Co-localization of TRPV2 and Insulin-Like Growth Factor-I Receptor in Olfactory Neurons in Adult and Fetal Mouse

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TRPV2, a member of the transient receptor potential family, has been isolated as a capsaicin-receptor homolog and is thought to respond to noxious heat. Here we show that TRPV2 mRNA is predominantly expressed in the subpopulation of olfactory sensory neurons (OSNs). We carried out histochemical analyses of TRPV2 and insulin-like growth factor-I receptor (IGF-IR) using in situ hybridization and immunofluorescence in the adult olfactory system. In olfactory mucosa, intense TRPV2 immunostaining was observed at the olfactory axon bundles but not at the soma. TRPV2-positive labeling was preferentially found in the olfactory nerve layer in the olfactory bulb (OB). Furthermore, we demonstrated that a positive signal for IGF-IR mRNA was detected in OSNs expressing TRPV2 mRNA. In embryonic stages, TRPV2 immunoreactivity was observed on axon bundles of developing OSNs in the nasal region starting from 12.5 d of gestation and through fetal development. Observations in this study suggest that TRPV2 coupled with IGF-IR localizes to growing olfactory axons in the OSNs.

Key words olfactory neuron; olfactory epithelium; olfactory bulb; insulin-like growth factor-I receptor; transient receptor potential V2

Olfactory sensory neurons (OSNs), which receive odorants, transmit odor information to the olfactory bulb (OB). Some volatile odorants having toxic effects physiologically damage the OSNs. In addition, because the olfactory organ is located at the peripheral region, physical damage is likely to occur in the OSNs. One of their particular features is that they are continuously renewed during adulthood to overcome these disadvantages. The life period of rat OSNs was about 30 d. To continuously renew during adulthood to overcome these disadvantages, the life period of rat OSNs was about 30 d. To

It has been suggested that the proliferation and differentiation of OSNs are controlled by several trophic factors. In the rodent or avian olfactory system, insulin-like growth factor (IGF)-1 is produced in the OB at the embryonic and postnatal stages, while the IGF-I receptor (IGF-IR) is expressed in both OB and olfactory epithelium (OE). These results suggest that IGF-I plays an important role in bulbo-epithelial interactions in the olfactory system.

In general, axon elongation and guidance depend on the localization, concentration, and temporal dynamics of cytoplasmic calcium signaling. Whereas increases in intracellular calcium levels lead to the activation of calcium-dependent signaling molecules, the location of a regulatory calcium-permeable channel remains unclear, as dose the way in which its activity is regulated. Transient receptor potential (TRP) ion channels form a large superfamily of non-selective cation channels that display a remarkable diversity of activation mechanisms. Members of the vanilloid receptor family (TRPV) are activated by a wide range of stimuli, including temperature, protons, phorbol esters, lipids, changes in extracellular osmolarity and pressure, and depletion of intracellular calcium stores. A trophic factor such as nerve growth factor increases TRPV1, which is activated by an increase in temperature or capsaicin, functions via phosphatidylinositol 3-kinase (PI3K)-regulated trafficking to the plasma membrane. TRPV2 (also known as VR1-1 or GRC), a TRPV1 homologue, is activated by high-intensity noxious heat. In a preliminary experiment, reverse-transcription polymerase chain reaction (RT-PCR) analysis using specific primers for each 22 plasma membrane-associated TRP family, TRPC1-7, TRPM1-8, TRPV1-6, and TRPA1 in mouse olfactory mucosa clearly detected amplification of fragment for TRPC1, TRPC6, TRPM3, TRPM4, TRPM5, TRPM6, TRPM7, TRPV2, TRPV6, and TRPA1 (data not shown). In situ hybridization showed that cell bodies labeled with molecular probes selectively directed against TRPM3, TRPM4, TRPM5, TRPM6, TRPM7, TRPV2, TRPV6, and TRPA1 mRNAs were located in the olfactory epithelium (data not shown). Ahmed et al. support that TRPV2 is expressed in mouse olfactory epithelium. Kanazaki et al. have reported that translocation of TRPV2 to the plasma membrane is augmented by IGF-1, although the physiological significance of IGF-1-regulated TRPV2 sensitization remains unknown. In this study, we carried out histochemical analyses of TRPV2 and IGF-1 receptor using in situ hybridization and immunofluorescence in the adult olfactory system to explore the essential calcium channel that regulates axon elongation and guidance. We also investigated the immunohistochemical localization of TRPV2 in the OSNs of the nasal regions during fetal development.

MATERIALS AND METHODS

Antibodies Purified rabbit anti-TRPV2 polyclonal immunoglobulin G (IgG), which was raised against amino acids 744–761 (KNSASEEDHLPLQVLQSP) of TRPV2, was obtained from Oncogene Research Products (San Diego, CA, U.S.A.). Mouse monoclonal antibody against βIII-tubulin and growth-associated protein-43 (GAP43) were obtained from Promega (Tokyo, Japan) and Sigma-Aldrich (Tokyo, Japan), respectively. Olfactory marker protein (OMP) antiserum immunized in goat was a gift from Dr F. Margolis (University of California, San Diego, CA, U.S.A.). The authors declare no conflict of interest.

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of Maryland School of Medicine). Alexa Fluor 488 donkey anti-rabbit IgG (H+L) and Alexa Fluor 546 donkey anti-goat IgG (H+L) were purchased from Molecular Probes (Tokyo, Japan). Rhodamine-conjugated donkey anti-mouse IgG (H+L) was from Alexis (Tokyo, Japan)

**Animals and Tissue Sample Collection for in Situ Hybridization and Immunohistochemistry**

The adult (8–12 weeks old) C57BL/6 male mice used for this study were purchased from Sankyo Laboratory (Sapporo, Japan). The mice were kept in a room at 22±0.5°C and 58% relative humidity, with a 12 h light:12 h dark cycle (light off at 20:00h). All mice had free access to food and water in the housing cage. The animal-use protocol was approved by the Ashihikawa Medical University Animal Experiment Committee, and we followed the Ashihikawa Medical University Guidelines for Animal Experiments throughout the study. Tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C until use. To prepare the frozen sections for immunofluorescence and in situ hybridization, mice were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), followed by intracardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Extracted nasal tissues were decalcified by agitating in 0.5 M ethylenediaminetetraacetic acid (EDTA)/PBS at 4°C for 3 d. Tiled pregnant mice at embryonic days (E) 10.5, 11.5, 12.5, 14.5, and 17.5 were fixed in 4% paraformaldehyde and stored in PBS until use. Tissues were cryoprotected by incubation in 30% sucrose/PBS at 4°C for 12 h, embedded in O.C.T compound, and cryosectioned (12 µm) using a JUNG CM3000 or CM3050 cryostat (Leica, Nussloch, Germany).

**In Situ Hybridization**

Antisense and sense probes were created by incubating 1 µg of linearized plasmid DNA harboring 346 base-pair mouse TRPV2 cDNA fragments (accession no. NM_011706, 2434–2779) obtained by polymerase chain reaction (PCR) with a 10×digoxigenin (DIG) RNA labeling mixture (Roche, Tokyo, Japan) and T7 or SP6 RNA polymerase (Roche) for 2 h at 37°C. The general cytoarchitecture of olfactory mucosa, we characterized the expression patterns of heterogeneous cells such as olfactory receptor, supporting, and basal cells. To determine the cellular localization of TRPV2 in olfactory mucosa, we used a previous described protocol. Briefly, cryosections were post-fixed in 4% paraformaldehyde in PBS for 10 min, washed twice with PBS, acetylated for 10 min with 0.25% acetic anhydride in 100 mM triethanolamine–HCl (pH 8.0) and again washed twice with PBS. Prehybridization was performed at room temperature with 200 µL hybridization buffer per slide for 3 h. The hybridization buffer was composed of 50% formamide (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 5×saline-sodium citrate (SSC) buffer (20×SSC; 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 5×Denhardt’s (Wako Pure Chemical Industries, Ltd.), 250 µg/mL yeast tRNA (Roche), and 500 µg/mL herring sperm DNA (Roche). The hybridization was carried out in hybridization buffer containing 200 ng/mL DIG-cRNA for 16 h at 72°C. Slides were washed three times for 20 min each with 0.2×SSC at 72°C. The hybridized DIG-labeled probe was detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche) at a dilution of 1:5000 and visualized with nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3′-indolylphosphatase p-toluidine salt (BCIP).

**Double-Fluorescent in Situ Hybridization**

The simultaneous expression of two genes, IGF-1R and TRPV2, was detected using DIG-labeled IGF-1R RNA probe (NM_010513, 5831–7210bp) and fluorescein-labeled TRPV2 RNA probe (NM_011706, 395–1435bp). These probes were created according to above, except for the fluorescein labeling with a 10× fluorescein RNA labeling mix (Roche, Manheim, Germany). Hybridizing and washing conditions were same as described above, except for applying of both DIG- and fluorescein-labeled probes to 5 µm coronal cryosections of olfactory epithelium. For amplification of the signal of the fluorescein-labeled probe, TSA-plus fluorescein system (PerkinElmer, Inc., Waltham, MA, U.S.A.) was employed after blocking with 1% blocking reagent (Roche) for 1 h and incubation with 1/400 anti-fluorescein antibody conjugated HRP (PerkinElmer, Inc.) for 1 h at 37°C. In addition, the tyramide–fluorescein signal was strengthened to Alexa Fluor 488 signal by two incubations, with 1/100 rabbit anti-fluorescein (AbD serotec, Oxford, U.K.) for 1 h at 37°C and with 1/50 donkey anti-rabbit IgG conjugated Alexa Fluor 488 (Molecular Probes, Eugene, OR, U.S.A.) for 1 h at 37°C. Each of the incubations with the antibodies was followed by washing with PBS containing 0.05% tween 20. The DIG-labeled signal detected with 1/5000 alkaline phosphatase-conjugated anti-DIG antibody was visualized with HNPP fluorescent detection set (Roche) according to the manufacturer’s instruction.

**Immunofluorescence**

Air-dried cryosections or cells plated on a glass-bottom dish were incubated in PBS containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA) (blocking solution), followed by overnight incubation at 4°C in blocking solution containing primary antibody. The sections were subsequently washed, and a positive immunoreaction was visualized by using Alexa Fluor 488 donkey anti-rabbit IgG, rhodamine-conjugated donkey anti-mouse IgG, and Alexa Fluor 546 donkey anti-goat IgG, diluted to 1:100 in blocking solution for 1 h. After washing, sections were counterstained with DAPI (Dojindo, Kumamoto, Japan) and mounted with Aqua Poly/Mount (Polysciences, Warrington, PA, U.S.A.). Microphotographs were taken using fluorescent optics on an IX71 inverted microscope (Olympus, Tokyo, Japan) with a C4742-95 camera (Hamamatsu Photonics, Hamamatsu, Japan).

**RESULTS**

**Cellular Localization of TRPV2 mRNA in Adult Mouse Olfactory Mucosa**

The olfactory mucosa consists of heterogeneous cells such as olfactory receptor, supporting, and basal cells. To determine the cellular localization of TRPV2 in olfactory mucosa, we characterized the expression patterns of TRPV2 mRNAs in the olfactory mucosa using in situ hybridization (Fig. 1). The general cytoarchitecture of olfactory epithelium (OE) with a laminar distribution according to the level of differentiation21) is shown in Fig. 1a staining for olfactory marker protein (OMP), a marker for mature olfactory sensory neuron (OSN).21) Olfactory stem cells (called basal cells) and mitotic progenitors lie in the deep OE. Several layers of mature OSNs that express OMP are present in the middle compartment of the OE. As shown in Fig. 1b, cell bodies labeled with molecular probes selectively directed against TRPV2 abounded in OSNs of the OE. In the OE, the supporting cells formed a single superficial layer clearly lacking TRPV2 mRNAs. No significant labeling in in situ hybridization analysis could be obtained with the sense probes (not shown).

**Immunolocalization of TRPV2 in Adult Mouse Olfac-
To determine the precise distribution of TRPV2 protein, we performed double-labeling immunofluorescence using antibodies against TRPV2 and OMP or growth-associated protein-43 (GAP43). Just above this level of proliferation, postmitotic immature OSNs are known to express GAP43\textsuperscript{23} and are OMP-negative. Olfactory nerve bundles lying in the lamina propria displayed intense anti-TRPV2 fluorescence (Figs. 2a, d). TRPV2-positive bundles were co-localized with OMP (Figs. 2b, c). TRPV2 immunoreactivities were also observed in axon bundles labeled with GAP43 (Figs. 2e, f). However, obvious staining on cell bodies and cilia of the OSNs was not found by immunofluorescence with the antibody against TRPV2. This result suggests that most TRPV2 protein is quickly transported to axons of GAP43-positive immature and OMP-positive mature OSNs, and that protein amounts in soma are too low to be detected by our immunofluorescence.

The axons of the OSNs from the olfactory mucosa form an OMP-positive olfactory nerve layer (ONL in Fig. 3a) at the surface of the olfactory bulb (OB). Consistent with the intense TRPV2 immunolabeling observed in the OMP-positive olfactory nerve bundles lying in the deep OE, the glomerular layer and olfactory nerve layer of the OB displayed immunoreactivity for TRPV2 (Fig. 3b). More abundant axons labeled with TRPV2 antibody were found to be concentrated in the olfactory nerve layer than in glomeruli, suggesting that TRPV2 localizes on olfactory axons and that the TRPV2-positive axons reach their target glomeruli.

**TRPV2 mRNA and Insulin-Like Growth Factor-I Receptor mRNA Expression in Mouse OSNs**  
Previous reports have indicated that translocation of TRPV2 is augmented by insulin-like growth factor (IGF)-I through PI3K and the Akt signaling pathway, leading to a gradual increase in calcium permeability.\textsuperscript{18} To explore the presence of the IGF system in olfactory mucosa, we examined the existence of mRNA of the IGF-IR in the OE. As shown in Fig. 1, subpopulation of OSNs expressed TRPV2 mRNA (Fig. 4a, green). Cell bodies of labeled with molecular probes selectively directed against IGF-I receptor abounded in OSNs (Fig. 4b, red). Most OSNs expressing TRPV2 mRNA expressed also IGF-IR mRNA (Fig. 4c, yellow).

**TRPV2 Immunolocalization during Fetal Development**  
If TRPV2 is confined to elongating axons of newly differentiated OSN, as expected from the localization of TRPV2 in the adult OB, TRPV2 may regulate the extension of axons of the OSNs in nasal regions during fetal development. To determine whether or not TRPV2 is involved in olfactory system development, we examined immunofluorescence using E10.5, E11.5, E12.5, E14.5, and E17.5 embryos. No significant signals in the nasal region were observed by immunofluorescence using antibodies against TRPV2 at E10.5 and E11.5 (not shown). We detected TRPV2-immunopositive staining in axon bundles labeled with βIII-tubulin, a marker of newly differentiated neurons\textsuperscript{24} at E12.5 (Figs. 5a–c), when OMP is still not expressed in the OSNs. This observation indicated that TRPV2 localizes to axons of immature OSNs in the fetal development.

At E14.5, when OMP-positive mature OSNs started to appear

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**Fig. 1. In Situ Hybridization of Olfactory Marker Protein and TRPV2 mRNA in Olfactory Mucosa of Adult Mice**  
DIGoxigenin-labeled RNA probes specific to olfactory marker protein (a) and TRPV2 (b) were hybridized with cryosections of olfactory mucosa and visualized with the colorant developed to brown precipitates. OSN, olfactory sensory neurons. The expression pattern shown here was confirmed in two independent experiments. Scale bar, 100μm.

**Fig. 2. Accumulations of TRPV2 Protein at Olfactory Axon Bundles**  
(a–c), Olfactory mucosa was double-labeled with anti-TRPV2 antibody (a) and olfactory marker protein antibody for staining of the mature olfactory neurons (b). TRPV2-immunopositive bundles are indicated by arrows in (a, c). Overlaid images of TRPV2 (green) and olfactory marker protein (red) (d–f). Double immunolabeling with TRPV2 and growth-associated protein-43, the latter of which is a typical marker of immature olfactory sensory neurons. TRPV2-labeled axons (d, arrows) are co-localized with growth-associated protein-43-immunoreactive axons (e). A merged image of TRPV2 (green) and growth-associated protein-43 (red) is shown in (f). The expression pattern shown here was confirmed in three independent experiments. Scale bar, 100μm.
axons derived from the OSNs appeared as nerve bundles stained with βIII-tubulin between developing OEs, as shown in Fig. 5d. TRPV2 immunoreactivity co-localized at neurons labeled with βIII-tubulin, clearly demonstrating that TRPV2 protein localizes on axon bundles of the OSNs during fetal development (Figs. 5d, e). At this stage, staining with TRPV2 antibody is visible on cell bodies and dendrites of olfactory neurons lying in developing OE. Double immunofluorescence using OMP and TRPV2 demonstrated staining of OMP-positive olfactory neurons with TRPV2 antibody (Fig. 5f–h), clearly showing that OMP-positive mature OSNs express TRPV2. Staining for TRPV2 using sagittal sections from E17.5 embryo was similar to that from E14.5 embryos (not shown).

DISCUSSION

In this study, we established that TRPV2 mRNA is expressed in a subset of OSNs and that protein is localized on olfactory axons. TRPV2-positive axons innervate to the olfactory nerve layer and glomerular layer in the OB. Localization of TRPV2 was also observed in olfactory axon bundles in embryonic stages, suggesting that TRPV2 has an essential role in processes related to the growth of olfactory axons during
fetal development.

**Restriction of TRPV2 Localization to Olfactory Axon Bundles in Vivo** In the adult olfactory system, TRPV2 distribution is restricted to the axons and is hardly observed in the soma and dendrites. Neurite outgrowth is regulated by cytoplasmic calcium-level elevation controlled by calcium-permeable channel activity on plasma membranes. The members of the TRP family are good candidates for the Ca\(^{2+}\) influx pathway. For example, TRPC1 is compartmentalized at the growth cone of *Xenopus* spinal neurons, and is required for the turning of growth cones induced by netrin-1 and brain-derived neurotrophic factor. The TRPC5 channel, which is localized to growth cones and gradually disappears as neurons mature and form stable connections with neighboring neurons, is a component of neurite extension by changing the growth cone morphology in rat hippocampal neurons. These results suggest that the TRP family plays a significant role in regulating the cytoplasmic calcium levels in axon guidance. The immunolocalization of TRPV2 on axon bundles in adult olfactory mucosa and in the GAP43-positive immature axons in the OB suggests that TRPV2 preferentially accumulates in the elongating axons of OSNs in the OE and OB. It is therefore possible that TRPV2 accumulated in elongating OSN axons is a driver of localized increases in intracellular calcium level as a means of extending the axons.

**Putative Functional Involvement of TRPV2 in the Event Controlled by IGF-I in Olfactory Sensory Neurons** IGF-I promotes myelination of neuronal axons, formation of synaptic networks, synaptic plasticity, cognitive function, and the survival and longevity of cells in developing and adult neurons. Evidence implicates IGF-I in the development and regeneration of neurons in the olfactory system. In the present study, we showed that IGF-IR mRNA exists in a subset of the mouse OSNs as well as in the rat OSNs. In the rat, mRNA encoding IGF-I and IGF-IR exist in the OB, and IGF-I promotes growth of the human OE has also been reported to contain IGF-I. In cultured olfactory neurons, IGF-I is a chemoattractant for axon growth cones. Therefore, it is possible that IGF-I generated in the OB and/or OE activates OSNs via the IGF-IR.

Mitril cell layer formation is disrupted in the olfactory bulb of IGF-I/−/− mice. In chick olfactory mucosa, IGF-I infusion suppresses the apoptotic cell death of OSNs, as well as transient effects on the maintenance of differentiation and mitosis stimulation of basal cells after olfactory axotomy. In the adult rat, only a few glomeruli were IGF-IR-positive, some of which were unusually small and strongly labeled. Some IGF-IR-positive fibers penetrated deeper into the external plexiform layer, even in adults. In developing tissues, IGF-IR staining co-localized with that for OMP and GAP43. TRPV2 has been identified as a channel regulated by IGF-I through PI3K and the Akt signaling axis in ectopically expressed mammalian cell lines. We have demonstrated that the distribution of the IGF-IR mRNA is consistent with the localization of TRPV2 mRNA-expressing neurons in the OE.

IGF-I induced increases in [Ca\(^{2+}\)]\(_e\), via the translocation of TRPV2 from intracellular pools to the plasma membrane in Chinese hamster ovary (CHO) cells expressing TRPV2. It is possible that IGF-I regulates both the rapid traveling of TRPV2 into axons of olfactory neurons and the local elevation in cytoplasmic calcium levels of olfactory axons and that TRPV2 mediates a mitotic, differentiation, and survival cues from IGF-I in the OSNs. It is particularly interesting that IGF-I has been shown to promote peripheral axonal regeneration in sensory neurons in vitro and in vivo. These findings illustrate that TRPV2 is an IGF-I-operated cytoplasmic calcium ion regulatory channel for axonal growth, and is required for the regeneration of sensory neurons expressing TRPV2, although further investigations of the role of TRPV2 against neurite outgrowth in vivo and in vitro are needed.

**TRPV2 Is Expressed in Developing Olfactory Neurons** TRPV2-expressing cells have been found in dorsal root ganglia during fetal development. In this study, we investigated TRPV2 expression in the olfactory system from E10.5 to E17.5. TRPV2-immunopositive axons existing from the presumptive OE were detected as early as E12.5. TRPV2 immunopositive neurons were co-labeled with βIII-tubulin starting from E12.5 and OMP starting from E14.5, and this showed TRPV2 protein is expressed in immature and mature olfactory neurons, and is localized in axons of the developing OSNs. The early stages of OE development in the mouse have been described at the morphological level. The placodes invaginate, forming the olfactory pits, and OSNs start to grow axons at E10.5. Proliferating OSN progenitors expressing Mash1 and proliferating OSN precursor expressing NeuroD emerge in developing OE at ca. E11.5. The OE becomes organized in layers around E11.5–12.5, with OSN progenitors becoming progressively localized to the basal side of the epithelium and post-mitotic neurons to an intermediate zone.

Olfactory pioneer axons first reach the OB anlagen at the anterior tip of the telencephalon at E12.5 and as shown in Fig. 5b by staining with βIII-tubulin in the anterior tip of the forebrain. From E14.5 to E17.5, axodendritic synapses start to form on the dendritic growth cones of mitral cells. We observed no significant signals in the anterior tips of the embryonic forebrain, prospective OB, at E12.5 and E14.5. Together, the spatiotemporal TRPV2 expression in the embryonic nasal region correlates with the growth and maintenance of olfactory axons but not with the commitment, differentiation, migration, and propagation of olfactory progenitor (Mash1-positive) and precursor (NeuroD-positive) cells, or with the formation of synaptic connection in the developing OB. IGF-IR immunoreactivities are found in the olfactory nerve layer of the rat OB at E18 and 19. Therefore, it is possible that TRPV2 coupled with IGF-IR also concerns with growing olfactory axons in the OSNs during development as similar to during regeneration. Obviously, further experimental studies are needed to verify this hypothesis.

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