Application of layer-specific markers in the evaluation of abnormal cytoarchitecture in the olfactory bulb of prokineticin receptor 2 deficient mice

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Abstract

Prokineticin receptor 2 gene mutant mice (Pkr2<sup>+/−</sup>) show deformity in the olfactory bulb (OB), they lose axonal invasion from the olfactory sensory neurons and olfactory interneurons fail to migrate in the OB. In the present study, we examined whether the mutant has an abnormality in the laminar structure in the OB by HE staining and immunohistochemistry using anti-tyrosine hydroxylase (TH), parvalbumin (PV), and reelin (RE) with special reference to the layered structure. In Pkr2<sup>+/−</sup> mice, TH-immunoreactive neurons were localized in the marginal area of the OB and the number and size of TH-expressing cells were markedly decreased. The distribution of PV-immunoreactive cells showed no apparent difference but the cells were significantly smaller in the mutant is wild-type mice (WT). RE-immunoreactive mitral cells showed abnormal alignment, the direction of neurite outgrowth was not well coordinated and they were much smaller than those in WT mice. The present findings suggest that not only the impairment of neuronal migration but also the loss of afferent axonal projection from the olfactory epithelium to the OB arrested the maturation and induced apoptosis of PV-, TH-, and RE-immunoreactive neurons in Pkr2<sup>+/−</sup>.

Key words: olfactory bulb, prokineticin, tyrosine hydroxylase, parvalbumin, reelin

Introduction

The olfactory bulb (OB) is located in the most rostral region of the brain and is involved in olfaction. The OB consists of two apparently distinct parts. The main olfactory bulb (MOB) occupies a large part of the OB and receives projections from olfactory neurons, receiving most of the odor signals while the accessory olfactory bulb (AOB) senses pheromones, receiving projections from the vomeronasal organ.

The MOB shows apparent layers: olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), and granular cell layer (GrL), from the surface to the center.1

Prokineticin is composed of Pkr1 (also named EG-VEGF) and Pk2 (also named Bv8). These are multifunctional secreted proteins with 10 cysteine and a molecular weights of 8kDa.2-4 The receptors for these PKs are Pkr1 and Pkr2,
both of which are G-protein coupled receptors, mediate signal transduction.\textsuperscript{2,4} The influences of PKs through the activation of PK receptors (PKRs) on several physiological events in the central and peripheral nervous systems, including intestinal contraction,\textsuperscript{5} hyperalgesia,\textsuperscript{6} spermatogenesis,\textsuperscript{7-9} neuronal survival,\textsuperscript{10} circadian rhythm,\textsuperscript{11,12} angiogenesis,\textsuperscript{13,14} digestive behavior,\textsuperscript{15} and hematopoiesis\textsuperscript{16,18} have been reported. Recently, the importance of Pk2 and its receptor Pkr2 in the development of the olfactory bulb has been demonstrated using Pk2- and Pkr2-deficient mutant mice. The Pk2-/- mice showed asymmetric deformity in the OB.\textsuperscript{17} Pk2 and Pkr2 mRNA are strongly expressed in the OB.\textsuperscript{18} Pkr2-/- mice showed symmetric OB reduction not only in the embryo but also in adult mice.\textsuperscript{9} Pkr2-/- mice lack axonal projection from the olfactory neurons to their targets in the MOB and, therefore, lack glomeruli in which axonal terminals of olfactory neurons make synaptic contacts on dendrites of the mitral cells.\textsuperscript{9} The above results suggest that the Pk2-Pkr2 system plays a crucial role in OB development.

In our present study, we examined whether the layered structure of the MOB is preserved in Pkr2 mutant mice. The MOB is known to have various chemical substances. Many chemical substances mark various types of neurons and layers in the OB. We examined the localization of reelin (RE),\textsuperscript{18} parvalbumin (PV)\textsuperscript{19,20} and tyrosine hydroxylase (TH)\textsuperscript{21,22} expressing neurons that are confined to one or a few layers in the MOB of adult mice.

### Materials and Methods

#### Pkr2 Gene-disrupted mice

Pkr2 disrupted mice were originally described by Matsumoto et al. in 2006.\textsuperscript{9} The original genetic background was changed from C57BL/6 strain to ICR strain (CLEA Japan, Tokyo) by 10 times backcross mating with the ICR strain to increase the ratio of the survival of neonates since the ratio of survival with the original C57BL/6 was very low. All experiments were performed in compliance with the regulations of the Animal Ethics Committee of Kinki University School of Medicine.

#### Immunohistochemistry

As the cerebral cortex in Pkr2-/- mice does not have any apparent deformities, we determined one plane by using the position of the rostral tip of the frontal lobe. The cutting plane is symmetrical in the right-to-left axis, vertical to the basal plane of the mouse brain, and is in tangential contact with the rostral tip of the frontal lobe (Fig. 1). The specificity of the anti-TH, anti-PV, and anti-RE antibodies used in the experiments has been described previously.\textsuperscript{23,24} Eight-week-old male wild-type mice (WT) and mutant were used for this study. Animals were anesthetized with the ether prior to transcardinal perfusion with 4% paraformaldehyde (PFA). The brain was removed from the skull, fixed with 4% PFA and then embedded in paraffin. The paraffin-embedded sections (40μm) were deparaffinized, and immunostained with mouse anti-TH antibody (1:2000; Millipore, Germany), mouse anti-PV antibody (1:4000; Millipore), and mouse anti-RE (1:2500; Calbiochem, Germany) as described below. The sections were activated by citric buffer (10 mM citric acid, pH 6.0) for 20 min at 95°C using a microwave, and then methylated by 3% H2O2 in 50% methanol for 30 min at 4°C. The sections were immersed in the primary antibodies (TH, PV and RE) overnight at 4°C. Subsequently, the sections were processed according to the avidin-biotin-peroxidase (ABC) method using Vectorstain ABC Kits (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After rinsing in PBS and 0.05 M Tris-HCl buffer (pH 7.4), antigens were visualized using 0.035% diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) in 0.05 M Tris-HCl buffer, containing 0.01% H2O2 for 5-10 min at room temperature. To identify the cytoarchitecture of the OB, the sections were counterstained with hematoxylin and eosin (HE).

#### Quantification of cell size

Images were observed under a microscope (BX43; Olympus, Tokyo, Japan) and size were captured by a CCD camera (DP25; Olympus). Twenty cells were chosen randomly per animal (n=3 in each phenotypes) and the cell body was measured using CellSens software (Olympus).

Results are expressed as the mean ± SEM. Differences between groups were examined for statistical significance using Student’s t-test.

### Results

#### Macroscopic observation

The OBs in the coronal section through the rostral end of the cerebral cortex, as defined in
Fig. 1  Schematic view of the olfactory bulb. To compare the cytoarchitectural structure of the olfactory bulb (OB) between wild-type (WT) and Pkr2−/− mice, the reference level is shown by a vertical dashed line. The reference level is the coronal section through the rostral end of the cerebral cortex and is vertical to the longitudinal antero-caudal axis of the brain. Other abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GrL, granule cell layer; GI, glomerulus.

Fig. 1, were compared between WT and Pkr2−/− mice (see Materials and Methods). All of the OB of WT were oval (Fig. 2a), whereas the OB of Pkr2−/− were rather round (Fig. 2d).

HE staining

In WT mice, the olfactory glomerulus was easily recognized by the oval neuropil containing a few cell bodies inside and densely packed cell bodies surrounding the vacant structure1 (Fig. 2c). The EPL was a cell sparse layer below the glomerular layer and the width of EPL was generally constant in the medial region (Fig. 2a, b, c) of the OB. The MCL was linearly arranged at the boundary between the EPL and the IPL (Fig. 2a, b, c). IPL is a cell-sparse narrow layer localized between the MCL and GrL (Fig. 2c). In the GrL, small cells were densely distributed, making tightly packed small clusters (Fig. 2c).

In Pkr2−/− mice, the outermost layer of the OB was recognized by the condensation of cell bodies, but the typical vacant oval structure of the olfactory glomerulus was not observed, so it was named the pseudo-glomerular layer (Fig. 2f). The EPL was also recognized even in the Pkr2−/− mice by its scarcity of cell bodies (Fig. 2f). Beneath the EPL, large clustered cells were present. The triangular shape of the somata of these cells suggests that they were mitral or tufted cells. Assuming that these cells are mitral/tufted cells, cells were smaller than those of WT mice (Fig. 2f). The extension of apical dendrites of mitral/tufted-like cells was not uniform as recognized in WT (Fig. 2c, f). Within the clustered mitral cells, there were small neurons packed as in the GrL but the IPL could not be distinguished (Fig. 2f). In the GrL, cells were well packed (Fig. 2f) and clusters of cells were also recognized; however, in Pkr2−/− mice, many neurons were strongly stained with hematoxylin. Most of the strongly stained neurons were small compared with the granule cells of WT mice.

Fig. 2  Hematoxylin-eosin (HE)-stained coronal sections of olfactory bulb (OB) of WT (a-c) and Pkr2−/− (d-f) mice. The rectangles in a and b are enlarged in b and c, respectively, and the rectangles in d and e are enlarged in e and f, respectively. In the OB of the mutant mouse, neither olfactory nerve layer (ONL) nor olfactory glomeruli (GL) were identified. Some small cells are scattered in the superficial layer corresponding to ONL, which is named the pseudo-glomerular layer (Pseudo-GL) in the present study. Large mitral cells are widely scattered, and therefore the mitral cell layer (MCL) does not form a unicellular arrangement as seen in the normal OB. The internal plexiform layer (IPL) is not identified in the mutant OB. Other abbreviations: EPL, external plexiform layer; IPL, internal plexiform layer; GrL, granule cell layer; GI, glomerulus; pseudo-GL, pseudo-glomerular layer. Figure 2c and 2f were rotated 90 degrees to the left from Figure 2b and 2e.

Scale bars: 500 µm (a, d); 200 µm (b, e); 50 µm (c, f).
Tyrosine hydroxylase (TH)-immunoreactive neurons
In WT mice, TH-immunoreactive neurons were densely present in the GL, surrounding the glomerulus (Fig. 3a, b) and immunoreactive neural fibers were observed inside the glomerulus. The strongly stained neural fibers were thick and well packed (Fig. 3b). In Pkr2−/− mice, a few TH-immunoreactive neurons were observed in pseudo-GL (Fig. 3c, d). The immunoreactive fibers were not well extended from the cell bodies and were sparse. The number and size of TH-immunoreactive neurons were apparently decreased (Fig. 3a, b, c, d).

Parvalbumin (PV)-immunoreactive neurons
In the MOB of WT mice, PV-immunoreactive neurons were present in the EPL as reported previously19,20 (Fig. 4a, b). A small number of weak PV-immunoreactive neurites were observed in the EPL (Fig. 4b). In Pkr2−/− mice, PV-immunoreactive neurons and neurites were also present in the EPL (Fig. 4c, d).

Reelin (RE)-immunoreactive neurons
In WT mice, RE was expressed by mitral cells in the MCL, tufted cells in the EPL and some...
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subsets of periglomerular neurons at the bottom of the GL (Fig. 5a, b). Mitral and tufted cells showed moderate to strong staining, but periglomerular cells showed moderate staining. In WT, RE-immunoreactive neurons were arranged in a regular row, and the neurite outgrowth has rather consistent direction (Fig. 5a, b, c). In Pkr2−/− mice, RE-immunoreactive neurons were also localized in both EPL and MCL but the linear arrangement of the RE-immunoreactive neurons and the consistency in the direction of neurite outgrowth was disrupted (Fig. 5c, d, e).

Quantitative analysis of cell sizes

We measured the cell bodies of TH-, PV- and RE-immunopositive neurons in the OB of normal and mutant mice and found that, in all immunoreactive neurons examined, the cell bodies were smaller in Pkr2−/− mice than in their normal counterparts. The mean size of TH-immunoreactive cell bodies located in the GL was 31.9±1.0 μm² (Mean ±SEM) in Pkr2−/−, significantly smaller than those (66.2±2.1 μm²) of WT (Student’s t test ; p<0.001). PA-immunoreactive neurons in the EPL were also significantly smaller (p<0.001) in Pkr2−/− (25±1.2 μm²) than in WT (44.2±3.2 μm²). RE was expressed in mitral cells, tufted cells and periglomerular cells, as described above, which have apparently different mean sizes of cell bodies. Therefore, we tried to identify mitral cells and to measure the cell bodies. Even in Pkr2−/−, the MCL was distinct from the EPL and pseudo-glomerular layer so that we were able to differentiate mitral cells by their specific location in the MCL and large cell bodies from other RE-immunopositive neurons. We measured the mean cell bodies of RE-immunopositive mitral cells in WT (247.5±5.8) and in Pkr2−/− (120.5±6.3), suggesting that

**Fig. 5** Distribution of reelin (RE)-immunoreactive neurons in coronal sections of olfactory bulb (OB) in WT (a, b, c) and Pkr2−/− (d, e, f) mice. Rectangles in a and d are enlarged in b and e, respectively. And rectangles in b and e are enlarged in c and f, respectively. The black arrows indicate RE-immunoreactive mitral cells, and black arrowheads indicate RE-immunoreactive periglomerular neurons. The white arrows and arrowheads indicate the neurites. In the normal OB, RE-immunopositive mitral cells are arranged in a laminar fashion and showed consistent direction of neurite outgrowth, whereas in the mutant OB they do not show any laminar fashion or the consistency in the direction of neurite outgrowth. Other abbreviations : GL, glomerular layer ; EPL, external plexiform layer ; MCL, mitral cell layer ; IPL, internal plexiform layer ; GrL, granule cell layer ; pseudo-GL, pseudo-glomerular layer. Figure 5b and 5e were rotated 90 degrees to the left from Figure 5a and 5d. Figure 5e and 5f were rotated 90 degrees to the right from Figure 5b and 5e. Scale bars, 200 μm (a, d) ; 50 μm (b, c, e, f).

**Fig. 6** The diagrams show the cell body size of tyrosine hydroxide (TH)-, parvalbumin (PV)- and reelin (RE)-immunopositive neurons in WT (gray bar) and Pkr2−/− (open bar) mice. Cell bodies size of 60 immunopositive neurons (20 neurons per slice ; n=3 for each phenotypes) were measured for each primary antibody. Values are the represent means±SE (n=3). TH-, PV-, and RE-immunopositive neurons were statistically smaller in the mutant OB compared with their normal counterparts (**p<0.001).
cell bodies of RE-immunopositive mitral cells were also significantly smaller in Pkr2−/− than in the WT.

Discussion

The present HE-stained sections demonstrated that mutation of the Pkr2 gene affects the laminar structures of the OB. The glomerulus was almost completely lost and mitral cells were not aligned in a row. In the GrL, chromatin condensation in the nuclei of granule cells suggests that many granule cells in the OB of Pkr2−/− mice undergo apoptosis. This is also supported by previous studies that demonstrated an increased number of apoptotic neurons by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate UTP nick-end labeling (TUNEL) analysis.17,25 Granule cells in the GrL of the OB make a dendro-dendritic synaptic connection with dendrites of mitral and tufted cells,26 therefore, it is possible that the impairment of granule cells leads to impaired dendrodendritic synaptic formation between granule cells and mitral cells, and triggers the cell death of granule cells. Therefore, the decreased volume of OB in Pkr2−/− mice could be attributed not only to the erroneous migration of interneurons26 but also to the increased number of apoptotic cell deaths of interneurons.

TH, a marker enzyme of dopaminergic neurons, is expressed by periglomerular neurons and external tufted cells situated in GL in the OB.27−29 In WT mice, strong TH-immunoreactive neurons were confined to the GL and TH-immunoreactive nerve fibers were clustered thickly in the glomerulus. The glomerulus was absent in the Pkr2−/− but a few TH-immunoreactive neurons were present in the pseudo-GL. Interestingly, TH-immunoreactive nerve fibers were also observed only in the pseudo-GL. This finding suggests that Pkr2 gene disruption does not affect the migration of TH-immunoreactive periglomerular neurons from their origin in the subventricular zone (SVZ) of the lateral ventricle to their final position, although we could not exclude the possibility that dopaminergic neurons that succeed in migrating to their final position can express TH immunoreactivity. PV, which is one of the neurochemical markers, is calcium-binding protein, and is expressed by neurons in EPL of the mice MOB. The present study has revealed that PV-immunoreactive neurons were distributed in the EPL in both WT mice and Pkr2−/− mice. This finding in WT mice is consistent with previous studies.20,20 PV-immunoreactive multipolar neurons, considered to be interneurons, may be generated in the SVZ, do not have any axons and make synaptic contacts with dendrites of mitral cells.29 In the present study, Pkr2 disruption did not seem to affect the distribution and cell number of PV-immunoreactive neurons, however, in the OB of Pkr2−/− mice, the cell size of PV-immunoreactive neurons was much reduced. These data suggest that Pkr2 gene disruption does not influence the migration of PV-immunoreactive neurons to EPL,29 but affects the maturation process of neurons, including enlargement of the cell size. PV-immunoreactive neurons have been reported to make synaptic contact with basal dendrites of mitral cells;29,35 therefore, the maturation process of PV neurons may be caused by the absence of normal retrograde factor from mitral cells. Since mitral cells appear to be abnormal in Pkr2−/−, it is possible that impaired synapse formation of PV neurons with mitral cells may affect the maturation process of PV neurons.

RE, the gene responsible for the reeler mutation, is a large extracellular matrix protein, expressed strongly by external tufted and mitral cells, and weakly in periglomerular cells in

### Table 1

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<th>RE</th>
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<td>401.4</td>
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<td>Mean</td>
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<td>44.2</td>
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The size of cell somata of TH-, PV-, and RE-immunopositive neurons in the OB of the normal (WT) and Pkr2-deficient mutant mice (Pkr2−/−).
In Pkr2-/- mice, we cannot distinguish mitral cells from tufted cells since the alignment of mitral cells is not clear, being scattered in a wider area, and the border between EPL and MCL is ambiguous. The findings suggest that synaptic formation of olfactory nerves with mitral cells is essential for the unicellular alignment of mitral cells. In addition, the present study clarified that tufted/mitral cells extend their neurites in almost random directions instead of a uniform direction, suggesting that the neurite outgrowth of tufted/mitral cells is regulated by synaptic contact between olfactory nerve endings and mitral cells. Some of the morphological phenotypes in Pkr2-/- mice are very similar to those of other mutant mice that lack axonal projection from the olfactory neurons. In fact, Arx, Dlx5 and fez1 (fez) mutant mice share abnormal features similar to those of Pkr2-/- mice. In these mutants, the ONL is absent due to the lack of projections from the olfactory neurons to the OB. The olfactory bulb is very small and the glomerulus is almost completely lost. The mitral cell layer is widened and mitral/tufted neurons marked by reelin expression are widely dispersed around the border of EPL and GrL. IPL is absent, possibly because of defects of the axonal extension of mitral cells. In contrast, interneurons in the OB are not necessary to arrange mitral cells in the OB. Dlx1/Dlx2 double knockout mice show a complete absence of GABAergic interneurons, including periglomerular cells and granular cells. The olfactory nerve layer was arranged neatly. Mitral cells did not show abnormal distribution, therefore, although impaired migration of olfactory interneurons of the OB also has been reported in Pkr2-/- mice, it is probable that the disorder of the layered structure is attributed to the loss of axonal projection from olfactory neurons. As Pk2-deficient mutant mice also show a deformity of the olfactory bulb, similar to those in Pkr2-/- mice, it is highly probable that the PK2 and Pkr2 ligand-receptor system is essential to make a normal layered structure. Thus, in mutants that show similar disorders, as shown in Pk2 and Pkr2 mutant mice, loss of axonal invasion may be the first event which causes the broad spectrum of abnormality in the olfactory bulb.

The reduction in the size of the neurons in Pkr2-/- mice observed in the present study could be attributed both to immature development of neurons and the apoptosis caused by impaired synaptic connection of the neurons. In the developing process of the neuron, the size of cell bodies of the neurons increases associated with synapse formation. And, moreover, the size of the neurons decreases in the process of apoptosis. Therefore, it is highly probable that the size reduction of the cell bodies was caused both by impaired development and apoptosis. We will further perform studies to investigate the maturation process of PV, RE, and TH-expressing neurons in future.

Conflict of Interest Statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript

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