

# Modification of Synapse Formation of Accessory Olfactory Bulb Neurons by Coculture with Vomeronasal Neurons

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## Abstract

Previously, a coculture system of accessory olfactory bulb (AOB) neurons and vomeronasal (VN) neurons was established for studying the functional roles of AOB neurons in pheromonal signal processing. In this study, the effect of VN neurons on the development of AOB neurons was examined in a coculture system. Spine density was quantitatively measured for various culture periods of 21, 28, 36, and 42 days *in vitro*. The densities of dendritic spines were lower in the coculture than in single culture for all periods *in vitro*. Synapse formation on spines was analyzed immunocytochemically using an anti-synaptophysin antibody. The ratio of the density of synaptophysin-immunopositive spine/total spine density was larger in the coculture than in the single culture. The volume of spine head was larger in the coculture than in single culture. These changes were not observed in the coculture in which there was no physical contact between AOB neurons and VN neurons. These observations suggest that synapse formation on the spines of AOB neurons is modified by physical contact with VN neurons.

**Key words:** accessory olfactory bulb, confocal laser microscope, EGFP-actin, spine, vomeronasal organ

## Introduction

The vomeronasal (VN) system consisting of the VN organ, the accessory olfactory bulb (AOB), and other central nervous system structures receiving axons from the AOB plays a critical role in the perception and processing of pheromonal signals (see reviews Halpern, 1987; Brennan, 2001; Halpern and Martinez-Marcos, 2003; Dulac and Torello, 2003). A pheromone is a conspecific chemical signal that provides information on the social and sexual states of other individuals within a mammalian species. The AOB has a simple laminar structure containing three main neuronal types: mitral/tufted (MT) cells, granule cells, and periglomerular cells. MT cells are the principal neurons of the AOB that receive VN neuron fibers from the VN organs forming the excitatory pathway for the transmission of pheromonal signals to the amygdala (see reviews Brennan, 2001; Halpern and Martinez-Marcos, 2003; Ichikawa, 2003). Granule cell dendrites receive excitatory inputs from MT cell dendrites via glutamate release, and granule cells provide a feedback inhibition to MT cells via  $\gamma$ -aminobutyric acid (GABA) re-

lease (Taniguchi and Kaba, 2001). Matsuoka *et al.* (1997, 2004) reported that dendrodendritic synapses in the AOB of female mice show plasticity changes after mating. It has been suggested that synaptic plasticity in the AOB provides an excellent model for studying the mechanism underlying long-term memory (Ichikawa, 2003).

We have developed a primary coculture system for the VN organ and AOB for studying the functional roles of VN and AOB neurons in pheromonal signal processing and synaptic plasticity (Ichikawa *et al.*, 1995; Muramoto *et al.*, 2003; Moriya-Ito *et al.*, 2005). Recently, we have utilized the system of continual culture of the VN organ established by Osada (1999) for coculture with the AOB. Cultured VN organs contain structures with a central cavity and show morphological characteristics similar to those of VN organs *in vivo*. Muramoto *et al.* (2003) have reported that the number of tyrosine hydroxylase-containing neurons significantly increases when cocultured with VN organs. Moreover, Moriya-Ito *et al.* (2005) have shown that the

maturation of VN neurons in cultured VN organs is induced by coculture with AOB neurons. It has been hypothesized that the differentiation or maturation of AOB neurons is modified by specific synaptic interactions between the axons of VN neurons and their targets. In the present study, we aim to determine whether the synapse formation of AOB is modified by VN neurons in coculture.

## Materials and methods

### Cell culture

AOB primary cultures were prepared according to a previously reported procedure with modifications (Muramoto *et al.*, 2003, Negishi-Kato *et al.*, 2003). Briefly, the AOB region, which was approximately one-quarter of the dorsal region of the entire olfactory bulb, was determined as in previous reports and was excised from a Wistar rat on embryonic day 21 (E21). The dissected AOB was dissociated with papain (Worthington Biochemical Corp., Freehold, NJ) and triturated through a pipette. Dissociated cells were suspended in a serum-free culture medium that consisted of Dulbecco's modified Eagles's medium and Nutrient Mixture F-12 (1:1) (DMEM/F-12, Gibco, Invitrogen, Carlsbad, CA) supplemented with 0.1 M L-ascorbic acid (Wako, Osaka, Japan), 0.025 M 2-mercaptoethanol (Sigma, St. Louis, MO), 50 U/ml penicillin G (Meiji Seika, Tokyo, Japan), 1.2 mg/ml NaHCO<sub>3</sub>, and 2% B27 serum-free additive (Gibco). The resulting cell suspension was plated at a density of  $2.5 \times 10^5$  cells/cm<sup>2</sup> on LabTek culture chamber slides (Nalge, Nunc, Rochester, NY) coated with polyethyleneimine (Sigma). Cultured neurons were maintained for 21–42 days in 5% CO<sub>2</sub> at 37°C.

VN organs were prepared as previously described (Moriya-Ito *et al.*, 2005). Briefly, the dissected VN organ from the head of Wistar rat on E15 was immersed in DMEM/F-12 containing 1 mg/ml collagenase/dispase (Boehringer, Mannheim, Germany) at 37°C for 60 min to remove surrounding tissues. After the inhibition of proteinase by DMEM/F-12 containing 10% fetal bovine serum (Gibco), one to three VN organs were plated onto LF1 plastic cell culture discs (diameter = 13.5 mm, Sumitomo Bakelite, Tokyo, Japan) covered with a feeder cell layer (Osada *et al.*, 1999). These VN organs individually formed spherical structures (Moriya-Ito *et al.*, 2005). The culture medium for the VN organ consisted of DMEM/F-12 containing 50 ng/ml hydrocortisone, 161 ng/ml putrescine, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml sodium selenite, 3 ng/ml fibroblast growth factor basic, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma–Aldrich). In this culture system, cultured VN organs survive for more than 1 year (Osada *et al.*, 1999).

All animals were treated according to the Guidelines for The Care and Use of Animals of Tokyo Metropolitan Institute for Neuroscience.

### Coculture

After the AOB was cultured *in vitro* for 2 h, a cultured VN organ within the first 1–3 weeks of culture was transferred into cultured AOB of 1cm<sup>2</sup> area and cocultured for 21, 28, 35, and 42 days *in vitro* (DIV). For the coculture, equal amounts of the medium the VN organs and AOB neurons were used.

To avoid direct contact between VN neurons and AOB neurons, they were separately plated on each half of the culture well, which was separated by 2% agarose gel (Moriya-Ito *et al.*, 2005).

### Imaging of enhanced green fluorescent protein–actin and synaptophysin

AOB neurons were transfected at 19, 26, 33, and 40 DIVs using the Lipofectamine 2000 reagent (Invitrogen). The plasmid vector for the expression of enhanced green fluorescent protein (EGFP)–actin was obtained from Clontech (Mountain View, CA). Transfected neurons were cultured for 48 h, the transfected neurons were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated with a rabbit polyclonal antibody against green fluorescent protein (GFP) (Medical and Biological Laboratories Co. [MBL], Nagoya, Japan) at 4°C overnight. After washing, the neurons were incubated at 37°C for 1 h with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Rockland, Gilbertsville, PA). Stained neurons were observed under a confocal laser microscope (LSM 510, Zeiss, Göttingen, Germany).

For other culture wells containing transfected neurons, double immunostaining was carried out using the antibodies to GFP and synaptophysin. The neurons were fixed in 4% paraformaldehyde in PBS and incubated with both a mouse monoclonal antibody of synaptophysin (Chemicon) and a rabbit polyclonal antibody of GFP (MBL) at 4°C overnight. After washing, the cells were incubated at 37°C for 1 h with both an FITC-conjugated anti-rabbit IgG antibody against anti-GFP antibody and Cy3.5-conjugated anti-mouse IgG (Rockland) against anti-synaptophysin antibody. Double-stained neurons were observed under a confocal laser microscope (LSM 510, Zeiss).

### Imaging analysis

All the data for statistical analysis were from more than two cultures.

### Spine density

The number of spines per unit length (10 μm) was measured at three sites of one dendrite in reconstructed three-dimensional (3-D) images obtained using a confocal laser microscope. The first site on one dendrite was selected between the cell soma and 20 μm from the cell soma, the second site on the dendrite was selected between 20 and 50 μm from cell soma, and the third site on the dendrite was selected

more than 50  $\mu\text{m}$  from the cell soma. Two dendrites from one neuron were selected for measurement. Spine densities for one neuron were obtained at three different sites along the dendrite, and spine density measurements were repeated on two dendrites per neuron. The spine density average was measured from more than four neurons for each of the two culture conditions.

#### Density of synaptophysin-immunopositive spines

The number of synaptophysin-immunopositive ( $\text{SY}^+$ ) spines was measured in the same manner as that of spine density. The ratio of  $\text{SY}^+$  spine density/total spine density was calculated.

#### Volume of spine head

The volume of a spine head was measured from more than four neurons for each of the two conditions at 28 DIV. The image of the spine head was three-dimensionally photographed using a confocal laser microscope (LSM 510, Zeiss). The longest and shortest diameters of spine head were measured on two-dimensional (2-D) images, and the  $z$ -axis length (length of orthogonal axis for a 2-D image) of the spine head was measured in the  $z$ -stacks of the 3-D image. The volume of the spine head was calculated for convenience using the following formula:

$$V = 4\pi/3(a/2 \times b/2 \times c/2),$$

where  $V$  = volume of the spine head,  $a$  = short diameter,  $b$  = long diameter, and  $c$  =  $z$ -axis length.

## Results

### Coculture

The VN organ started growing axon bundles from VN neurons 1 day after coculture (Moriya-Ito *et al.*, 2005). The axon bundle growth progressed for 7 days. After 1 week, the rapid growth stopped. Numerous axon bundles and fascicles surrounded AOB neurons. It has not been clarified yet whether the axon terminals of VN neurons form synaptic contacts with AOB neurons.

### Identification of EGFP-actin-transfected neurons

The images of cultured AOB neurons transfected with EGFP-actin were obtained using a confocal laser microscope. Two types of neuron were identified on the basis of their morphological characteristics. One type was multipolar neurons, which had a large soma (more than 100  $\mu\text{m}^2$ ) and multipolar and thick dendrites (Figure 1B). The other type was bipolar neurons, which had a small soma (less than 100  $\mu\text{m}^2$ ) and thin bipolar dendrites (Figure 1A). On the basis of their morphological characteristics, we conclude that multipolar neurons are MT cells and bipolar neurons are granule cells.

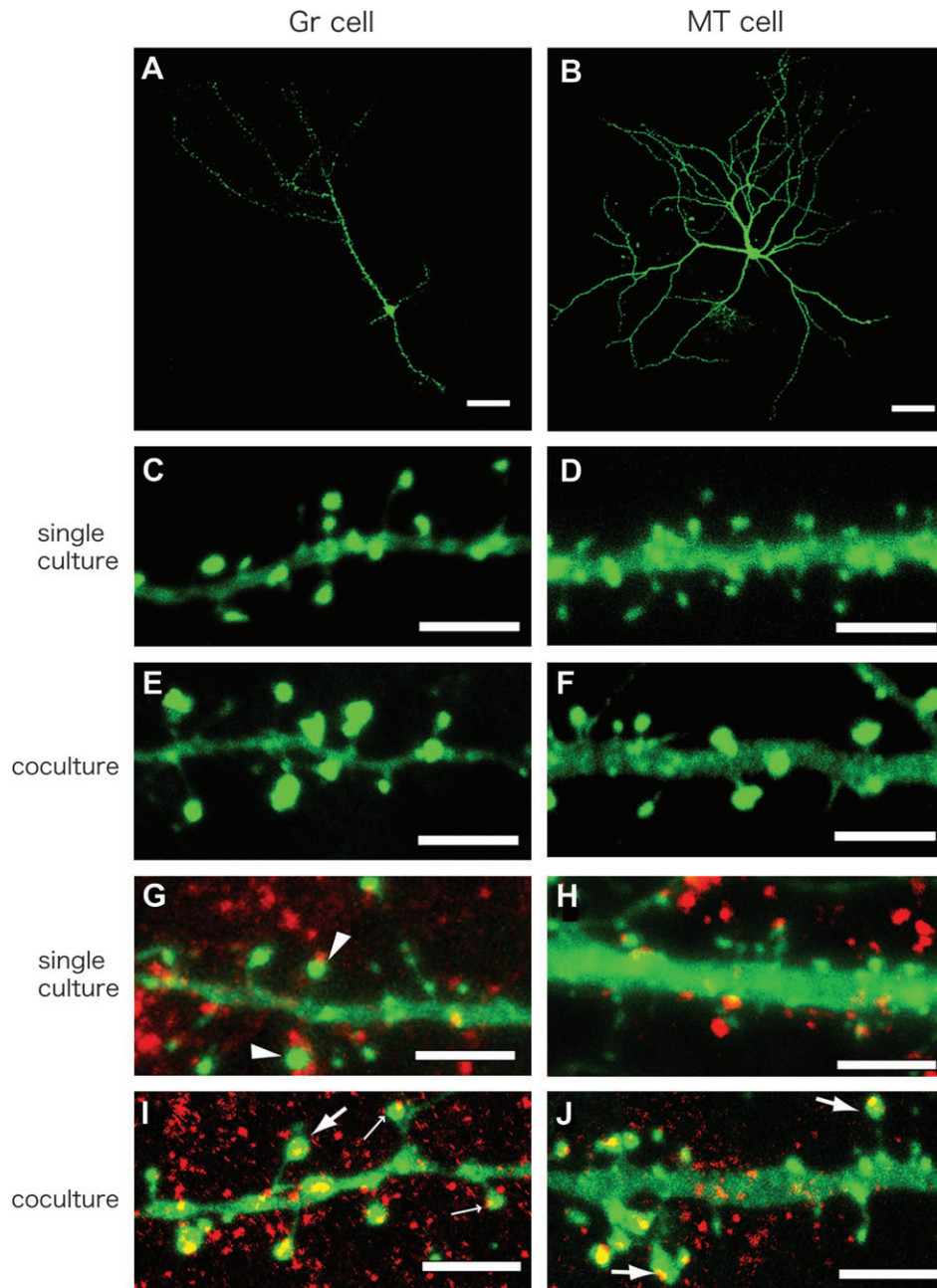
### Changes in spine density during culture period

In the confocal images of AOB neurons transfected with EGFP-actin, we observed numerous spiny processes on their dendrites (Figure 1C–F). It is not easy to distinguish spines from filopodia. In this study, we identified the spines for convenience on the basis of the following criteria. A spine contains a spine head whose shortest diameter is larger than 1  $\mu\text{m}$  and a spine neck whose thickness is smaller than the short diameter of the spine head. By this classification, most of the filopodia and immature (stubby) spines were not included in this analysis.

The spine density of MT and granule cells at each DIV was measured. Spine density changed during the culture periods from 21 to 42 DIVs. Spine density on three different sites along one dendrite ( $\leq 20$   $\mu\text{m}$  from the soma,  $\geq 20$   $\mu\text{m}$   $\leq 50$   $\mu\text{m}$  from the soma, and  $\geq 50$   $\mu\text{m}$  from the soma) was measured. However, because there was no significant difference in spine density among the three sites in each culture condition [analysis of variance (ANOVA),  $P \geq 0.1$ ], we analyzed spine density without discrimination of dendritic sites. In granule cells, spine density increased from 21 DIV ( $4.0 \pm 0.33$  in single culture,  $2.7 \pm 0.22$  in coculture) to 28 DIV ( $5.1 \pm 0.40$  in single culture,  $3.8 \pm 0.35$  in coculture) and then decreased at 35 DIV [ $3.8 \pm 0.34$  in single culture;  $2.7 \pm 0.22$  in coculture; two-way ANOVA:  $F(3,184) = 10.77$ ,  $P < 0.0001$ ; *post hoc* Scheffe test:  $P < 0.05$ ]. At 42 DIV, the spine density was the same ( $3.4 \pm 0.35$  in single culture and  $2.2 \pm 0.18$  in coculture) as that at 35 DIV (Figure 2A). In MT cells, the spine density was  $3.7 \pm 0.50$  in single culture and  $1.7 \pm 0.17$  in coculture at 21 DIV and increased at 28 DIV ( $5.0 \pm 0.51$  in single culture and  $3.3 \pm 0.34$  in coculture; two-way ANOVA:  $F(3,190) = 9.19$ ,  $P < 0.0001$ ; *post hoc* Scheffe test:  $P < 0.01$ ). At 35 and 42 DIVs, the spine density was the same as that at 28 DIV ( $4.9 \pm 0.27$  in single culture,  $3.1 \pm 0.21$  in coculture and  $5.1 \pm 0.34$  in single culture,  $3.2 \pm 0.25$  in coculture) (Figure 2B).

### Effect of coculture with VN organs on AOB spine density

In granule cells, spine density was decreased by their coculture with VN organs at all culture periods (Figures 1C,E and 2A). At 21, 35, and 42 DIVs, spine density was significantly different between the single culture and the coculture (two-way ANOVA:  $F(1,184) = 31.28$ ,  $P < 0.001$ ; *post hoc* Scheffe test:  $P = 0.002$  at 21 DIV,  $P = 0.02$  at 28 DIV,  $P = 0.009$  at 35 DIV, and  $P = 0.003$  at 42 DIV). In MT cells, spine density was also decreased by their coculture with VN organs at all culture periods (Figures 1D,F and 2B). At all DIVs, spine density was significantly different between the single culture and the coculture [two-way ANOVA:  $F(1,190) = 63.16$ ,  $P < 0.001$ ; *post hoc* Scheffe test:  $P < 0.0001$  at 21 DIV,  $P = 0.006$  at 28 DIV,  $P < 0.0001$  at 35 DIV, and  $P < 0.0001$  at 42 DIV]. The coculture of AOB neurons with VN neurons induced a decrease in the number of spines in the AOB neurons.

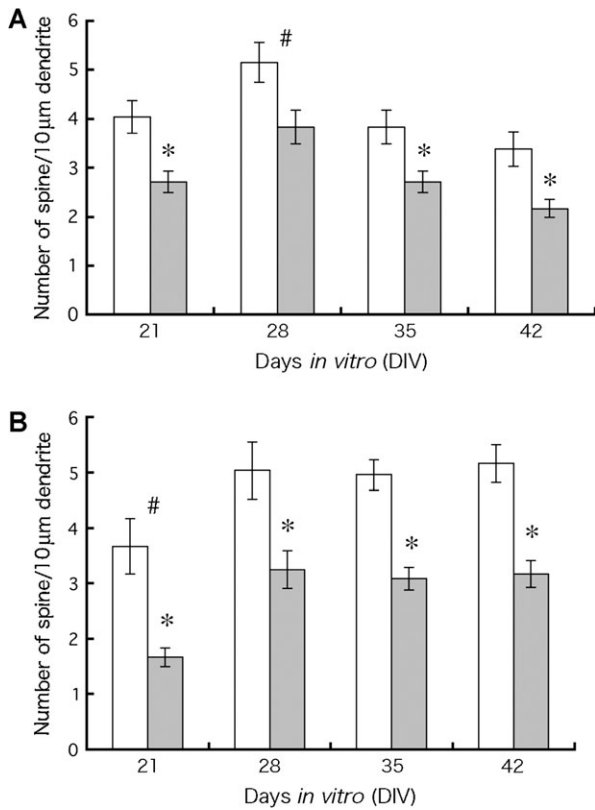


**Figure 1** Morphology of EGFP-actin-transfected AOB cells at 28 DIV. Low-magnification images of granule cell (**A**) and MT cell (**B**), scale bar, 50  $\mu\text{m}$ . Immunofluorescence images of dendrite of granule cell (**C**) and MT cell (**D**) in single culture or granule cell (**E**) and MT cell (**F**) in coculture; scale bar, 5  $\mu\text{m}$ . Double immunofluorescence images of granule cell (**G**) and MT cell (**H**) in single culture or granule cell (**I**) and MT cell (**J**) in coculture after staining with anti-EGFP antibody (green) and anti-synaptophysin antibody (red); scale bar, 5  $\mu\text{m}$ . Arrows indicate three types of SY<sup>+</sup> spines, SY<sup>+</sup> spots: within the spines (thick arrows), outside, but attached to the spines (arrowheads), and extended from the spines (thin arrows).

#### Effect of coculture with VN organs on AOB synapse formation

Spines have been considered as the postsynaptic sites of synapses (Harris and Kater, 1994; Yuste and Denk, 1995; Matus, 2000). Spine density, thus, reflects synaptic density. However, in culture, it is difficult to distinguish between spine and filopodia (or immature spine). Synaptophysin is

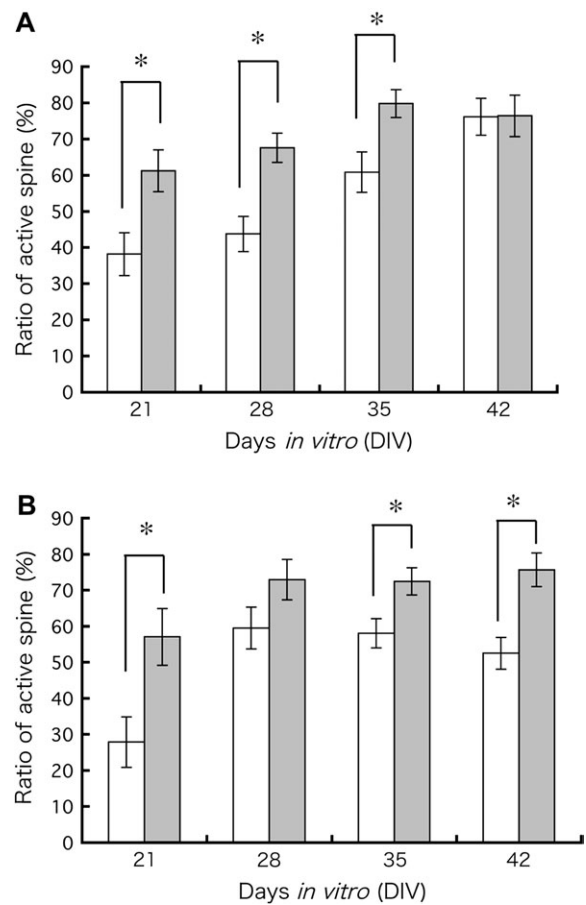
a good marker of synapses because it localizes in the synaptic vesicle membrane. Thus, to identify synapses in this study, the presynaptic terminal was labeled immunocytochemically using an anti-synaptophysin antibody. The granule and MT cells of the AOB produce dendrodendritic synapses (Matsuoka *et al.*, 1997; Ichikawa, 2003). Thus, the dendrites of these neurons have both presynaptic and postsynaptic



**Figure 2** Chronological changes in AOB dendrite. Spine density is indicated as the mean number of spines on a 10- $\mu$ m dendrite of a granule cell (**A**) or a MT cell (**B**). Sharps indicate significant differences from the DIV groups (two-way ANOVA:  $P < 0.0001$ ; *post hoc* Scheffe test:  $P < 0.01$ ). Asterisks indicate significant differences between the single culture (white) and coculture (gray) (two-way ANOVA:  $P < 0.0001$ ; *post hoc* Scheffe test:  $P < 0.01$ ;  $n = 24$  sites/4 neurons/DIV).

functions. It is necessary to identify whether SY<sup>+</sup> spots localize inside or outside (at least the areas were attached to the spine) of spines. However, their identification was very difficult in the present study, even with the use of a confocal laser microscope, because most of the SY<sup>+</sup> spots extended from a spine (see Figure 1G,J). However, it was clear that these SY<sup>+</sup> spines made synaptic contacts and were “active spines.” Thus, in this analysis, the classification of presynaptic or postsynaptic sites was not performed. Three types of spine were identified as SY<sup>+</sup> spines, SY<sup>+</sup> spots: 1) within the spines, 2) outside, but attached to the spines, and 3) extended from the spines. SY<sup>+</sup> spines were measured per unit length of dendrites and the ratio of the number of SY<sup>+</sup> spines to the total number of spines was calculated.

In the granule cells of the single culture, the ratio of the number of SY<sup>+</sup> spines to the total number of spines increased gradually from 21 to 42 DIVs ( $38.1 \pm 5.9$  at 21 DIV,  $43.7 \pm 4.8$  at 28 DIV,  $60.8 \pm 5.6$  at 35 DIV, and  $76.1 \pm 5.1$  at 42 DIV) (Figure 3A). This observation indicates that synapse formation depends on the culture period. At 21, 28, and 35 DIVs, the ratio of SY<sup>+</sup> spines increased significantly in the coculture ( $61.2 \pm 5.7$  at 21 DIV,  $P = 0.01$ ;  $67.5 \pm 4.0$  at 28 DIV,



**Figure 3** Chronological changes in ratio of number of active spines to total number of spines. An active spine is defined as a spine that colocalized and/or associated with anti-synaptophysin-immunopositive spot in a granule cell (**A**) or a MT cell (**B**). Asterisks indicate significant difference between single culture (white) and coculture (gray) ( $P < 0.01$ , Mann–Whitney *U*;  $n = 24$  sites/4 neurons/DIV).

$P < 0.001$ ;  $79.8 \pm 3.8$  at 35 DIV,  $P = 0.01$ ; and  $76.4 \pm 5.7$  at 42 DIV,  $P = 0.60$ —Mann–Whitney *U*) (Figure 3A). Thus, granule cells showed enhanced synapse formation by coculture with VN organs. On the other hand, in the MT cells, the ratio of the number of SY<sup>+</sup> spines to the total number of spines increased from 21 to 28 DIVs ( $27.9 \pm 6.9$  at 21 DIV and  $59.4 \pm 5.8$  at 28 DIV), and then at 35 and 42 DIVs, it was the same as that at 28 DIV ( $58.0 \pm 4.0$  at 35 DIV and  $52.4 \pm 4.3$  at 42 DIV) (Figure 3B). At 21, 35 and 42 DIVs, the ratio of SY<sup>+</sup> increased significantly in the coculture ( $57.0 \pm 7.8$  at 21 DIV,  $P = 0.01$ ;  $72.9 \pm 5.5$  at 28 DIV,  $P = 0.08$ ;  $72.4 \pm 3.7$  at 35 DIV,  $P = 0.01$ ; and  $75.6 \pm 4.6$  at 42 DIV,  $P = 0.02$ —Mann–Whitney *U*) (Figure 3B). These observations suggest that the MT cells also showed enhanced synapse formation by coculture with VN organs.

#### Effect of coculture with VN organs on size of spine head

Spines are the postsynaptic sites of synapses and have important roles in synaptic function. It has been observed that

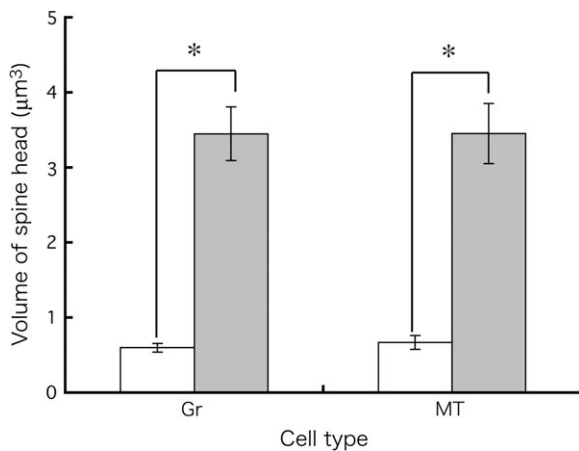
there is a relationship between synaptic function and the size of the spine head. To examine the effect of coculture with VN organs on the spine head, the size of spine heads was measured in EGFP-actin-transfected AOB neurons at 28 DIV. In both granule and MT cells, the size of spine heads increased approximately fivefold in the coculture ( $0.60 \pm 0.06 \mu\text{m}^3$  in single culture and  $3.45 \pm 0.36 \mu\text{m}^3$  in coculture of granule cells,  $P < 0.0001$ ; Mann-Whitney  $U$ ;  $0.67 \pm 0.09 \mu\text{m}^3$  in single culture and  $3.45 \pm 0.40 \mu\text{m}^3$  in coculture of MT cells,  $P < 0.0001$ ; Mann-Whitney  $U$  ( $n = 36$ , Figure 4). Coculture with VN organs induced an enlargement of the spine heads in AOB neurons.

### Coculture of AOB neurons and VN organs without physical contact

To examine whether a direct contact with VN organs is necessary to induce changes in the densities of spines and synapses, we examined the coculture in which VN organs were physically separated from AOB neurons by an agarose gel divider. Under this coculture condition, in which there was no physical contact between VN neurons and AOB neurons, spine density and ratio of the number of SY<sup>+</sup> spines were not different from those of in single culture of granule and MT cells at 28 DIV (Figure 5). This result suggests that changes in the density of spines and synapses were not induced by soluble trophic factors but by physical contacts between VN organs and AOB neurons ( $P = 0.71$  of granule cells and  $P = 0.35$  of MT cells; Mann-Whitney  $U$ ). This strongly suggests that the synaptic contacts between the axons of VN neurons and AOB dendrites modulate dendrodendritic synapse formation among AOB neurons.

### Discussion

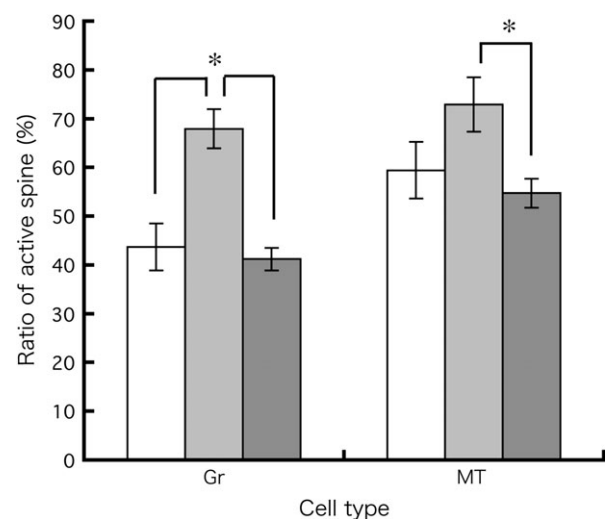
In the past decade, we developed primary culture systems for VN organ and AOB for studying the functional roles



**Figure 4** Difference in volume of spine head. The volume of the spine head was calculated from 3-D images. Asterisks indicate significant difference between single culture (white) and coculture (gray) ( $P < 0.0001$ , Mann-Whitney  $U$ ;  $n = 36$ ).

of VN 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, 2004; Negishi-Kato et al., 2003; Moriya-Ito et al., 2005). We identified two types of neuron in the AOB culture system. One was a medium-sized (approximately  $180 \mu\text{m}^2$ ) multipolar neuron with several thick dendrites, and the other type was a unipolar or bipolar neuron with a small round soma (about  $90 \mu\text{m}^2$ ) and thin dendrites. Most neurons of the former type showed no immunoreactivity to the anti-glutamate decarboxylase (GAD) antibody, whereas neurons of the latter type showed immunoreactivity to the anti-GAD antibody. Takami et al. (1992) reported on the morphology of GABAergic neurons and MT cells in AOB. GABAergic neurons are found in the periglomerular region and granule cell layer and have a small soma. By contrast, MT cells are non-GABAergic and have several dendrites and a large soma *in vivo*. Considering these properties, we have classified the multipolar neurons as MT cells. As dopaminergic periglomerular cells of AOB both *in vivo* (Mugnani et al., 1984) and *in vitro* (Muramoto et al., 2003, 2004) were rarely observed, unipolar or bipolar neurons were suggested to be mainly granule cells. In this study, bipolar and multipolar neurons were observed. We therefore classified the bipolar and multipolar neurons as granule cells and MT cells, respectively. Multipolar neurons in this culture have dendrites covered with spines. In general, MT cells *in vivo* do not bear many spines in adults. It is considered that the appearance of spines is a specific peculiarity of cultured cells.

This study showed that the spine density of AOB neurons cocultured with VN neurons was decreased. The elimination



**Figure 5** Effect of physical contacts between VN neurons and AOB neurons. No difference between coculture without physical contact and single culture. An active spine is defined as a spine that immunoreacted and colocalized with synaptophysin. Asterisks indicate significant difference between coculture (light gray) and single culture (white) or separated coculture (dark gray) ( $P < 0.01$ , Mann-Whitney  $U$ ;  $n = 24$  sites/4 neurons/DIV).

of spines during the development of the brain is called “pruning” (Segal *et al.*, 2000; Segal, 2005). An important process in dendritic development involves spine pruning. Cortical spine density increases with age and then decreases at maturity (Rakic *et al.*, 1986; Lund and Hollbach, 1991). The activation of synaptic receptors can cause a rapid elongation of filopodia (Maletic-Savatic *et al.*, 1999), formation of spines (Engert and Bonhoeffer, 1999; Shi *et al.*, 1999), or the shrinkage of existing spines (Halpain *et al.*, 1998; Zhou *et al.*, 2004). The enhancement of network activity by manipulation of receptor functions can delay spine formation (Papa and Siegel, 1996) or cause the disappearance of existing spines (Drakew *et al.*, 1996; Jiang *et al.*, 1998). This suggests that the spine pruning of AOB dendrites is promoted by their specific interaction with VN neurons and/or input from VN neurons. Moreover, the ratio of SY<sup>+</sup> spine in AOB dendrites increased, despite a reduction in spine density, suggesting that spines without presynaptic terminals are selective eliminated. However, it is not clear whether spine pruning is an active process associated with an increase in synaptic activity or a passive process caused by the lack of afferent activation. Recently, high-resolution imaging methods have been used in studying spine formation (see reviews Dunaevsky and Mason, 2003; Yuste and Bonhoeffer, 2004; Carlisle and Kennedy, 2005; Segal, 2005). The mechanisms of spine formation and pruning might be elucidated in the near future.

The volume of spine heads in the coculture was larger than that in the single culture. Recent studies indicated that spine head volume correlated with the expression of functional AMPA receptors in the hippocampus (Matsuzaki *et al.*, 2001). Moreover, it was reported that large spines are structurally stable for more than 1 year *in vivo* (Grutzendler *et al.*, 2002; Trachtenberg *et al.*, 2002). It is suggested that AOB neurons in coculture with VN neurons with many large spines are more active and more stable than those in single culture, indicating that AOB neurons cocultured with VN neurons are more mature.

It is not clear whether the axons of VN neurons form synaptic contacts with AOB neurons in a coculture system by confocal laser microscopy. However, our recent study indicates that some AOB neurons are activated by the electrical stimulation of a VN organ in coculture. Moreover, our preliminary study suggests that axons that have similar morphological characteristics to those of VN neuron terminals *in vivo* formed synaptic contacts with AOB neurons in coculture as observed by electric microscopy (data not shown). This suggests that functional synaptic contacts between AOB neurons and VN neurons are formed in this coculture system. Moriya-Ito *et al.* (2005) have reported that the maturation of VN neurons in VN organs cocultured with AOB is induced by their physical contact with AOB neurons. This agrees with our present study that suggests that the modification of AOB neurons is induced only by their physical contact with VN neurons. It has also been hypothesized that the synapse formation of AOB

neurons can also be modified by their physical contact with VN neurons.

A large number of synapses have been observed on granule and MT cells in the culture of AOB neurons only (Negishi-Kato *et al.*, 2003). These granule and MT cells form dendrodendritic synaptic contacts with each other. Thus, the dendrites of these neurons have both presynaptic and postsynaptic functions. It is necessary to determine whether SY<sup>+</sup> spines have presynaptic or postsynaptic functions. However, this was very difficult to carry out in this study even with the use of a confocal laser microscope. It was clear that SY<sup>+</sup> spines form synapses, although the identification of presynaptic or postsynaptic sites was not performed. The ratio of SY<sup>+</sup> spines on AOB neurons was increased by their coculture with VN neurons, and this effect was dependent on the physical contact of AOB dendrites with the axons of VN neurons. This suggests that synapse formation of AOB neurons is induced by the inputs of VN neurons to AOB neurons. As the next step of this study, the localization of SY<sup>+</sup> spots will be identified using other markers of synaptic contacts, such as the antibodies of synaptic transmitters and receptors of neurotransmitters.

A coculture system of VN and AOB neurons has been established in our laboratory. Activity-dependent synapse formation on spines has been reported *in vivo* and *in vitro* by several authors (see reviews Yuste and Bonhoeffer, 2004; Carlisle and Kennedy, 2005; Segal, 2005). In the culture of AOB neurons, the bicuculline-induced disinhibition of cultured AOB neurons enhanced synapse formation. It is not however clear whether the effect of the coculture is activity dependent. Thus, to study activity-dependent synaptic plasticity using the present coculture system, electrical stimulation or the application of pheromonal substances to VN organs is necessary. Only few substances have so far been identified as real pheromones, although many candidate pheromones have been reported. If the application of chemical substances to cultured VN neurons can be manipulated, this system can be very useful in studying the assay system of pheromonal substances.

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