weeks.

Immunocytochemistry

The cocultured cells were fixed for 30 min in 4% paraformaldehyde in PBS. After fixation, the cells were incubated in a buffer containing 0.1% saponin (Sigma-Aldrich) and 10% Block Ace (Dainippon Seiyaku, Japan) in PBS for 15 min at room temperature. Without washing, the fixed and permeabilized cells were then incubated with primary antibodies, the mouse monoclonal anti-β-tubulin III antibody (1:500, Sigma-Aldrich) and the rabbit polyclonal anti-OMP antibody (1:500, refer to Takigami *et al*., 2004), at 4°C for 2 days. These primary antibodies were diluted with 10% Block Ace in PBS. Following three washings in PBS for at least 10 min each, the cells were incubated with secondary antibodies: Cy3.5-conjugated donkey anti-mouse IgG antibody (1:200, Rockland) and FITC-conjugated donkey anti-rabbit IgG antibody (1:200, Rockland) in PBS containing 10% Block Ace for 1 h at room temperature. Finally, after washing the cells five times, the cells were mounted with a water-based mountant (FluoroGuardTM, Bio-Rad). Stained immunofluorescent cells were observed by confocal laser microscopy (LSM510, Zeiss, Germany).

To identify the proportion of VRNs in VN pockets, dissociated VN pocket cells were plated onto LabTek chamber slides coated with Celltak (BD Biosciences) for 2 h and fixed for 30 min in 4% paraformaldehyde in PBS. After fixation, the cells were stained with the anti-β-tubulin III antibody, anti-OMP antibody and DAPI (0.5 ng/ml, Molecular Probes). The number of anti-β-tubulin III- and anti-OMP-immunopositive cells and the number of DAPI-stained nuclei were determined, and the proportion of mature neurons relative to the total neurons in the VN pocket and of total neurons relative to total cells in the VN pocket were calculated.

RT-PCR

VN pockets were excised from the culture surface using a microhook (Fine Science Tool, Canada) and removed using a micropipette. Total RNA was prepared from three VN pockets, grown for 3 weeks in either single cultures or cocultures with AOB, using an RNeasy minikit (Qiagen, Germany). In these steps, genomic DNA was degraded by RNase-free DNase I (Qiagen). cDNAs were constructed from the total RNA using ReverTra Ace α (Toyobo, Japan) with an oligo-dT primer. The resulting cDNA was amplified by PCR using a KOD-plus, high-fidelity and hot-start system, (Toyobo) using primers for the following markers: actin, 5′-AACGGCTCCGGCATGTGCAA, 3′-CATTGTAGAAAGTGTGGTGCC; OMP, 5′-GAAGCAGGATGGTGAGAAGC, 3′-GTCCAGAACCACGTTCCAGT; β-tubulin III 5′-TGAGGCCTCCTCTCACAAGT, 3′-TGCAGGCAGTCACAATTCTC; TRPC2, 5′-TGGGCAACAGACTGACAGAG, 3′-CAGCCTCTCCTGTAGCATCA; GTP-binding protein αi2 subunit (Gαi2), 5′-CATCTTCTGTGTCGCCTTCA, 3′-CTTGTTGGCCCCTGTGTACT; and GTP-binding protein αo subunit (Gαo), 5′-CACCCTTGACCATCTGCTTT, 3′-GCAATGATGATGTCGGTGAC. The amplification protocols were 95°C for 5 min (95°C for 30 s, 55°C for 30 s and 68°C for 1 min) for 24 (actin) or 35 (OMP, β-tubulin III, TRPC2, Gαi2 and Gαo) cycles and 68°C for a 10 min final extension. The reaction products were separated by electrophoresis on 2% agarose gels and visualized with ethidium bromide. The cDNA concentrations of the template for all the samples were made the same by the densitometric quantification of the actin product gel image that was analyzed using NIH image. The products had the expected sizes and their identities were confirmed by sequencing (data not shown).

Electron microscopy

Whole-cultured VN pockets were fixed for 1 h in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB). The fixed VN pockets were then washed in PB and postfixed with 1% osmium tetroxide in PB for 1 h. The fixed samples were then dehydrated and embedded in epoxy resin (Quetol 812). Then, 1-μm-thick sections stained with toluidine blue were prepared to determine tissue orientation by light microscopy. Ultrathin sections with silver-gray interference were cut and mounted on formval-coated, one-hole copper grids. After staining with uranyl acetate and lead citrate, VN pocket tissues were examined with a Hitachi H7500 electron microscope. Negative photomicrographs were prepared by standard darkroom methods, and then digitally reverse-scanned. Brightness and contrast were adjusted with Photoshop 7.0.

The length of VRN surface membranes and the circumference of the central cavity were measured on the basis of the NIH image, and the rate for which VRN surface membranes account on the total circumference of the central cavity was determined. The number of protruding microvilli was determined in the surface-exposed VRNs.

Results

VN pockets in coculture with AOB

The system for the coculturing of VN pockets with AOB cells was designed to resemble the normal *in vivo* development. In the single-cultured AOB cells, synaptic connections were not formed at 2 DIV. These cultured neurons should be the same as those in the early postnatal stage when VRN axons and

AOB dendrites start forming glomerular structures *in vivo*. AOB neurons had many neurites at 7 DIV (Figure 1b). For the coculture of VN pockets with AOB cells, VN pockets were excised from the feeder layer and transferred to the AOB culture at 2 DIV.

The axon bundles of VN pockets were severed during transplantation, but they regenerated and regrew on AOB cells. VN pockets started to grow axon bundles at 1 day after coculture (Figure 1c), and axon growth was maintained until 7 days after coculture (Figure 1d,e). The extension of the axon bundles of VN pockets was faster in the coculture with AOB cells than in the single-culture on the feeder layer (data not shown).

OMP expression in VN pockets by coculture

To investigate the maturation of VRNs, we examined the OMP immunoreactivity of VRNs in VN pockets. Both the single-cultured and AOB-cocultured VN pockets were immunocytochemically stained using anti-β-tubulin III, a specific marker for both mature and immature sensory neurons, and anti-OMP antibodies. In the single culture of VN pockets, few OMP-immunopositive cells were observed (Figure 2). When VN pockets were cocultured with AOB cells, many OMP-immunopositive cells were observed (Figure 2). β-Tubulin III-immunopositive cells were observed in VN pockets in both the single culture and coculture. These results indicate that the proportion of mature VRNs increases in the cocultured VN pockets.

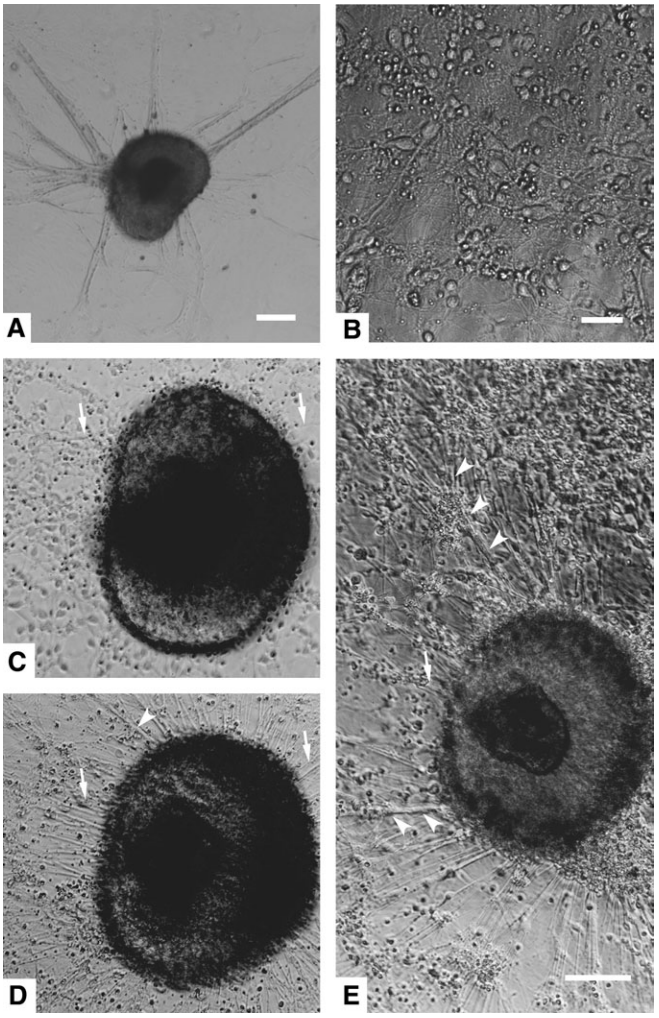

Figure 1 Light micrographs of VN pocket, AOB and VN pocket cocultured with AOB. **(A)** Single-cultured VN pocket at 14 DIV. Scale bar = 100 μm. **(B)** Cultured AOB cells at 7 DIV. Scale bar = 20 μm. **(C-E)** VN pocket cocultured with AOB cells at 1 (C), 4 (D) and 7 (E) days after transplantation. The arrows indicate extending axon bundles of VRNs. The arrowheads indicate thick fasciculated axon bundles. Scale bar = 100 μm.

Induction of OMP expression in VN pockets by nontarget neurons in CNS

To investigate whether the number of OMP-immunopositive VRNs increases only when cocultured with their target, the AOB, we examined the cocultures of VN pockets with other CNS tissues, namely the MOB and cerebral cortex. In cocultures containing VN pockets and both the MOB and cerebral cortex, the number of OMP-immunopositive VRNs also increased (Figure 2). Since our culture systems for CNS cells contain many astrocytes (data not shown), we postulated that the increase in the number of OMP-immunopositive VRNs is affected by astrocytes. Thus, a culture consisting of only astrocytes was prepared, and cocultured with VN pockets. However, OMP immunoreactivity was not observed in VN pockets cocultured with astrocytes (Figure 2). This result indicates that the presence of CNS neurons is important for the OMP expression of VRNs.

To confirm whether direct contact with CNS neurons is necessary for VRN maturation, we examined cultures in which VN pockets were physically separated from AOB cells by an agarose gel divider, and single VN cultures to which was added conditioned medium from separately cultured AOB cells. After 3 weeks, the number of OMP-immunopositive VRNs did not increase under both conditions (data not shown). This result suggests that the OMP expression of VRNs is not induced by trophic factors during AOB cell culture.

Comparison of cocultured and single-cultured VN pockets

To confirm the maturation of VRNs, we analyzed the mRNA expression of OMP and other VRN-specific markers, such as the signal transduction-related protein, by reverse-transcript polymerase chain reaction (RT-PCR) analysis. mRNA was isolated from only VN pockets in the coculture and single culture. After the reverse transcription of mRNA, specific primers for actin, OMP, β-tubulin III, transient receptor potential channel 2 (TRPC2), Gαi2 and Gαo were used to amplify the cDNA by PCR. OMP mRNA was expressed in only the cocultured VN pockets (Figure 3). Other VRN-specific markers were expressed under each condition, thus these signal transduction molecules are expressed before VRN maturation. The densitometric

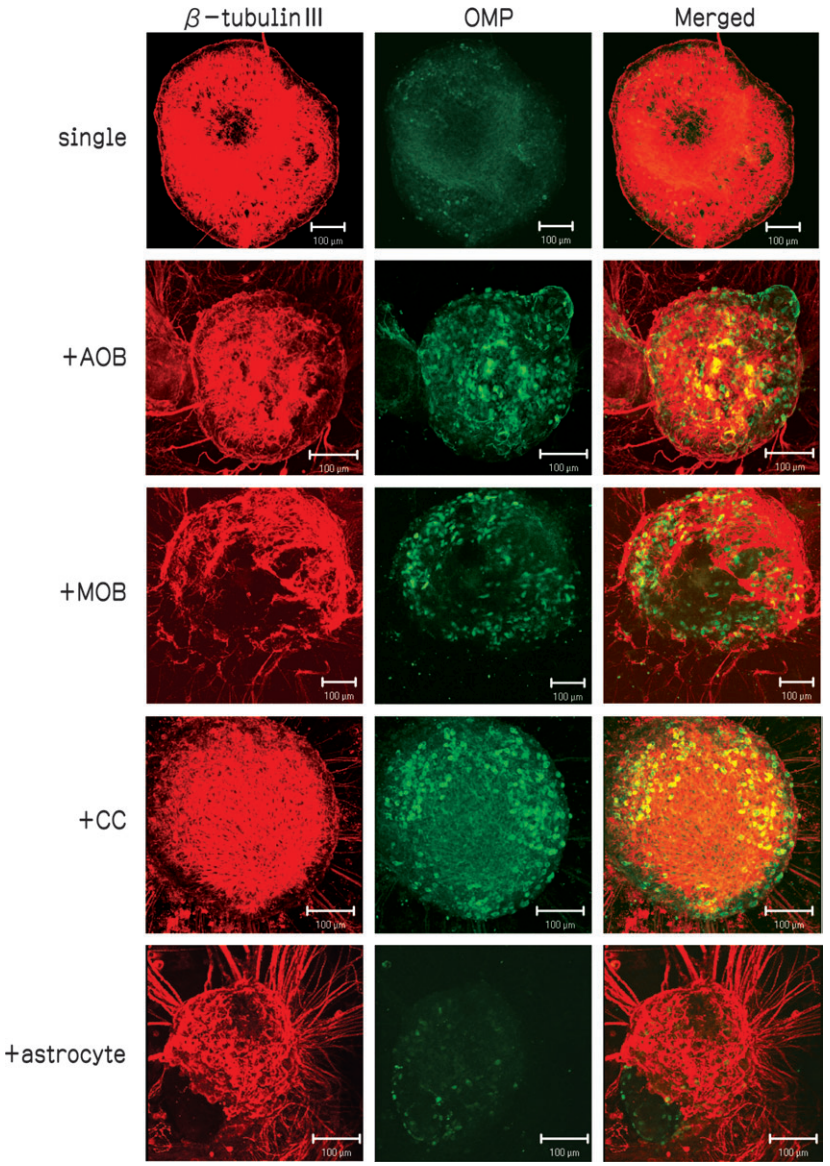

Figure 2 Immunocytochemical images of VN pockets using anti-β-tubulin III and anti-OMP antibodies. Single-cultured VN pocket (as a control) and VN pockets cocultured with AOB neurons, main olfactory bulb (MOB) neurons, cerebral cortex (CC) neurons or astrocytes at 21 days after transplantation. Scale bar = 100 μm.

quantification of electrophoresis gel images revealed that the expression levels of β-tubulin III, TRPC2, Gαi2 and Gαo in the cocultured VN pockets were approximately one-half those in the single-cultured VN pockets (Figure 3). Due to the reduction in the expression levels of all VRN-specific mRNAs, we postulated that the expression of these mRNAs is not down-regulated, but the number of VRNs in a cocultured VN pocket is less than that in a single-cultured VN pocket.

Moreover, to determine why the expression of β-tubulin III and other VRN-specific molecule changes, we examined other details of both cocultured and single-cultured VN pockets. The diameter of a cocultured VN pocket was smaller than that of a single-cultured VN pocket (Table 1, $P < 0.001$). To determine whether this difference was attributed to a change in the total number of cells or cell density, the total number of cells was determined. The total number of cells in a cocultured VN pocket was markedly less than that in a single-cultured VN pocket (Table 1, $P < 0.001$). Moreover, we identified the proportion of VRNs in VN pockets by immunocytochemical staining using anti-β-tubulin III and DAPI. The proportions of β-tubulin III-immunopositive cells relative to the total number of cells in a VN pocket were 54.4 ± 3.62% ($n = 4$) in the single-cultured VN pockets and 44.4 ± 2.27% ($n = 4$) in the cocultured ones (Table 1). These results indicate that the total number of VRNs in VN pockets was significantly reduced by coculture with AOB neurons ($P < 0.005$). However, the proportion of

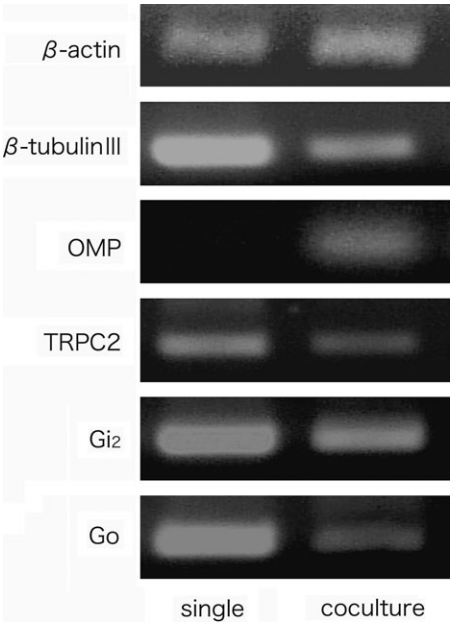

Figure 3 RT-PCR analysis of VRN-specific mRNAs using mRNA from single-cultured VN pockets and VN pockets cocultured with AOB neurons. The primer pairs were specific for β-actin, β-tubulin III, OMP, TRPC2, Gαi2) and Gαo. The sizes of the product are 0.25, 0.24, 0.11, 0.27, 0.22 and 0.19 kb for β-actin, β-tubulin III, OMP, TRPC2, Gαi2 and Gαo.

OMP-immunopositive cells relative to the total number of neurons was dramatically higher in the cocultured VN pockets (39.3 ± 7.11%, $n = 4$) than in the single-cultured ones (1.5 ± 0.53%, $n = 4$). This result suggests that the number of OMP-immunopositive cells in a VN pocket is ~10-fold more in coculture than in single culture, although the total number of cells is less.

Microvillus development in cocultured VN pockets

Previously, we observed the structure of VN pockets by electron microscopy (Osada *et al*., 1999). The internal epithelial surface of the VN pockets is composed of VRNs and supporting cells. In the single-cultured VN pockets, a few dendritic knobs of VRNs appear on the luminal surface (Figure 4d), and VRNs have no or very few short microvilli (Figure 4c,e). These structural characteristics were similar to those of VRNs observed in VNO during postnatal days 1–7 *in vivo (*Ichikawa and Osada, 1995). In this study, in the cocultured VN pockets expressing the OMP, we examined the microvilli of VRNs. Figure 4b shows an electron micrograph of a VRN. The cell organelles in VRNs, such as microtubules and centrioles in the distal end of dendritic knobs, are well developed, and many microvilli protrude on the luminal surface. In the cocultured VN pockets, ~80% of VRNs had more than five protruding microvilli, whereas ~80% of VRNs were without microvilli in the single-cultured VN pockets (Figure 4e). The number of mature VRNs with many protruding microvilli markedly increased in the cocultured VN pockets. Moreover, the occupation rate of dendritic knobs of VRNs on the luminal surface was larger in the coculture (24.3 ± 2.07%, $n = 5$) than in the single culture (9.6 ± 2.13%, $n = 5$, $P < 0.005$; Figure 4d).

Table 1 Characterization of VN pockets

	Diameter (μm)	Total cell number	Ratio of neurons (%)[a]	Ratio of matures (%)[b]
Single culture	423.5 ± 24.6 ($n = 5$)	22456 ± 2019 ($n = 4$)	54.4 ± 3.62 ($n = 4$)	1.5 ± 0.53 ($n = 4$)
Coculture	324.9 ± 15.1 ($n = 5$)	8735 ± 1938 ($n = 4$)	44.4 ± 2.27 ($n = 4$)	39.3 ± 7.11 ($n = 4$)

[a] Ratio of neurons in total cells (%).
[b] Ratio of mature cells in total neurons (%).

Discussion

Osada *et al*. (1999) reported that VRNs in VN pockets undergo continuous degeneration and regeneration. They showed that the proliferation of precursor cells and differentiation into neurons occur in VN pockets since VN pockets do not regenerate their axon bundles in the presence of an inhibitor of cell division. In the transplantation of VN pockets to other cell cultures, the axon bundles of VN pockets are severed, but new axon bundles extend from them onto new set of culture cells. This phenomenon indicates the ability of VRNs in VN pockets to regenerate. This characteristic of VN pockets is useful in the establishment of a coculture system.

In this study, we observed the expression of OMP and the protrusion of microvilli in VRNs cocultured with AOB cells *in vitro*. These phenomena observed upon coculture with AOB cells thus indicate the maturation of VRNs. Since the development of microvilli is coincident with axonal termination in the AOB *in vivo*, we postulate that the maturation of VRNs *in vitro* is similarly induced by the contact between VRNs and AOB neurons. Approximately 80% of VRNs on the luminal surface in the cocultured VN pockets had many protruding microvilli, supporting the notion that VRNs in VN pockets can receive pheromonal signals.

We found that the histological characteristic of VN pockets in coculture differs from that of VN pockets in single-culture. Both β-tubulin III expression level and the number of VRNs decreased in the cocultured VN pockets. During development of the olfactory epithelium *in vivo*, as ORNs mature, β-tubulin III expression level decreases, while OMP expression level increases (Roskams *et al*., 1998). Therefore, our results may show that the development of the VNO *in vitro* closely resembles that of the olfactory epithelium *in vivo*. Interestingly, Simpson *et al*. (2003) proposed a model of the regulation of neurogenesis and mature neuron survival in the olfactory epithelium in which some unclear factors from mature ORNs inhibit the proliferation of neuronal precursors via mitogen-activated protein kinase and the axonal outgrowth of immature neurons via phospholipase C.

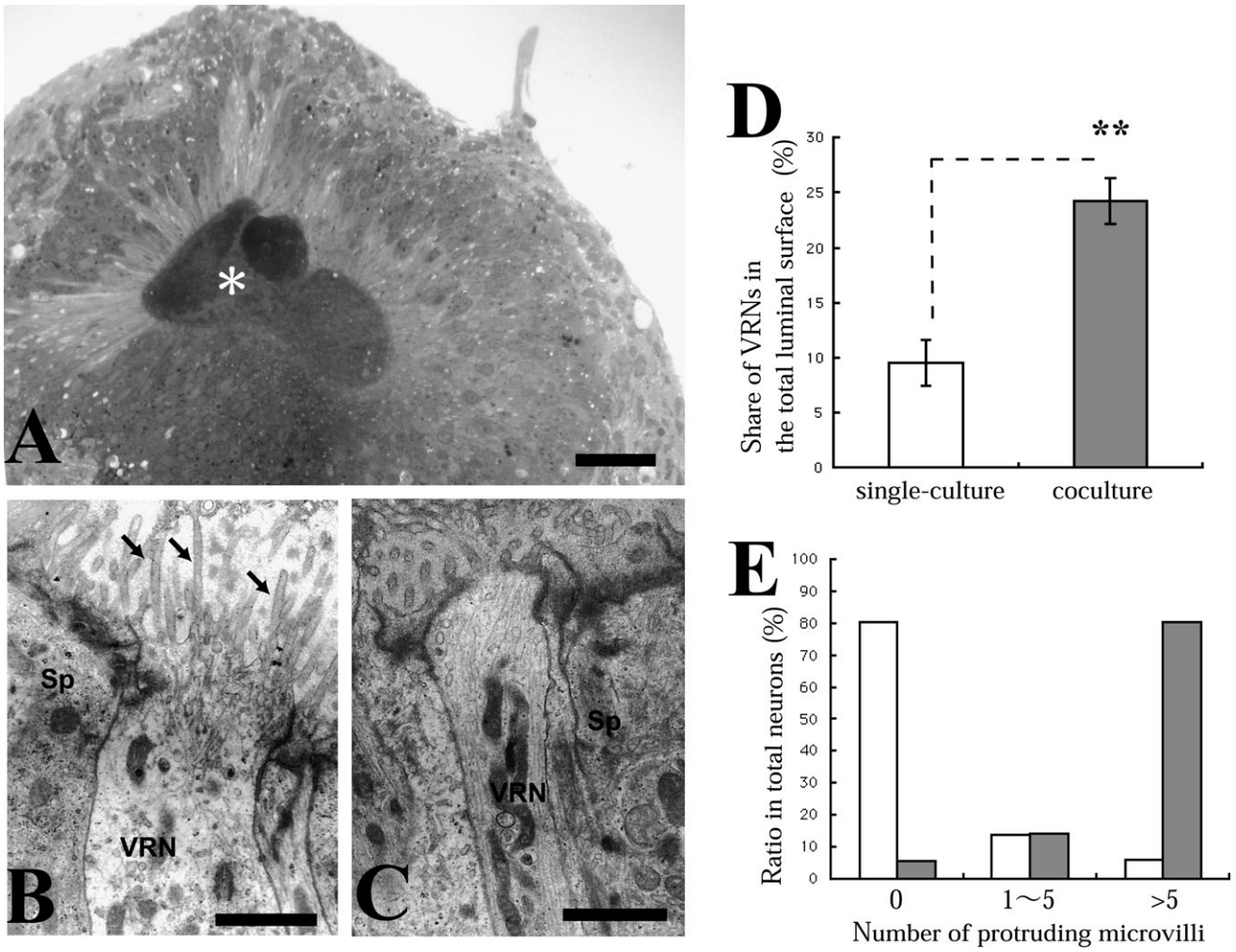

Figure 4 Light and electron micrographs of VN pockets. **(A)** Light micrograph of a VN pocket cocultured with AOB neurons 1 μm vertical sectional view to the culture disc. The asterisk indicates a central cavity. Scale bar = 20 μm. **(B)** Electron micrograph of VRN in (A). Scale bar = 1 μm. Many microvilli (arrow) are observed on the dendritic knob of VRN in the central cavity. **(C)** Electron micrograph of VRN in single-cultured VN pocket. The VRN does not have microvilli on its luminal surface. Scale bar = 1 μm. **(D)** Share of dendritic knobs of VRNs in the total luminal surface of one VN pocket. Electron micrographs using this analysis were taken at ×7000 magnification, and the total circumferences of the central cavity were reconstructed. The rates for which VRN surface membranes account on the total circumference of the central cavity were determined ($n = 6$). The asterisk indicates a statistically significant difference ($P < 0.005$). **(E)** Percentage of protruding microvilli of VRNs in VN pockets. 157 VRNs in single-cultured and 271 VRNs in cocultured VN pockets were observed.

Because the total number of cells and the proportion of neurons were decreased in the cocultured VN pockets, we consider that a similar mechanism may be present in the vomeronasal system, and our results may be attributed to the inhibition of the proliferation of precursors and differentiation of precursors to neurons in cocultured VN pockets. It seems that cocultured VN pockets are organized in the same manner as mature VNOs *in vivo*.

In our preliminary studies, we used cultured AOB cells containing a few astrocytes for coculture with VN pockets, with the result that VRNs in VN pockets were almost OMP-immunonegative (data not shown). This result indicates that the capability of neuron-enriched cultured AOB cells to induce VRNs maturation is low. In this study, the AOB cultures contained many astrocytes, and the number of OMP-immunopositive VRNs increased. Since the main difference between preliminary and present conditions is the number of containing astrocytes, we presumed that CNS astrocytes are critical for VRN maturation. It has been reported that CNS glial cells, mainly astrocytes, support the differentiation of ORNs (Pixley, 1992). However, in the case of using only astrocyte layers for the VN pocket coculture, very few OMP-immunopositive VRNs were observed. It seems that astrocytes do not directly induce OMP expression in VRNs. Recent findings suggest that astrocytes are implicated in the dynamic regulation of synaptic network formation and electrical neuronal activity (Newman, 2003; Slezak and Pfrieger, 2003). AOB neurons under astrocyte-rich conditions may form synaptic connections more easily and have higher neuronal activities than those under astrocyte-poor conditions. Therefore, AOB culture with many astrocytes may be appropriate in inducing VRN maturation via the synaptic

formation between VRNs and AOB neurons or the activity-dependent secretion of neurotrophic factors of CNS neurons.

OMP expression was induced by not only coculture with AOB but also coculture with other parts of the CNS. Muramoto *et al*. (2003) reported that VN pockets affect the differentiation and maturation of only AOB neurons, concretely the increase in the number of tyrosine hydroxylase-containing interneurons, but not those of MOB neurons in each coculture, and that the differentiation and maturation of AOB neurons are not influenced by the olfactory epithelium. It seems likely that the differentiation and maturation of AOB neurons are strictly regulated by their sensory neurons, that is, VRNs. On the other hand, this study indicates that VRNs do not need specific neurons of the CNS for maturation. We hypothesized that the maturation of AOB neurons is induced by VRN projection and/or activation via specific interactions with the axons of VRNs, whereas the maturation of VRNs is induced by the universal trophic effect in the CNS. In the olfactory system, it has been reported that the atrial C-type natriuretic peptide (CNP) and neurotrophin-3 in the glomeruli of the MOB promote the survival of more mature ORNs (Simpson *et al.*, 2002, 2003). However, our results of separate coculture and coculture in a conditioned medium suggest that the maturation of VRNs is not induced by only trophic molecules. It seems that the maturation of VRNs also requires direct contact with CNS cells.

Does the contact between VRNs and nontarget CNS neurons have any functional significance? Morrison and Graziadei (1996) demonstrated that VRNs have the ability to form glomeruli that contain ectopic synapses with CNS neurons by the transplantation of neonatal VNOs into the parietal cortex of littermates. They also reported that ectopic glomeruli lack periglomerular cells. It seems that the differentiation and maturation of CNS neurons are strongly regulated by loci in the brain and/or developmental stage. Muramoto *et al*. (2003) have also demonstrated these phenomena. We postulate that the maturation of VRNs is induced by contact stimulation with CNS neurons, and that ectopic synapses between VRNs and CNS neurons may be formed in our coculture systems.

However, the synaptic formation between VRNs and CNS neurons in the coculture system has not yet been clarified. In the olfactory system, MOB-ORN contacts provide trophic support for immature ORNs, preventing premature ORN death and allowing ORNs to live long enough to express OMP (Carr and Farbman, 1992, 1993; Schwob *et al*., 1992; Simpson *et al*., 2002). It has been demonstrated in an olfactory explant culture that trophic support for the survival and maturation of ORNs is specifically provided by MOB tissues and requires physical contact with the MOB (Chuah and Farbman, 1983; Chuah *et al*, 1985; Chuah and Au, 1988). These reports support the notion that the synaptic contacts between VRNs and CNS neurons can be formed in our coculture system due to the induction of VRN maturation. After ascertaining the synaptic formation between VRNs and AOB neurons, our future studies will focus on the analysis of the reception and processing of pheromonal messages using this coculture system.

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