Profile of tyrosine hydroxylase-expressing neurons in the olfactory bulb of prokineticin type 2 receptor-deficient mice during embryonic development

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Abstract

Dopamine neurons in the olfactory bulb play essential roles in the maintenance of olfactory function. The maturation of these neurons involves mitosis, cell migration and the transcriptional regulation of specific neurotransmitters and is an intensively studied process. In our present study, we investigated the embryonic development of tyrosine-hydroxylase (TH)-expressing neurons in the main olfactory bulb (MOB) of Pkr2-/- mice that lack axonal infiltration from the olfactory neurons (ON) and wild-type littermates (WT) at E16.5, E18.5 and P0 using immunohistochemical techniques. Even at E16.5, we observed two populations of neurons in the MOB of WT mice, one scattered in the primitive glomerular layer and another population of TH-positive neurons in the surface region of the primitive granular cell layer. The number of TH-positive neurons had increased markedly by P0. Pkr2-/- mouse embryos and neonates showed disorganization of the layered structure, mainly due to the loss of glomerular layer in the olfactory system. The number of TH-immunoreactive cells per slice was markedly lower at P0 but not at E16.5 and E18.5. Interestingly, even in Pkr2-/- mice, TH-immunoreactive neurons were still present in the periphery of the MOB. Hence, it is highly probable that the lack of Pkr2-/- does not affect the migration of TH-containing neurons from the subventricular zone to the periphery of the MOB during embryonic development but strongly impairs the migration of TH-expressing neurons or TH expression in the MOB around birth.

Key words: olfactory bulb, tyrosine hydroxylase, prokineticin, periglomerular cell, embryo, dopamine, tufted cell

Introduction

The olfactory bulb (OB) is the most rostral region of the brain and is generally divided into two parts. The main OB (MOB) occupies a large part of the OB and receives projections from the olfactory neurons, receiving most of the odor signals, whilst the accessory OB receives projections from the vomeronasal organ and plays the principal role in sensing pheromones. The MOB has the appearance of a layered structure comprising a glomerular cell layer, external plexiform layer, mitral cell layer, internal plexiform layer, and internal granular cell layer, from the surface to the center.

Dopaminergic neurons in the MOB play crucial roles in the maintenance of olfactory function. In the matured MOB, dopamine-contain-
ing neurons exist almost exclusively in the glomerular layer.\textsuperscript{4,5} Two types of neurons, periglomerular and tufted neurons, are present in the glomerular layer and contain dopamine as a neurotransmitter.\textsuperscript{4} Tyrosine hydroxylase (TH) is one of the rate-limiting enzymes in dopamine synthesis and is regarded as a marker of dopamine-producing neurons in the MOB.\textsuperscript{6} TH expression in the MOB depends on axonal innervation from olfactory neurons. Deafferentation causes widespread downregulation of TH transcription in the neurons in the glomerular layer, suggesting that afferent projections from the olfactory nerve are essential drivers of TH expression\textsuperscript{37-40}. Although TH expression in the MOB is strongly influenced by projections from olfactory neurons, it remains to be investigated whether all of the neurons expressing TH are dependent on innervation from the olfactory neurons.

The prokineticins (PKs), PK1 (also known as EG-VEGF) and PK2 (also denoted Bv8), are secreted bioactive proteins.\textsuperscript{12-14} Two endogenous prokineticin receptors, termed Pkr1 and Pkr2, are members of G-protein coupled receptors that initiate signal transduction by PKs.\textsuperscript{12-14} Pkr1 shows preferential distribution in the peripheral tissues, whereas Pkr2 expression is mostly confined to the central nervous system. From previous analysis using receptor transfected mammalian cell lines, PK2 binds prokineticin receptors (PKR) with higher affinity than PK1, suggesting that PK2 is a stronger agonist of the PK/PKR system under physiological condition.\textsuperscript{12-14} Pkr2-deficient mutant (Pkr2\textsuperscript{−/−}) exhibited hypoplastic OBs and hypogonadism due to the lack of GnRH neurons in the hypothalamus,\textsuperscript{16,16} whereas Pkr1\textsuperscript{−/−} exhibited no apparent morphological abnormality.\textsuperscript{16} Pkr2\textsuperscript{−/−} mice fail to form afferent projections from olfactory neurons to their targets in the MOB and therefore lack a glomerulus in which axon terminals of olfactory neurons and dendrites of mitral cells form synapses.\textsuperscript{12,18} In our present study, we analyzed the embryonic development of TH-expressing neurons in the MOB of wild-type (WT) mice. In addition we compared the OB of Pkr2 mutant mice and control WT littermates to investigate whether loss of axon terminal projection from olfactory neurons affects TH expression in the OB.

### Experimental Procedure

#### Experimental animals

The generation of Pkr2\textsuperscript{−/−} mice is described by Matsumoto \textit{et al.}\textsuperscript{16} Originally, the background of the mice was C57/BL but, with the low survival ratio of neonates, the genetic background was changed. The colony used in the present study was established by 11 backcrosses with C57BL/129SvJae (Japan, Tokyo). The resulting strain was subsequently maintained by interbreeding for at least 10 generations. Genotypes were determined using an established PCR method. For comparison, embryos and neonates of WT and mutant mice (n = 3) at E16.5, E18.5 and P0 were collected. This study was performed in compliance with the Rules and Regulations of the Animal Care and Use Committee of Kinki University School of Medicine, and adhered to the Guide for the Care and Use of Laboratory Animals, Kinki University School of Medicine.

#### Tissue preparation

The day on which a check plug was evident was counted as embryonic day 0.5 (E0.5) and the day of birth was defined as postnatal 0 (P0). Pregnant mice were deeply anesthetized using ether and the embryos were removed by cesarean section. E16.5 and E18.5 fetuses were fixed in 0.1 M PB, pH 7.4, containing 4% paraformaldehyde (PFA) at 4°C for 24 h. P0 neonatal mice were anesthetized by hypothermia and intracardially perfused with 4% PFA. The brains were then removed and immersed in the same fixative for 24 h at 4°C. Subsequently, embryonic and neonatal mice were blocked in the coronal plane and embedded in paraffin. The paraffin-embedded brains were sectioned at 4 μm thickness in the frontal plane of the entire OB and mounted on silane-coated slides.

#### Immunohistochemistry

The specificity of the anti-TH antibodies used in the experiments has been described previously.\textsuperscript{19} As the cerebral cortex of Pkr2\textsuperscript{−/−} 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).

Sections were deparaffinized in xylene and rehydrated in an ethanol series. For antigen retrieval, the slides were immersed in citrate
buffer (pH 6) and heated in a microwave oven for 15 min. The slides were then bleached for 30 min at 4°C with 50% methanol containing 1.5% H2O2 to block endogenous peroxidase activity. The sections were next incubated with a primary antibody against TH (MAB318; Merck Millipore, Germany) at 4°C overnight (diluted 1:2000 in PBS containing 0.3% Triton X-100). After rinsing with PBS, the sections were incubated in biotinylated anti-mouse IgG (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBS at room temperature for 1 h, washed with PBS and incubated in avidin-biotin complex (Vectastain ABC kit) diluted 1:500 in PBS at room temperature for 1 h. After rinsing with PBS, the sections were washed with 0.05 M Tris-HCl buffer, pH 7.4 and treated with 0.035% diaminobenzidine (DAB) and 0.05 M Tris-HCl buffer in the presence of 0.003% hydrogen peroxide for 10-15 min at room temperature. After the DAB reaction, the samples were rinsed with 0.05 M Tris-HCl buffer, stained with hematoxylin and dehydrated with a graded series of ethanol, immersed in xylene, and embedded in Entellan (Merck, Darmstadt, Germany).

**Statistical analysis**

The cell size and distribution patterns were determined using ImageJ analysis software. To compare cell size, we measured the length of the short axis of the cells. The number of TH-immunoreactive neurons per slice was counted manually under a microscope. Results are expressed as the mean ± SEM. Differences between groups were examined for statistical significance using two-way ANOVA (analysis of variance) and individual values were analyzed for significant differences using one-way ANOVA Tukey’s post hoc test.

**Results**

**Analysis of developing olfactory structures in mice using hematoxylin and eosin stained sections**

At E16.5, the MOB in WT mouse embryos shows evidence of layered structures. In the MOB, the olfactory nerve layer, consisting of olfactory neuron axons, is already established at this stage (Fig. 2A). The external plexiform layer and mitral cell layer are also recognized, although they are in an immature state. The glomerular layer and external plexiform layer are not well separated by E16.5 so the outer most...
layer in OB should be named the primitive glomerular and external plexiform layer, as described previously. In addition, the internal plexiform layer and internal granular layer are also not well divided and the layer is referred to as the intermediate zone. In the central area, there is a subependymal zone distinguished by its typical radial cell arrangement that has been reported to contain proliferating neurons.

At E18.5 of WT (Fig. 2B), the appearance of an established mitral cell layer was the most striking observation. Mitral cells are distinguishable from other cells by their larger size and were found to be arranged in a line. Due to the formation of the mitral cell layer, the glomerular/external plexiform layer became distinguishable by E18.5.

At P0 in WT mice (Fig. 2C), all of the layers in the adult brain besides the internal plexiform layer were distinguishable; however, the structure of the glomerulus was found to be immature as we did not observe the spherical shapes that are typical of this structure and the internal plexiform layer was not discernible at this stage.

In the MOB of the Pkr2−/− mouse embryo, the layered structure including the olfactory nerve layer was lost at all of the developmental stages examined; however, a cell-dense area surrounding the ventricle and a gradient of cell density from the center to the periphery was observed throughout development in the mouse (Fig. 2D-F). At all of the ages, the subependymal zone, distinguished by its typical radial cell arrangement, was also observed in both Pkr2−/− as well as in WT mice (Fig. 2A, B, D, E; Fig. 5A, D). Cell density was high in the central region, and low in the periphery of the MOB.

Analysis of TH-positive neurons in the OB of wild-type littermates and Pkr2−/− mice

In the MOB of WT mice at E16.5, TH-immunopositive cells were observed. These cells were generally divided into two populations (Fig. 3A-C). Strongly stained TH-immunoreactive neurons were found to be scattered in the primitive glomerular and external plexiform layer. Moreover, a few weakly stained TH-immunoreactive neurons were detected in a scattered pattern in the intermediate zone. In Pkr2−/− mutant mice (Fig. 3D-F), TH-immunoreactive neurons were scattered from the core to the surface, with the exception of the subependymal zone.

At E18.5 in WT mouse embryos, strongly stained TH-immunopositive neurons were found to be scattered (Fig. 4A-C). Immunoreactive fibers were barely detectable, but a small number of neurons showed weakly labeled neural fiber extensions. In the internal granular layer, a few moderately stained small neurons were detected. In Pkr2−/− embryos, TH-immunopositive neurons were found to be scattered in all layers apart from the subependymal zone, in a randomly distributed pattern that was similar to that observed at E16.5 (Fig. 4D-F).

At P0 in WT mice, most of the TH-immunopositive neurons were localized in the glomerular layer and the majority of TH neurons were arranged in a line in the glomerular layer (Fig. 5A-C). The neurons were larger than at E16.5 or E18.5 (Fig. 6A-C). Weakly labeled smaller neurons were also recognizable in the granular layer. In Pkr2−/− neonatal mice, TH-immunopositive neurons were scattered and did
not show a linearized arrangement; however, most of these cells were generally situated in the periphery of the MOB, close to its edge in an area that should be the glomerular layer (Fig. 5D-F). No apparent increase in cell size was observed (Fig. 6D-F). The glomerular layer was not easily detectable in PKR2 mutants because the cell-sparse region, the external plexiform layer, which is recognizable beneath the linearized TH-immunopositive neurons in the MOB of WT embryos, was lacking. In both WT and mutant embryos at P0, TH-immunopositive fibers were found to be abundant. WT-immunoreactive fibers were mainly extended to the outer side of the cell bodies (Fig. 5C). In contrast, TH-immunoreactive neural fibers were found to be extended randomly in mutant mice (Fig. 5F).

**Statistical analysis of cell size and number**

We performed ANOVA to determine whether there were significant differences in the number and size of TH-immunopositive neurons per tissue section between WT and Pkr2 mutant mice. Using Tukey's multi-comparison analysis, we did not find any differences between the number of TH-immunopositive neurons in WT and Pkr2<sup>−/−</sup> embryos at E16.5, but a significantly larger number of these cells was found in WT embryos both at E18.5 (<i>P</i> < 0.05; Fig. 7A) and at P0 (<i>P</i> < 0.01; Fig. 7A). Using ANOVA, we found that the size of the TH-immunopositive neurons was significantly greater in WT at P0 but not at E16.5 or E18.5 (<i>P</i> < 0.001; Fig. 7B).

**Discussion**

In our present study, we found that TH is expressed by E16.5 in the primitive glomerular and external plexiform layer and intermediate zone of the mouse embryo. This finding is consistent with previous studies in mice that
Table 2 Average size of TH-immunopositive neurons during development (Mean±SEM) 60 neurons in three slices (normal 3; mutant 3) were measured for statistical analysis.

<table>
<thead>
<tr>
<th>Age</th>
<th>Mean length (µm)±SEM</th>
<th>WT</th>
<th>Pkr2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>E16.5</td>
<td>6.56 ±0.11</td>
<td>6.54 ±0.1</td>
<td></td>
</tr>
<tr>
<td>E18.5</td>
<td>6.79 ±0.20</td>
<td>6.22 ±0.2</td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>9.06 ±0.29</td>
<td>7.33 ±0.3</td>
<td></td>
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</tbody>
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Fig. 7 Cell number per slice (A) and length of the short axis (B) of TH-immunoreactive neurons in the OB of WT and Pkr2−/− mice. Values are the mean ±SEM (n = 3). *P<0.05, **P<0.01; Tukey's multi-comparison analysis (B) values are the mean ±SEM (n = 60). ***P<0.001.

have shown that TH mRNA and protein expression are detectable by E16.5 in the primitive glomerular and external plexiform layer and outer portion of the intermediate zone,25,26 providing a clear contrast to the observation in rats that TH-immunoreactive neurons are recognized in the MOB only after birth.27,28

TH-immunoreactive neurons showed virtually no increase in number from E16.5 to E18.5 in our present analysis but demonstrated a marked increase by P0. Further, a difference in the number of TH-immunoreactive neurons between Pkr2−/− and wild-type embryos became apparent at P0. These observations suggest that two types of TH-immunoreactive neurons with distinct developmental processes are present in the OB; one appearing by E16.5 and the other around birth. Previous studies have also demonstrated that two types of dopaminergic neurons, tufted
Development of TH Neurons in Pkr2 Mutant Mice

and periglomerular cells (PGC), are localized in the glomerular layer. In mice, tufted cells develop between E13 and E18 locally in the subventricular zone (SVZ) of the primitive OB, whilst PGCs in OB develop later in the SVZ, and appear in the glomerular layer around P1. Hence, it is probable that TH-expressing neurons in the glomerular layer of embryonic OB at E16.5 are external tufted cells, and that a rapid increase in PGCs follows their development.

Pkr2-/- and WT mice showed no difference in the number and cell size of TH-immunoreactive neurons at E16.5, suggesting that the mutation hardly affects the phenotype of TH-immunoreactive neurons by this age. The Pkr2-/- mouse phenotype includes a complete loss of axon invasion arising from olfactory neurons into the OB. In WT mice, axons from olfactory epithelial neurons invade the OB from E14 and begin to form synapses in the glomerular layer. Furthermore, in the glomerular layer, the number of synapses formed between olfactory neuron axons and mitral/tufted neurons markedly increased from E15, therefore, it is highly probable that TH mRNA and protein expression at E16.5 and E18.5 in the MOB are independent of the innervation of projections from olfactory neurons. Taken together, the present findings suggest that mitosis, migration, and TH transcription in TH-immunoreactive neurons are maintained even in Pkr2-/- in embryos.

In contrast, the effects of a Pkr2 mutation became apparent around birth. The number of TH-immunoreactive cells increased markedly around the time of birth in WT mice but not in Pkr2-/- mice. What causes the difference in cell number between WT and Pkr2-/- mice? Most of the increase in neurons in WT mouse is possibly due to the increase of PGCs that arise later than external tufted cells. Previous studies have shown that cell proliferation is not impaired in the SVZ of cerebral hemispheres in pkr2-/- mice. In WT mice, neurons expressing Pkr2 are observed both in the SVZ and rostral migratory stream (RMS). These earlier findings together suggest that Pkr2 is necessary to enable primordial neurons to leave the RMS to a more superficial position of the OB. It is therefore possible that, in Pkr2-/- mice, neuronal migration from the SVZ via the RMS to the OB is impaired due to the lack of Pkr2; however, whether dopamine neurons in the OB express Pkr2 remains to be elucidated. One other possibility is that a decrease in TH expression is due to the suppression of TH transcription, which, in PGCs, is also regulated by unknown factors possibly from the olfactory nerve layer. Previous reports demonstrated that denervation of olfactory nerve projections and naris closure produce a dynamic decrease in TH expression; however, in both of these cases, the dopamine neurons survive and localize in circumference areas of the OB, which suggests that migration from the SVZ via the RMS to the periphery of the OB is preserved even after loss of afferent projections from olfactory neurons. Further studies are necessary to elucidate the fate of dopamine neurons in the OB of Pkr2-/- mice.

We have previously reported that Pkr2-/- mice exhibit hypogonadotropic hypogonadism. Therefore, it is possible that the hypogonadism have some effects on the morphological abnormality of the OB. However, a previous study suggests that it is unlikely that the hypogonadotropic hypogonadism contributes to the abnormal formation of the OB. In the Ebf2-/- mice that also develop hypogonadotropic hypogonadism, the authors found no abnormality in the OB. The finding suggests that the hypogonadotropic hypogonadism in Pkr2-/- mice was not involved in the formation of the abnormal OB.

Interestingly, TH-immunoreactive neural fibers extend from TH-immunoreactive neurons in the deep layer of the glomerular layer toward the outer region of the MOB. This suggests that some attractants are present in the outer superficial layer of the glomerular layer or olfactory nerve layer. In Pkr2 mutant mice, TH-immunostained fibers were observed to extend randomly around the neurons, suggesting that the appropriate axon guidance or preservation of axonal architecture of the dopamine neurons requires input from olfactory neurons and, possibly also synapse formation between olfactory neuron axons and mitral/tufted cells.

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References


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