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Expression of Maf family proteins in glutamatergic neurons of the mouse olfactory bulb

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Abstract

The fate of neurons in the developing brain is largely determined by the combination of transcription factors they express. In particular, stem cells must follow different transcriptional cascades during differentiation in order to generate neurons with different neurotransmitter properties, such as glutamatergic and GABAergic neurons. In the mouse cerebral cortex, it has been shown that large Maf family proteins, MafA, MafB and c-Maf, regulate the development of specific types of GABAergic interneurons but are not expressed in glutamatergic neurons. In this study, we examined the expression of large Maf family proteins in the developing mouse olfactory bulb by immunohistochemistry and found that the cell populations expressing MafA and MafB are almost identical, and most of them express Tbr2. Since Tbr2 is expressed in glutamatergic neurons in the olfactory bulb, we further examined the expression of glutamatergic and GABAergic neuronal markers in MafA and MafB positive cells. The results showed that in the olfactory bulb, MafA and MafB are expressed exclusively in glutamatergic neurons, but not in GABAergic neurons. We also found that few cells express c-Maf in the olfactory bulb. These results indicate that, unlike the cerebral cortex, MafA and/or MafB may regulate the development of glutamatergic neurons in the developing olfactory bulb. This study advances our knowledge about the development of glutamatergic neurons in the olfactory bub, and also provides insight into the mechanism by which the cortex and olfactory bulb, although both generated from the telencephalon, generate projection and interneurons with different properties.

Keywords

Maf family proteins; olfactory bulb; glutamatergic neurons; development; compartment

Introduction

Glutamatergic excitatory and GABAergic inhibitory neurons are two major neuronal types in the brain. In the embryonic brain, the pallium (dorsal telencephalon) generates the Emx1-expressing precursor cells that give rise to the glutamatergic neurons (Gorski et al., 2002), while the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) of the

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subpallium (ventral telencephalon) produce the precursor cells of inhibitory interneuron of the cerebral cortex (Wonders & Anderson, 2006). The development of these glutamatergic and GABAergic neurons in the cortex is regulated by a subset of specific transcription factors that have little overlap with each other. For example, sequential expression of transcription factors, Pax6 → Neurogenin2 → Tbr2 → NeuroD → Tbr1, is a core cascade for the development of glutamatergic neurons (Mihalas & Hevner, 2017). On the other hand, Nkx2.1 is specifically expressed by progenitor cells in the MGE and activates Lhx6 that is required for the differentiation and migration of cortical interneurons (Du et al., 2008; Sandberg et al., 2016; Sussel et al., 1999).

The olfactory bulb (OB) is the first relay station of olfactory information in the brain. During development, the anterior end of the telencephalon evaginates to form the OB, which contains two types of glutamatergic projection neurons, mitral and tufted cells, and several GABAergic interneurons, including periglomerular cells and granule cells. The precursors of mitral/tufted cells are generated in the subventricular zone of the presumptive OB (pOB) (Blanchart et al., 2006; Imamura et al., 2011), while, like cortical interneurons, periglomerular and granule cells are mainly generated in the lateral ganglionic eminence (LGE), a part of the subpallium (Wichterle et al., 2001). Due to their similar developmental origin, mitral/tufted cells often are recognized as OB counterparts of cortical pyramidal neurons. However, while Pax6, Neurogenin2, Tbr2, NeuroD, and Tbr1 are all expressed in developing mitral cells (Hebert et al., 2003; Imamura & Greer, 2013; Roybon et al., 2015; Winpenny et al., 2011), we previously found that the transcriptional cascade, Pax6 → Tbr1 → Tbr2, is used for mitral cell development, which is different from core cascades for pyramidal neuron development (Imamura & Greer, 2013). These results show that the development of OB projection neurons has some transcription factors in common with cortical pyramidal neurons, but with an idiosyncratic cascade.

Musculoaponeurotic fibrosarcoma (Maf) family proteins are part of the basic leucine zipper (bZIP) transcription factor and are classified into two subfamilies, small-Maf, and large-Maf, based on their molecular size (Blank & Andrews, 1997) (Fujikawa et al., 1993). The large-Maf family is composed of four members; MafA, MafB, c-Maf, and NRL (Neural retina-specific leucine zipper protein), and regulates cell fate determination, differentiation, and maturation in many tissues (Yang & Cvekl, 2007). In the mouse brain, MafB and c-Maf are expressed in medial ganglionic eminence (MGE) lineages and regulate the development of somatostatin-positive (SST+) and parvalbumin-positive (PV+) cortical interneurons (Chen et al., 2017; Cobos et al., 2006; Pai et al., 2020). A recent report showed that while conditional knockout (cKO) of either MafB or c-Maf in MGE cells did not change the number of SST+ nor PV+ cells whereas double cKO of MafB and c-Maf significantly reduced the number of PV+ cells (Pai et al., 2019). This suggests that MafB and c-Maf have redundant and compensatory functions in the development of cortical interneurons.

In this study, we focused on the roles of Maf family proteins in the OB since our RNA-seq analysis indicated the expression of large-Mafs in developing mitral cells (Kawasawa et al., 2016). Here, we report the spatiotemporal expression pattern of large-Mafs in the developing OB. Surprisingly, OB interneurons did not express MafA, MafB, or c-Maf. In contrast, MafA and MafB are found in developing mitral/tufted cells. These results suggest that MafA

and MafB are involved in the transcriptional cascade regulating the development of OB projection neurons and that Maf family proteins have different functions in the developing cerebral cortex and OB.

Material and Methods

Animals

CD1 mice (Charles River; Wilmington, MA; strain code 022; RRID:IMSR_CRL:22) were used for all the experiments in this study. The day on which we found a copulation plug was determined as embryonic day (E) 0, and the succeeding days of gestation were numbered consecutively. Prenatal embryos were harvested and fixed in 4% paraformaldehyde (PFA) overnight after pregnant mothers were euthanized with CO_2 inhalation. Postnatal pups were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and were subsequently transcardially perfused with phosphate-buffered saline (PBS) followed by 4% (wt/vol) PFA in PBS. The brains were removed and post-fixed in the same fixative at 4°C overnight. All protocols were approved by and all methods were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Penn State College of Medicine.

Immunohistochemistry

The fixed brains were cryopreserved in 30% sucrose (wt/vol) in PBS and embedded in optimal cutting temperature compound (Sakura Finetek USA; Torrance, CA). The brains were sectioned using a cryostat into 20 μm slices and stored at −80 °C until use. The slices were pretreated for 30 min in 25 mM HCl at 65°C and rinsed with 40 mM borate buffer (pH 8.5), PBS and TBS-T (10 mM Tris-HCl (pH 7.4), 100 mM NaCl with 0.3 % Triton X-100 (vol/vol)). The slices were then blocked with a blocking buffer (5 % normal donkey serum (vol/vol) in TBS-T) at 20 – 25 °C for 1 hour and incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. Sections were washed with TBS-T and then incubated with secondary antibodies with 4’6-diamino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific; Waltham, MA; RRID:AB_2629482) for nuclear staining for 1 hour. The immunoreacted sections were washed and coverslipped with Fluoro-Gel mounting medium (Electron Microscopy Science; Hatfield, PA).

Primary antibodies used are, rabbit anti-MafA (1:500; Bethyl Laboratory; Montgomery, TX; #A700–067; RRID:AB_2891864), rabbit anti-MafB (1:500; Sigma-Aldrich; St. Louis, MO; #HPA005653; RRID:AB_1079293), rabbit anti-c-Maf (1:500; Bethyl Laboratory #A700–045; RRID:AB_2891844), rat anti-Tbr2 conjugated with PE (1:500; Thermo Fisher Scientific #12-4875-80; RRID:AB_1603278), guinea pig anti-parvalbumin (1:500; Synaptic Systems #195004; RRID:AB_2156476), mouse anti-calretinin (1:400; Millipore #MAB1568; RRID:AB_94259), mouse anti-Tbx21 conjugated with Alexa 488 (1:300; BioLegend; San Diego, CA; #644830; RRID:AB_2566019), mouse anti-reelin (1:200; MBL International; Woburn, MA; #D223–3; RRID:AB_843523), chicken anti-tyrosine hydroxylase (TH) (1:2000; Abcam; Cambridge, United Kingdom; #ab76442; RRID:AB_1524535), goat anti-neurog2 (1:500; Santa Cruz Biotechnology; Dallas, TX; #sc-19233; RRID:AB_2149513), mouse anit-pax6 (1:100; DSHB; #PAX6;

RRID:AB_528427), rabbit anti-Tbr1(1:5000; Abcam; #ab31940; RRID:AB_2200219), and goat anti-OMP (1:1000; Wako; Richmond, VA; #544–10001; RRID:AB_2315007).

Secondary antibodies used are goat anti-mouse IgG1 Alexa Fluor 488 (1:300; Thermo Fisher Scientific #A-21121; RRID:AB_2535764), goat anti-mouse IgG1 Alexa Fluor 555 (1:300; Thermo Fisher Scientific #A-21127; RRID:AB_141596), donkey anti-rabbit Alexa Fluor 488 (1:300; Thermo Fisher Scientific #A-21206; RRID:AB_2535792), donkey anti-rabbit Alexa Fluor 555 (1:300; Thermo Fisher Scientific #A-31572; RRID:AB_162543), donkey anti-guinea pig Cy5 (1:300; Jackson ImmunoResearch Labs; West Grove, PA; #706-175-148; RRID:AB_2340462), or donkey anti-Chicken Cy3 (1:300; Jackson ImmunoResearch Labs #703-165-155; RRID:AB_2340363).

To double label with two rabbit antibodies, anti-Tbr1 and anti-MafB, the sections were first labeled with anti-Tbr1 detected with donkey anti-rabbit Alexa Fluor 555. Then the sections were fixed with 4% PFA at 20 – 25 °C for 15 min and incubated with anti-MafB labeled with Alexa Fluor 488 using Zenon IgG labeling kit (Thermo Fisher Scientific) for 2 hours at 20 – 25 °C.

In situ hybridization

In situ hybridization was performed by RNAscope technology using the Fluorescent Multiplex Assay kit v2 (Advanced Cell Diagnostics (ACD)). OB and cerebral cortex were sectioned using a cryostat into 14 μm slices and stored at −80 °C until use. Sections were pretreated according to the manufacturer's protocol and hybridized with probes specific for *mafa*, *mafb*, *c-maf*, *vglut1*, and *gad65* at 40 °C for 2 hours. After hybridization, the signals were amplified and fluorescently developed with Opal 520 and Opal 570 (1:1500; Akoya Biosciences; Marlborough, MA). The sections were counterstained with DAPI for 30 sec and mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). All the probes were designed by ACD and purchased from them (*mafa*: Cat# 556931; *mafb*: Cat# 438531-C2, *c-maf*: Cat# 412951; *vglut1*: Cat# 416631-C3; and *gad65*: Cat# 439371-C3).

Imaging and data analysis

Images were captured with Zeiss Axio Imager.M2 with/without using Zeiss Apotome.2 (Carl Zeiss AG; Oberkochen, Germany). Levels were adjusted using Photoshop software (Adobe; RRID: SCR_014199), but the images were otherwise unaltered. To quantify the distribution of MafA+ and MafB+ cells in the OB, the number of MafA and MafB positive cells in each layer of the OB were manually counted in the coronal sections of the OB in P0, P7, and P15 mice using Fiji (n = 3 animals per age). Percentages of MafA+ and MafB+ cells expressing Tbr2 were calculated by manually counting the number of MafA+, MafB+, and Tbr2+ cells in the coronal sections of P0, P7, and P15 OBs (n = 3 animals per age). All results are presented as mean ± standard deviations (SD).

Results

Expression of Maf family proteins in cortical interneurons

We first examined the expression of large-MafB family proteins, MafA, MafB, and c-Maf, in the developing cerebral cortex since it has been shown that MafB and c-Maf are responsible for the development of cortical interneurons originating from the MGE (Chen et al., 2017; Cobos et al., 2006; Pai et al., 2019). While no significant MafA signal was observed in the cerebral cortex, MafB+ and c-Maf+ cells were found in the marginal and subventricular zones as well as in the subventricular zone of the MGE (data not shown) in E14 mice (Fig. 1A–C), indicating that both MafB and c-Maf are expressed by migrating cortical interneurons. MafA, MafB, and c-Maf were all found in layers II–V at postnatal day (P) 7, but the numbers of MafB+ and c-Maf+ cells were higher than that of MafA+ cells (Fig. 1D–F). Previously, it was demonstrated that MafB and c-Maf regulate differentiation of PV+ and SST+ interneurons in the cerebral cortex (Cobos et al., 2006; Pai et al., 2019). Our immunohistochemical analysis further showed that a portion of MafA+, MafB+, and c-Maf+ cells were PV+ (Fig. 1G–I). In addition, *in situ* hybridization analysis showed that cells expressing *mafa*, *mafb*, or *c-maf* mRNA co-expressed *gad65* mRNA but not *vglut1* mRNA in the P7 cerebral cortex (Fig. 1J–O). These results show that MafA, MafB, and c-Maf are expressed in a subset of interneurons, but not in the glutamatergic pyramidal neurons, in the mouse cerebral cortex.

Spatiotemporal expression pattern of Maf family proteins in the postnatal olfactory bulb

Figure 2A shows the expression of MafB in the mouse brain at P0. In addition to the cerebral cortex, a strong MafB signal was observed in the OB. However, there were no MafB+ cells in the rostral migratory stream (RMS), where OB interneuron progenitors migrate toward the OB after being generated in the dorsal LGE (Fig 2A’). Next, we investigated the expression of large-Maf family proteins in the OB of the mouse at different time points in the neonatal stages. Interestingly, MafA and MafB showed almost identical expression patterns, but few cells expressed c-Maf (data not shown). The *in situ* hybridization experiment showed that cells expressing *mafa* mRNA are mostly positive for *mafb* mRNA and vice versa in the P0 OB (Fig. 2B). Co-expression of *mafa* and *mafb* mRNAs in the same cells are observed also at P7 (data not shown). Therefore, at least in OB from P0 to P7, MafA and MafB are likely to be co-expressed in the same cells. At P0, both MafA+ and MafB+ cells were mostly found in the mitral cell layer (MCL) and in the superficial part of the granule cell layer (GCL) (Fig. 2C, D). At P4, cells strongly expressing MafA and MafB were found also in the superficial external plexiform layer (EPL), or the deep glomerular layer (GL), in addition to the MCL (Fig. 2E, F), whereas the signals in the MCL became weaker by P7 (Fig. 2G, H) and in the EPL by P15 (Fig. 2I, J). Finally, there were only a few cells strongly expressing MafA and MafB in P30 OB (Fig. 2K, L). The distribution of MafA+ and MafB+ cells in the OB at P0, P7, and P15 was quantified by calculating the percentage of cells expressing MafA and MafB in each layer, respectively (Fig. 2M, N). The results showed that the expression peaks of MafA and MafB gradually shifted from deep (MCL and superficial GCL) to superficial (GL) layers in the neonatal period. This peak shift is due to the appearance and disappearance of MafA+ and MafB+ cells in the order of MCL, EPL, and GL. At P0, there are few OB projection neurons in the

EPL, and the late-generated OB projection neurons, such as middle and external tufted cells, later migrate to the EPL and GL, respectively (Hirata et al., 2019; Imamura et al., 2020; Nguyen & Imamura, 2019). These results indicate that MafA and MafB are expressed by the developing projection neurons in the OB and disappear upon maturation.

Expression of MafA and MafB in olfactory bulb projection neurons

Although the locations we observed the MafA+ and MafB+ cells in the OB correspond to the location of mitral and tufted cells, many interneurons also exist in these layers (Huang et al., 2013; Imamura et al., 2006; Lepousez et al., 2010). Thus, we next examined whether the MafA+ and MafB+ cells possess the properties of OB projection neurons. Examining the expression of Tbr2 that is expressed by glutamatergic neurons in the OB (Brill et al., 2009; Bulfone et al., 1999; Winpenny et al., 2011), we found that most of MafA+ and MafB+ cells expressed Tbr2 (Fig. 3A, B). Our quantification revealed that more than three quarters of both MafA+ and MafB+ cells co-expressed Tbr2 in OBs at P0, P7, and P15 (Supplementary Table 1). We further examined the expression of Tbx21 and reelin, markers for mitral/tufted cells. Interestingly, MafA+ and MafB+ cells in the MCL were mostly positive for Tbx21 and reelin while some of them in the EPL and GL were negative at P7 (Fig. 3C–F). These results strongly suggest that cells expressing MafA and MafB in the MCL and EPL are mitral and tufted cells.

There were many MafA+ and MafB+ cells that do not express mitral/tufted cell markers in the EPL and GL. On the other hand, they did not express any molecules that are expressed periglomerular interneurons such as PV (Fig. 3G, H), calretinin (Fig. 3I, J), and TH (Fig. 3K, L), either. Giving that they express Tbr2 (Supplementary Table 1), both MafA+ and MafB+ cells in the EPL and GL are also likely the glutamatergic interneurons (Brill et al., 2009). The *in situ* hybridization analysis showed that cells expressing *mafa* or *mafb* mRNA co-expressed *vglut1* mRNA but not *gad65* mRNA in the P7 OB (Fig. 3M–P). Taken together, these data indicate that MafA and MafB are specifically expressed by glutamatergic neurons, but not by GABAergic interneurons, in the postnatal mouse OB.

Spatiotemporal expression patterns of MafA and MafB in the embryonic olfactory bulb

Next, we examined the expression of large Maf family proteins in the OB at prenatal stages. At E13, many MafA+ and MafB+ cells were found in the intermediate zone (IZ) of the pOB, but only a small numbers were found from the ventricular zone (VZ) where progenitor cells of mitral/tufted cells are found. We found that no MafA+ and MafB+ cells co-expressed Pax6 or Neurog2 progenitor cell markers in the VZ (Winpenny et al., 2011) (Fig. 4A–D). We previously showed that Pax6 and Tbr1/Tbr2 were mutually exclusive in their expression in developing mitral cells and that Tbr1 expression preceded Tbr2 (Imamura & Greer, 2013). Therefore, we also compared the expression of MafA/MafB with Tbr1/Tbr2. In the pOB of E13 mice, which is defined as the region receiving OMP+ olfactory sensory neuron (OSN) axons (Fig. 4E), almost all MafB+ cells were Tbr1+ (Fig. 4F). However, in E14 pOBs, MafA+ and MafB+ cells that did not express Tbr2 were found, especially in the lateral and medial regions (Fig. 4G, H). Focusing on the spatial expression pattern, we found that expression of MafA and MafB were high in the cells located at the IZ of medial and lateral regions of the pOB, but low in the anterior part (Fig. 4G2, H2). In contrast, a strong

Tbr2 signal was observed in the anterior pOB (Fig. 4G3, H3). By E17, strong Tbr2 signals extended to the lateral and medial regions, and the majority of MafA+ and MafB+ became Tbr2+. Figure 4I shows that majority of MafA+ cells co-expressed Tbr2 at E17; 71.6 ± 9.3 % and 74.4 ± 5.5 % of MafA+ and MafB+ cells were Tbr2+, respectively, which is consistent with the values at P0 (Supplementary Table 1). We also should note that neither MafA nor MafB is expressed in mitral cells of the accessory olfactory bulb, where both Tbr1 and Tbr2 are expressed. Thus, whereas MafA, MafB, and Tbr2 are expressed in developing glutamatergic neurons in the OB, their expressions may start from different regions in the pOB (Fig. 4J).

Discussion

It has been known that MafB and c-Maf transcription factors regulate the development of GABAergic interneurons in the cerebral cortex (Chen et al., 2017; Cobos et al., 2006; Pai et al., 2019). In this study, we found that MafA is also expressed in a subset of interneurons in the postnatal mouse cortex, but not in the embryonic brain. Although we could not determine whether MafA is co-expressed with MafB/c-Maf, MafA+ cells were more sparsely distributed in the cortex than MafB+/c-Maf+ cells, suggesting that MafA+ cells and MafB+/c-Maf+ cells in the cortex are different interneuron populations. In addition, in the spinal cord, it has been shown that MafB and c-Maf are expressed by mixed populations of inhibitory and excitatory neurons, whereas MafA is restricted to excitatory neurons (Del Barrio et al., 2013). Therefore, the combination of expression of large-Mafs in the nervous system may be different in each tissue. An important finding of this study is that MafA and MafB are selectively expressed in glutamatergic neurons in the developing mouse OB without significant c-Maf expression.

The cerebral cortex and OB both originate from the telencephalon, and the developmental mechanisms of pyramidal neurons and mitral/tufted cells, which are glutamatergic neurons in respective regions, have been found to be quite similar. Both pyramidal neurons and mitral/tufted cells originate from Pax6+ Neurog2+ progenitor cells in the VZ, and T-box transcription factors, such as Tbr1 and Tbr2, play an important role in their differentiation into glutamatergic neurons (Brill et al., 2009; Englund et al., 2005; Hevner et al., 2001; Sessa et al., 2008; Winpenny et al., 2011). The core transcriptional cascade, Pax6 → Neurogenin2 → Tbr2 → NeuroD → Tbr1, is also used for the generation of granule cells in the dentate gyrus and glutamatergic interneurons in the OB. In contrast, differences in developmental mechanisms have been reported between cerebral pyramidal neurons and mitral/tufted cells. Tbr2 is expressed in intermediate progenitor cells in the cerebral cortex and is required for the generation of Tbr1+ mature neurons, whereas developing mitral cells express Tbr1 prior to Tbr2 (Imamura & Greer, 2013; Nguyen & Imamura, 2019), indicating that the OB projection neurons are generated through the direct neurogenesis that is seen in the development of reptile brains (Cardenas et al., 2018). Tbr2 also regulates the dendritic specification of mitral/tufted cells during postnatal stages (Mizuguchi et al., 2012). Moreover, some transcription factors such as Tbx21 and AP2ɛ are only expressed in mitral/tufted cells, but not in the cerebral cortex (Faedo et al., 2002; Feng & Williams, 2003), and NeuN, a well-known neuronal marker, is absent from mitral/tufted cells in the OB (Imamura et al., 2006). These results suggest that the transcriptional cascade used

for the generation of mitral/tufted cells is somewhat similar but different from that of cortical pyramidal neurons. In this study, we found an interesting new case where the same transcription factor was expressed only in GABAergic interneurons in the cortex and only in glutamatergic projection neurons in the OB. Depending on the combination of transcription factors, large-Maf family proteins may be able to regulate both the transcription of genes required for GABAergic neuron development and those required for glutamatergic neuron development. Also, elucidating the mechanism of the lack of expression of large-Maf family proteins in cortical pyramidal neurons and OB interneurons is an important issue.

Another interesting observation is the almost identical expression patterns of MafA and MafB in the OB, raising the question of whether their functions complement each other. In the cortex, MafB and c-Maf are both involved in the generation of SST+ and PV+ interneurons at the embryonic stage and compensate for each other (Pai et al., 2019), and are necessary to promote expression of Mef2c to specify PV+ interneurons in the developing cortex (Pai et al., 2020). That said, Pai et al. (2019) also showed that MafB and c-Maf have opposite functions in synaptic maturation. Our observations indicate that MafA and MafB play important roles in the development of OB glutamatergic neurons, but the loss of either MafA or MafB may not cause serious defects in the development of OB projection neurons. However, in this study, we also observed that MafB begins to disappear from the OB slightly earlier than MafA. This slight difference in temporal expression pattern may be important for morphological maturation of OB projection neurons, such as the formation and pruning of synapses. Critical next steps are to elucidate whether MafA and MafB complement each other's functions in OB projection neurons and to identify the genes whose expression is regulated by these transcription factors, which include the investigation of MafA and/or MafB knockout mice (Blanchi et al., 2003; Bourane et al., 2009).

Large Mafs are members of bZIP transcription factors, which usually form homo and heterodimers with other bZIP proteins to regulate the expressions of other genes (Yang & Cvekl, 2007). Heterodimerization between large Mafs has also been proposed (Vinson et al., 2002). The dimeric Maf factors recognize palindromic sequences referred to as Maf recognition elements (MARE) (Kurokawa et al., 2009). Importantly, it has been shown that large Mafs are key regulators for the generation of the specific types of cells, including pancreatic β cells and lens cells. In pancreatic β cells, MafB and MafA play important roles during development and adult life, respectively, and a switch in expression from MafB to MafA occurs between these time points(Hang & Stein, 2011). Moreover, Mafs usually work together with other transcription factors to determine cell fates. For example, binding of NeuroD, Pdx1, and MafA to E1, A3, and RIpE3b/C1 elements, respectively, is necessary for the production of insulin in mature β cells (Zhu et al., 2017). On the other hand, the expression of crystallin genes for lens specification is determined by the interactions between large Mafs, Pax6, and Sox2 (Muta et al., 2002; Yang et al., 2004; Yang & Cvekl, 2005). Therefore, it is important to elucidate the other transcription factors expressed with large Mafs to understand the mechanisms of how large Mafs regulate the development of both GABAergic and glutamatergic neurons in different brain regions.

We also found that both MafA+ and MafB+ cells initially show different expression patterns with Tbr2+ cells in the embryonic pOB, whereas their expressions are overlapped quite well

in the postnatal OB. Tbr2 is first expressed by mitral/tufted progenitor cells in the anterior region of the pOB, whereas expressions of both MafA and MafB are strong in the lateral and medial regions of the pOB. We have previously shown regional differences in mitral cell development in the OB. In that study, we reported that the expression of Tbx21 begins in the anteromedial region of the OB, followed by the dorsomedial and then vsentrolateral areas, which is similar to the expression pattern of Tbr2 (Nguyen & Imamura, 2019). On the other hand, the expression of vGluT1 is first detected in the ventrolateral region during development. MafA and MafB are probably the first transcription factors that show a temporal expression pattern similar to that of vGluT1. OSNs project their axons to the pOB in a compartmentalized manner as well. During development, OSNs in the dorsal region mature earlier and direct their axons to the pOB earlier than the OSNs in the ventral region (Takeuchi et al., 2010). Moreover, OSNs expressing Neuropilin-1 (Nrp1) and Semaphorin 3A (Sema3A) project complimentarily to the posterior and anterior pOB, respectively, while Robo2 and Sema3F/Nrp2 control OSN projections along the dorsal-ventral axis (Cho et al., 2007; Imai et al., 2009; Nguyen-Ba-Charvet et al., 2008; Takeuchi et al., 2010). The compartmentalized development of OB projection neurons might be regulated by the factors secreted from the OSN axons that project to different regions of the OB.

In summary, this study suggests that large-Maf family proteins play different roles in the development of glutamatergic and GABAergic neurons in the OB and cerebral cortex. Future studies on the detailed functions of the large-Maf family proteins may help to elucidate the mechanisms that give rise to the wide variety of neurons in the brain. Furthermore, investigating the mechanism of compartmentalization of the OB may provide insights into the mechanism that generates diversity among OB projection neurons, leading to a better understanding of multifaceted olfactory information processing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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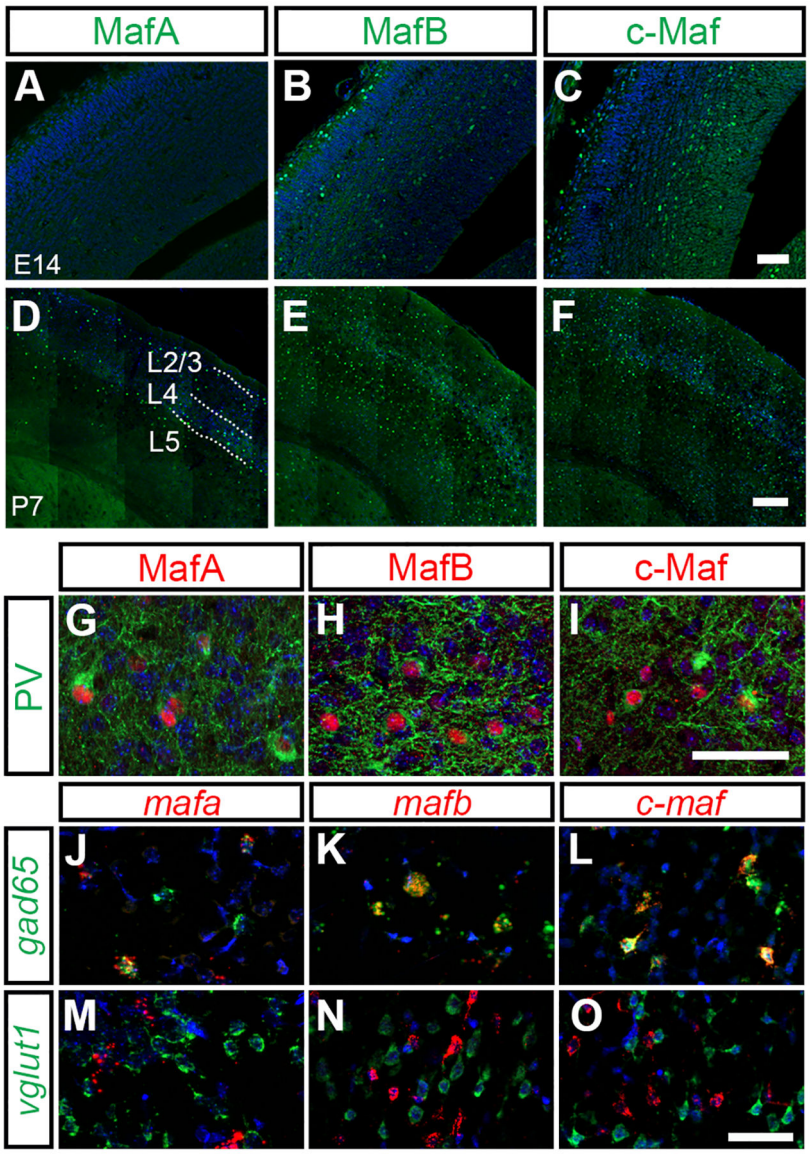

Figure1. Expression of MafA, MafB, and c-Maf in GABAergic interneurons in the cerebral cortex

(A–C) Coronal sections of E14 mouse cortex immunostained with antibodies for anti-MafA (A), anti-MafB (B), and anti-c-Maf (C) (green). Few MafA positive cells are detected. **(D–F)** Expression of MafA (D), MafB (E), and c-Maf (F) in P7 mouse cortex (green). **(G–I)** Expression of MafA (G; red), MafB (H; red), and c-Maf (I; red), in PV+ interneurons (G–I; green) in P7 mouse cortex. **(J–O)** mRNAs of large Maf family proteins are detected in P7 mouse cortex with *in situ* hybridization analysis. Cells expressing *mafa* (J, M; red), *mafb* (K, N; red), and *c-maf* (L, O; red) mRNAs co-express *gad65* mRNA (J-L; green), but not *vglut1* mRNA (M-O; green). All nuclei are stained with DAPI (blue). Scale bars:100 μm in (A–C); 200 μm in (D–F); and 50 μm in (G–O).

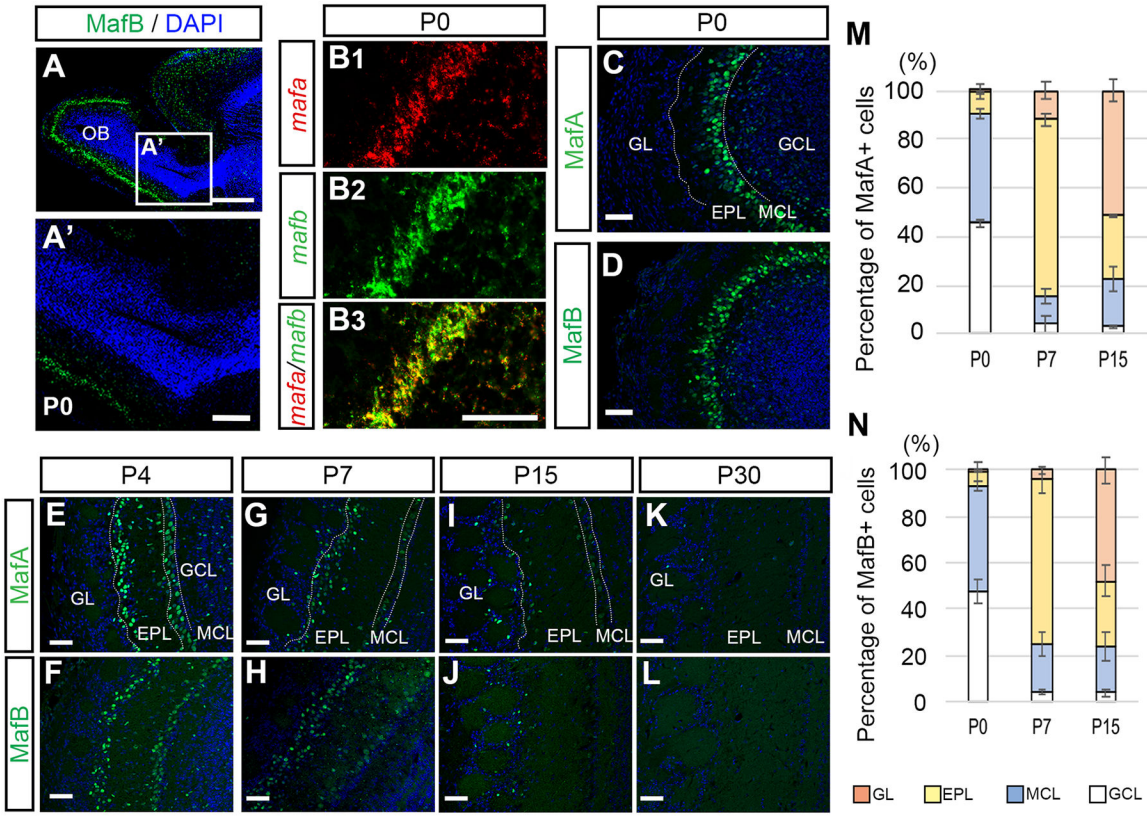

Figure2. Expression of MafA and MafB in the olfactory bulb

(A) MafB immunostaining in a sagittal section of P0 mouse brain (green). MafB signal is not detected in the RMS (A'). **(B)** *In situ* hybridization analysis shows co-expression of mafa (red) and mafb mRNAs (green) in P0 mouse OB. **(C–L)** Expression of MafA (B, D, F, H, J; green) and MafB (C, E, G, I, K; green) are examined in the coronal sections of mouse OBs at P0 (C, D), P4 (E, F), P7 (G, H), P15 (I, J), and P30 (K, L). All nuclei are stained with DAPI (blue). **(M, N)** Graphs showing distributions of MafA- (L) and MafB-positive cells (M) in the OB at P0, P7, and P15. Percentages of MafA or MafB-positive cells in each layer, GCL, MCL, EPL, and GL, are shown. GCL, granular cell layer; MCL, mitral cell layer; EPL, external plexiform layer; GL, glomerular layer. Scale bars: 400 μm in (A); 200 μm in (A'), 50 μm in (B), and 100 μm in (C–L).

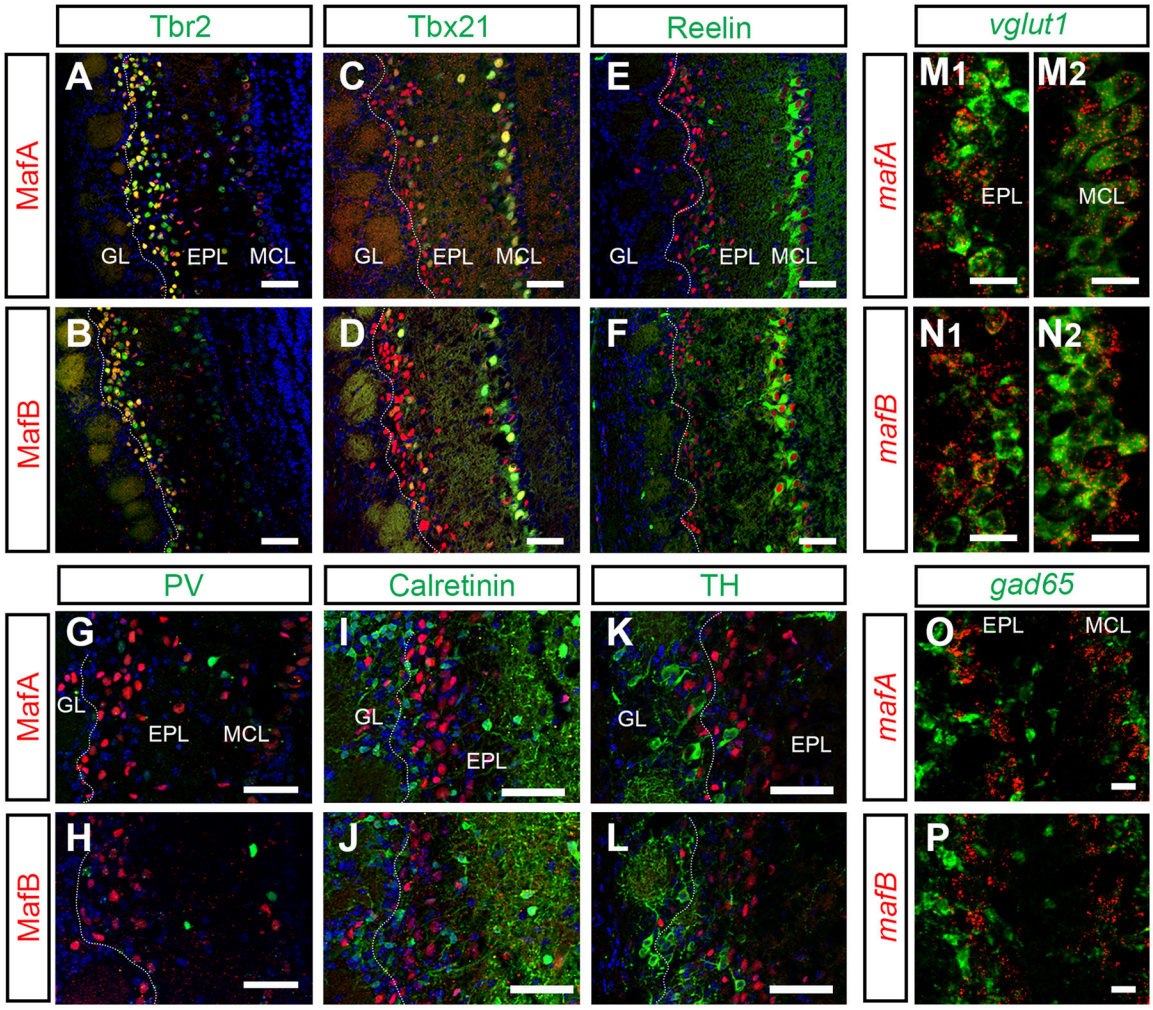

Figure3. Expression of MafA and MafB in glutamatergic neurons in the olfactory bulb

(A–F) Coronal sections of P7 mouse OB. Expression of OB glutamatergic neuron markers in MafA+ (A, C, E; red) and MafB+ (B, D, F; red) are examined with anti-Tbr2 (A, B; green), anti-Tbx21 (C, D; green), and anti-Reelin (E, F; green) antibodies. **(G–L)** Expression of OB interneuron markers in MafA+ (G, I, K; red) and MafB+ (H, J, L; red) are examined with anti-PV (G, H; green), anti-calretinin (I, J; green), and anti-TH (K, L; green) antibodies. **(M–P)** mRNAs of large Maf family proteins are detected in P7 OB with *in situ* hybridization analysis. Cells expressing *mafa* (M, O; red) and *mafb* (N, P; red) co-expressed *vglut1* mRNA (M, N; green) but not *gad65* mRNA (O, P; green). Scale bars: 50 μm (A-L) and 20 μm (M-P).

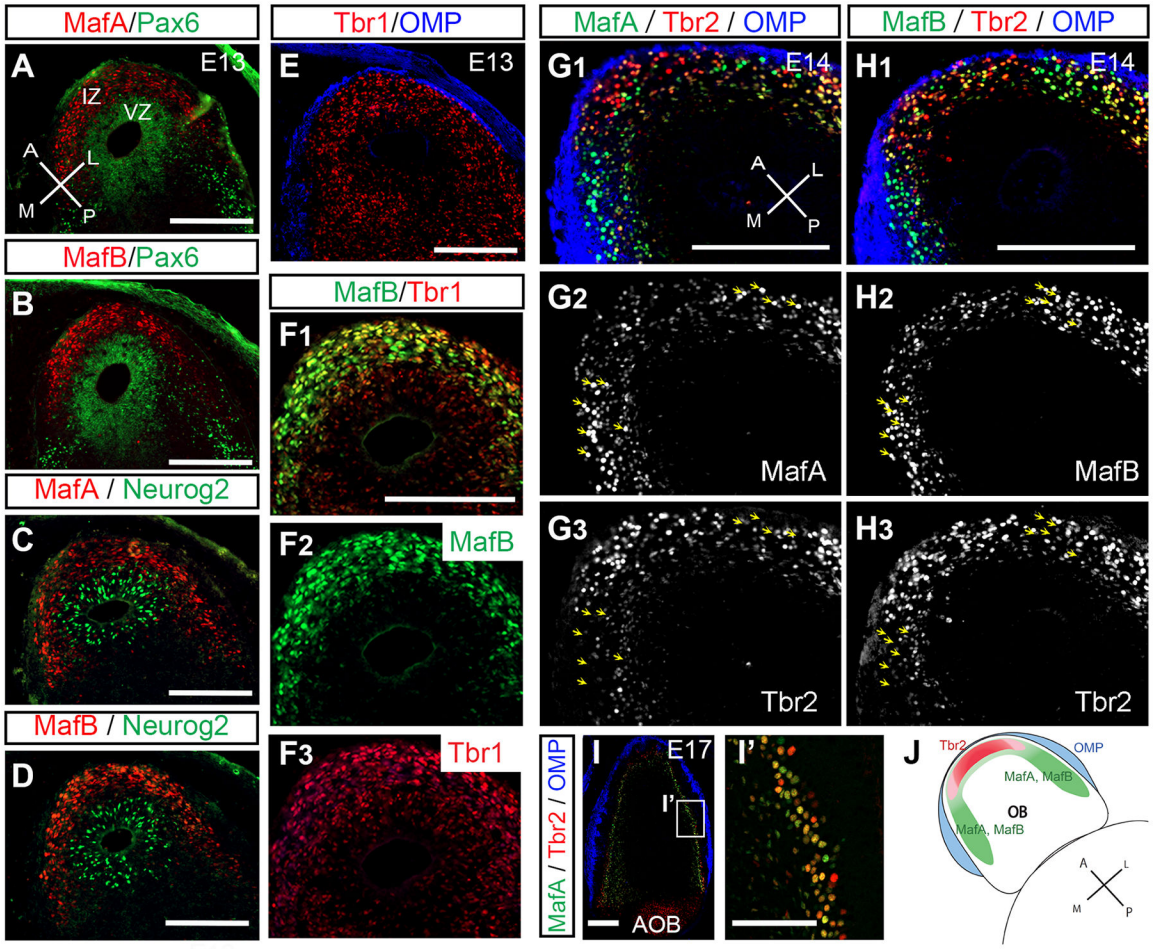

Figure 4. Spatiotemporal expression patterns of MafA/MafB and Tbr2 in the embryonic olfactory bulb

(A–F) Horizontal section of E13 OBs. MafA+ (A, C; red) and MafB+ cells (B, D; red) are predominantly localized in the intermediate zone (IZ) and are not colocalized with Pax6+ (A, B; green) or Neurog2+ cells (C, D; green) that are found in the ventricular zone (VZ). On the other hand, in the pOB innervated by the OMP+ olfactory sensory neuron axons (E; blue), most of the MafB+ cells (F; green) co-express Tbr1 (E, F; red). **(G, H)** Horizontal sections of E14 OBs stained with anti-MafA (G; green) or anti-MafB (H; green) antibodies together with anti-Tbr2 (red) and anti-OMP (blue) antibodies. Cells strongly expressing MafA and MafB are abundant in the IZ of lateral and medial regions, while cells strongly expressing Tbr2 are observed in the anteromedial IZ of the pOB. Representative cells positive for MafA or MafB but negative for Tbr2 are indicated by yellow arrows. **(I)** Horizontal section of E17 OB stained with anti-MafA (green), anti-Tbr2 (red), and anti-OMP (blue). Majority of MafA+ cells are Tbr2+ (I'). In contrast, MafA signals are not observed in the accessory olfactory bulb (AOB) that are positive for Tbr2. Scale bars: 200 μm (A-I) and 100 μm (I'). **(J)** Schematic diagram of MafA and MafB expression in the embryonic mouse OB. MafA and MafB expressions begin from the IZ of medial and lateral pOB, while Tbr2 expression in the IZ is initially high in the anteromedial regions.