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Segregated labeling of olfactory bulb projection neurons based on their birthdates

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Abstract

Mitral and tufted cells are the projection neurons of the olfactory bulb (OB). We previously reported that somata location and innervation patterns were different between early- and late-born mitral cells (Imamura *et al.*, 2011). Here, we introduced a plasmid that drives the expression of a GFP gene into the mouse OB using *in utero* electroporation, and demonstrated that we can deliver the plasmid vectors into distinct subsets of OB projection neurons by changing the timing of electroporation after fertilization. The electroporation performed at embryonic day (E) 10 preferentially labeled mitral cells in the accessory OB and main OB mitral cells in dorsomedial mitral cell layer (MCL). In contrast, the E12 electroporation introduced the plasmid vectors preferentially into main OB mitral cells in the ventrolateral MCL and tufted cells. Combining these data with BrdU injections, we confirmed that E10 and E12 electroporation preferentially labeled early- and late-born projection neurons, respectively. This work introduces a novel method for segregated labeling of mouse olfactory bulb projection neurons based on their birthdates. With this technique we found that early- and late-born projection neurons extend their secondary dendrites in the deep and superficial external plexiform layer (EPL), respectively. Although a similar segregation has been suggested for mitral versus tufted cell dendrites, we found mitral cells projecting secondary dendrites into the superficial EPL in E12 electroporated main OB. Our observations indicate that timing of neurogenesis regulates not only somata location and innervation patterns, but also the laminar organization of projection neuron dendrites in the EPL.

Keywords

olfactory bulb; development; projection neuron; in utero electroporation; neuronal circuit

Introduction

Following detection of an odor, olfactory sensory neuron axons relay that information to the olfactory bulb (OB) where they synapse with projection neurons, which in turn innervate the

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olfactory cortex. The projection neurons in the main olfactory bulb (MOB) have canonically been classified into two types based on the location of their cell bodies: 1) mitral cells are found in a monolayer of the OB, the mitral cell layer (MCL); while 2) tufted cells are stochastically distributed throughout the external plexiform layer (EPL). Nevertheless, both mitral and tufted cells extend a single primary dendrite into a glomerulus where they receive afferent synapses from the olfactory sensory neuron axons as well as interacting in local glomerular-specific circuits. Of interest here, both mitral and tufted cells also have secondary dendrites that spread widely in the EPL where dendrodendritic reciprocal synapses are formed with a population of interneurons, granule cells.

Despite some similarities, mitral and tufted cells can be defined with distinguishing features and roles in odor information processing. For example; *in vivo* physiological studies suggested differences in their molecular receptive ranges and firing rates (Nagayama *et al.*, 2004; Fukunaga *et al.*, 2012; Kikuta *et al.*, 2013). Also, secondary dendrites of mitral and tufted cells preferentially extend in the deep and superficial portions of the EPL, respectively (Mori *et al.*, 1983; Orona *et al.*, 1984; Mori, 1987); and axonal tracing studies revealed that tufted cells innervated predominantly the olfactory tubercle, whereas mitral cell axons spread widely in the olfactory cortices (Haberly & Price, 1977; Scott, 1981; Nagayama *et al.*, 2010; Ghosh *et al.*, 2011; Sosulski *et al.*, 2011; Igarashi *et al.*, 2012).

More recent studies have begun to elaborate differences among mitral cells, suggesting that they too are a heterogeneous population. For example, subpopulations of mitral cells have distinct electrophysiological properties that likely reflect differences in the expression of membrane channels (Padmanabhan & Urban, 2010; Angelo *et al.*, 2012). Innervation of the olfactory cortices is not homogeneous among mitral cells and correlates, at least in part, with the location of the cell body in the OB (Yan *et al.*, 2008; Miyamichi *et al.*, 2010; Imamura *et al.*, 2011). Despite these provocative findings, the mechanisms yielding different subpopulations of mitral cells have been enigmatic. We previously reported that early-born and late-born mitral cells differently localized their cell bodies in the dorsomedial and ventrolateral MCL, and that the olfactory tubercle receives heavier axonal input from late-born mitral cells (Imamura *et al.*, 2011). In addition, we established an *in utero* electroporation strategy with which we can introduce the plasmid vectors into subpopulations of OB projection neurons (Imamura & Greer, 2013; 2014). Here, we further developed the *in utero* electroporation to label the distinct subsets of OB projection neurons based on their birthdates.

Materials and Methods

Animals

All the experiments were carried out using the CD-1 mouse strain (Charles River Laboratories; Wilmington, MA). The day on which we found a copulation plug was designated E0, and the succeeding days of gestation were numbered in order. Prenatal embryos were harvested and fixed in 4% paraformaldehyde (PFA) overnight after pregnant mothers were euthanized with CO₂ asphyxiation. All protocols were approved by the Yale University Animal Care and Use Committee.

In utero electroporation

A plasmid that drives the expression of a GFP gene under the CAG enhancer, pGFP (pCAG-GFP; Plasmid #11150) (Matsuda & Cepko, 2004), was obtained from Addgene (Cambridge, MA).

In utero electroporation was performed according to the procedure previously reported (Imamura & Greer, 2013; 2014). Pregnant mothers were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and the uterine horns were carefully taken out from the abdominal cavity. Approximately 0.5 μ l of DNA solution (1.5 – 4 μ g/ μ l in 50% TE) was injected into the lateral cerebral ventricle of embryos by inserting a glass pipette. The DNA solution was mixed with 200 μ g/ml of Fast Green to visibly confirm the injection site. Then, electroporation was carried out by applying square electric pulses: 2 pulses of 30 V, 50 ms duration with a 950 msec interval. To efficiently label the mitral cell precursors in the presumptive OB, positive current was given from posterior to anterior. Following electroporation, the uterine horns were repositioned in the abdominal cavity, and following suturing the animals recovered in a warm environment.

BrdU injection

5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich; St. Louis, MO) was intraperitoneally injected into pregnant mothers at E10, E11, or E12 (50 mg/kg). For the same day injection, BrdU was administered immediately after the *in utero* electroporation. For the injections performed at different days, BrdU was injected at 24 or 48 hours prior to/after the electroporation.

Immunohistochemistry

The fixed brains were cryopreserved in 30% sucrose (wt/vol) in 0.1 M phosphate buffer (pH 7.4), and embedded in optimal cutting temperature compound (Sakura Finetek; Torrance, CA). The olfactory tissues were cut on a cryostat into 20 μ m slices, mounted on slides and stored at -20°C until use. The slices were pretreated for 30 min in 0.025 M HCl at 65°C , and rinsed with 0.1 M borate buffer (pH 8.5), PBS and TBS-T (10 mM Tris-HCl (pH 7.4), 100 mM NaCl with 0.3% Triton-X100 (vol/vol)). The slices were then blocked with blocking buffer (5% normal donkey serum (vol/vol) in TBS-T) at $20 - 25^{\circ}\text{C}$ for 1 h and incubated with primary antibodies diluted in blocking buffer overnight at 4°C . Sections were washed with TBS-T, then incubated with secondary antibodies with 4'-diamino-2-phenylindole dihydrochloride (DAPI; Life Technologies; Carlsbad, CA) or DRAQ5 (Biostatus Ltd.; Leicestershire, United Kingdom) for nucleus staining for 1 h. The immunoreacted sections were washed and coverslipped with Fluoro-Gel mounting medium (Electron Microscopy Science; Hatfield, PA).

Primary antibodies used for immunohistochemistry were anti-BrdU (Accurate Chemical & Scientific Corporation; Westbury, NY; rat, 1:100), anti-GFP (Abcam; Cambridge, MA; chicken, 1:1000), anti-PGP9.5 (Cedarlane; Ontario, Canada; rabbit, 1:2000), anti-neuropilin1 (R&D Systems; Minneapolis, MN; goat, 1:500) and anti-Tbx21 (kindly provided by Dr. Yoshihiro Yoshihara at RIKEN; Saitama, Japan; rabbit, 1:10000). Donkey anti-species IgG conjugated with Alexa 488, Alexa 555, Alexa 674 (Life Technologies), or

Cy2 (Jackson ImmunoResearch; West Grove, PA) were used as secondary antibodies. Detailed information and the specificity of primary antibodies are summarized in Table 1.

Image acquisition and statistical analysis

Images were acquired with a laser scanning confocal microscope (Leica TCS SL; Leica Microsystems; Wetzlar, Germany). Levels were adjusted in Photoshop software (Adobe; San Jose, CA), but the images were otherwise unaltered. To quantify the percentages of BrdU+ cells among electroporated projection neurons in the OBs, images of three coronal slices taken every 1,440 μm from anterior to posterior were acquired, and numbers of GFP+/Tbx21+ and BrdU+/GFP+/Tbx21+ were manually counted to calculate the percentages of BrdU+ among GFP+ projection neurons. Since Tbx21 labels the nuclei of OB projection neurons, we only included cells that had more than four BrdU-positive dots in the Tbx21+ nuclei in the BrdU+/GFP+/Tbx21+ category. To analyze the distribution of GFP+ mitral and tufted cells in the MOB, a coronal slice was taken from the middle OB. By dividing in halves both vertically and horizontally, the OB slice was separated into four regions, dorsomedial, dorsolateral, ventrolateral, and ventromedial. Then numbers of electroporated, GFP+/Tbx21+, mitral cells in the MCL and tufted cells in the EPL were separately counted in each quarter region. The absolute numbers of cells analyzed are summarized in Supplementary Tables 1 and 2. All results are presented as mean and standard error of the mean (SEM). Statistical analyses were carried out using GraphPad Prism 4 software (GraphPad Software; La Jolla, CA).

To analyze the distribution of secondary dendrites in the EPL, two 100 μm wide regions that include the EPL were extracted from lateral and medial parts of a coronal section of middle OB. The region where Tbx21+ secondary dendrites of mitral/tufted cells were observed was defined as the EPL, and equally subdivided into superficial, middle, and deep sublamina. GFP signal (green channel) in the EPL was dichotomized and the numbers of positive pixels were counted in total EPL and each sublamina. Percentages of GFP signals were calculated by dividing the number of positive pixels in each sublamina with that of total EPL.

Results

Differential labeling of projection neurons based on the birthdates

In utero electroporation introduces plasmid vectors into the cells lining the ventricular wall of the embryonic brain. In the developing cerebral cortex, it was suggested that ventricular cells that were in S and M phases of cell cycle preferentially incorporated the electroporated plasmids (Stancik *et al.*, 2010). Since cells in M phase are also positioned at the ventricular wall of the developing OB (Imamura & Greer, 2013), we tested the use of *in utero* electroporation to label the subsets of OB projection neurons based on their birthdates. Mouse OB projection neurons are generated in the developing brain at specific time points in embryonic development; mitral cells in the MOB are generated between embryonic day (E) 9 and 13 (Imamura *et al.*, 2011). Using electroporation we found that at E11 plasmid vectors predominately transfected MOB mitral cells (Imamura & Greer, 2013).

In the current study, we electroporated plasmids driving the expression of a GFP gene into developing OB cells at E10, E11, or E12. At all ages, we found significant numbers of GFP + projection neurons in the OB at postnatal day (P) 7. To analyze the relationship between GFP labeling and birthdate, BrdU was intraperitoneally injected into the electroporated mother at either E10, E11, or E12. We focused our analyses on cells that co-expressed Tbx21, a transcription factor selectively expressed in OB projection neurons (Faedo *et al.*, 2002; Mitsui *et al.*, 2011). When electroporation and BrdU injection were both performed at E10 there were abundant double-labeled cells (Figure 1A). However, with electroporation performed at E10 and BrdU injected 48 hours later at E12, double labeling was rare (Figure 1B). In contrast, when both the electroporation and BrdU injection were performed at E12, a larger number of GFP+ projection neurons were double-labeled for both markers (Figure 1C, D).

To quantify the relationship between GFP labeling and birthdate, we calculated the percentages of BrdU-labeled cells (BrdU+/GFP+/Tbx21+) among projection neurons expressing GFP (GFP+/Tbx21+) (Figure 1E). To perform this analysis, we counted all Tbx21+ cells including MOB mitral and tufted cells as well as the accessory olfactory bulb (AOB) mitral cells. As expected from our qualitative observations, when electroporation was performed at E10, a significantly larger percentage of GFP+ cells were co-labeled with BrdU injected at E10 ($36.5 \pm 3.6\%$) than when the BrdU was injected 48 hours later at E12 ($8.9 \pm 2.6\%$) (one-way ANOVA [F(2, 6) = 16.80, $p = 0.0035$] followed by Tukey HSD test). In contrast, there was extensive double labeling of projection neurons following simultaneous electroporation and BrdU injection at E12 ($40.1 \pm 2.2\%$), while there was less evidence of double-labeling when the BrdU was injected at E10 ($12.3 \pm 1.2\%$) or E11 ($20.5 \pm 0.5\%$), 48 or 24 hours prior to electroporation at E12, respectively. These percentages were significantly different (one-way ANOVA [F(2, 6) = 74.38, $p < 0.0001$] followed by Tukey HSD test). The largest number of cells electroporated at E11 were co-labeled with BrdU injected at E11 ($41.8 \pm 1.0\%$) compared to E10 ($26.5 \pm 4.8\%$) or E12 injection ($19.9 \pm 2.7\%$). These data corroborate that *in utero* electroporation predominantly labels neuronal populations in the OB that are being generated at the time of electroporation. We do note that there were GFP+ projection neurons labeled with BrdU injected at a day different from electroporation. For example, we did not find a significant difference in the percentages of E10 electroporated projection neurons labeled with BrdU injected at E10 and E11. We speculate that some plasmids may be introduced into progenitor cells that are not currently dividing and therefore may remain viable until the division occurs at a later time. Despite this minor caveat, there was a clear and definitive correlation between the timing of electroporation and the birthdates of labeled cells as confirmed with BrdU labeling. Hereafter, we performed *in utero* electroporation at E10 or E12 for the preferential labeling of early- or late-born olfactory bulb projection neurons, respectively.

Of interest, we also note that mitral cells in the AOB were predominately labeled with electroporation performed at E10, with far less labeling when electroporation was performed at E11 or E12 indicating that the timeline of mitral cell generation in the AOB is earlier than that of the MOB (Figure 1F–H). In contrast to the AOB mitral cells, many AOB granule cells were labeled following E12 electroporation (Figure 1H).

Relation between cell location and electroporation timing

Next, we analyzed the distribution of projection neurons expressing GFP in the MOB. We previously reported that localization of mitral cell bodies in the MCL is regulated in part by the timing of cell division; early-born mitral cells are localized in dorsomedial region of the MOB and late-generated in ventrolateral region of the MOB (Imamura *et al.*, 2011). Consistent with our earlier finding, we observed more GFP+ cells in dorsal portion of the MCL in the MOB following electroporation at E10 (Figure 2A, C), while GFP+ mitral cells were preferentially found in ventrolateral MCL following E12 electroporation (Figure 2B, D). In the MOB, not only mitral cells but also tufted cells, which are Tbx21+ cells localized in the EPL, are labeled with our *in utero* electroporation protocol. Consistent with an earlier report using tritiated-thymidine as a marker of cell division (Hinds, 1972), we found that tufted cells are generated later than mitral cells. The proportion of GFP+ tufted cells to GFP + mitral cells in the MOB was larger following electroporation at E12 ($44.3 \pm 3.1\%$) than E10 ($22.0 \pm 0.9\%$) or E11 ($28.8 \pm 3.9\%$) (Figure 2E).

We divided the MOB into four equal quadrants, dorsomedial (DM), dorsolateral (DL), ventromedial (VM), and ventrolateral (VL), and then calculated the proportion of GFP+ mitral and tufted cells localized in each quadrant (Figure 2F, G). In the MOB electroporated at E12, we found significantly more GFP+ mitral cells in VL ($39.0 \pm 3.1\%$), especially compared to DM ($12.4 \pm 1.2\%$) and VM ($23.0 \pm 3.5\%$) (one-way ANOVA [F(2.133, 14.93) = 11.06, $p = 0.0010$] followed by Tukey HSD test). Of interest, regional differences in the distribution of tufted cells were less obvious. However, since tufted cell generation peaks later than E12 (Hinds, 1972), analyses of tufted cells generated at later time points may reveal a similar but as yet unidentified distribution rule in the MOB.

Relation between dendrite extension and electroporation timing

Projection neurons in the MOB extend an apical dendrite radially into a single glomerulus where they receive afferent input from the axons of olfactory sensory neurons. Their secondary dendrites distribute in the EPL perpendicular to the plane of the apical dendrite. The secondary, or lateral, dendrites of mitral cells have been of long interest due to their integration of local circuit synaptic interactions with MOB granule cells. When electroporation was performed at E10, the GFP signal in the secondary dendrites was predominantly in the deep EPL, proximal to the MCL, in the P7 MOB; labeling of secondary dendrites was less evident in the superficial EPL (Figure 3A). In sharp contrast, the GFP signal in the secondary dendrites was predominantly found in the superficial EPL of following E12 electroporation of the MOB (Figure 3B). To quantify our observation, we radially subdivided the EPL into sublamina. Since Tbx21 stained dendrites of mitral and tufted cells, we defined the EPL as Tbx21+ area, and divided it into three sublamina; superficial, middle, and deep. By measuring the GFP+ area in each sublamina, we calculated the proportions of GFP signals localized in each area (Figure 3C). The proportion of GFP signal found in the superficial EPL was the largest when the MOB was electroporated at E12 ($43.7 \pm 2.9\%$) and the least following E10 electroporation ($24.8 \pm 2.0\%$). These data suggest that in addition to localizing in different regions of the MCL, early- and late-born projection neurons extend their dendrites preferentially in the deep and superficial EPL, respectively.

We further examined whether the dendritic segregation of early- and late-born projection neurons in the EPL was preserved in the MOB of mice at 3 weeks of age. The EPL can be molecularly subdivided into sublamina using the neuronal marker protein PGP9.5, which is expressed mainly by the somata and thick dendrites of mitral cells (Taniguchi *et al.*, 1993; Imamura *et al.*, 2006). Because of its selective expression pattern, strong PGP9.5 immunoreactivity was found in the MCL and the deep half of the EPL and less so superficially. When we double-labeled with PGP9.5 the dendrites of GFP+ projection neurons labeled with E12 electroporation overlapped with the PGP9.5-negative, superficial, region of the EPL. In contrast, the majority of GFP+ dendrites were found in PGP9.5-positive, deep, region of the EPL when electroporation was at E10 (Figure 3D, E). It has been generally acknowledged that mitral and tufted cells separately extend their lateral dendrites in deep and superficial EPL sublayers, respectively (Mori, 1987). Since E12 electroporation labeled a larger proportion of tufted cells than electroporation at E10, it is reasonable to assume that GFP+ dendrites localized in the superficial GFP were derived in part from tufted cells. To confirm, we reconstructed the morphologies of GFP+ cells in E12 electropolated MOB and found tufted cells projecting their dendrites throughout the superficial EPL, as expected (Figure 3F). However, in contrast to widely held conventions, we also found many mitral cells that projected their secondary dendrites into the superficial EPL when electroporation was at E12 (Figure 3G). This result implies that even among mitral cells, the target region of the secondary dendrites in the EPL is in part regulated by the timing of neurogenesis.

Relation between axonal pathway and electroporation timing

Finally, as our understanding of how the timing of neurogenesis was affecting the organization of mitral cells locally within the MOB, we next asked if these differences also extended to the efferent axonal projections of the early- and late-born MOB neurons. All projection neuron axons pass through the lateral olfactory tract (LOT), located at the antero-ventro-lateral portion of the mouse brain, en route to innervating the olfactory cortices. It was previously suggested that newly generated immature axons of projection neurons were added successively to the most superficial part of LOT during embryonic development (Inaki *et al.*, 2004). However, whether this subdivision is preserved in the mature LOT was not pursued. Here, we used our electroporation strategy to examine the GFP labeled axonal pathways of projection neurons in the LOT to determine if the timing of neurogenesis contributed to a preserved pattern of axon lamination. When we labeled the early-born projection neurons with E10 electroporation and observed at P7, GFP-labeled axons seen throughout the LOT, as defined by neuropilin-1+ (Figure 4A). In contrast, GFP-labeled axons were restricted to the most superficial region of the LOT when electroporation was performed at E12 (Fig 4B). These data support the notion that the timing of neurogenesis establishes sublamina in the LOT that are preserved in the mature LOT at P7. We previously showed that the olfactory tubercle received heavier axonal input from late-born mitral cells as well as tufted cells. Taken together, we can conclude that axons projecting to different regions of the olfactory cortices are segregated into different sublamina of the LOT.

Discussion

Here, we introduced plasmid that drives the expression of a GFP gene into projection neurons of the mouse OB using *in utero* electroporation. By changing the timing of electroporation after fertilization, subpopulations of AOB mitral cells, MOB mitral cells, and tufted cells were differentially labeled. AOB mitral cells were predominantly labeled with E10 electroporation, while the largest proportion of labeled MOB tufted cells was seen with E12 electroporation. Since mouse MOB mitral cells are generated in embryonic brain with a peak at E11, and generation of AOB mitral cells occurs prior to MOB mitral cells while the peak of tufted cell generation comes around E15 (Hinds, 1972; Blanchart *et al.*, 2006; Imamura *et al.*, 2011), the plasmid vectors were introduced into distinct subsets of OB projection neurons based on their timing of neurogenesis. Combining electroporation with BrdU injection, we further confirmed that E10 and E12 electroporations preferentially labeled early- and late-born projection neurons, respectively.

Birthdate-dependency is a widely applied rule influencing the organization of neuronal circuits in the nervous system. In the cerebral cortex, cortical pyramidal neurons having different birthdates migrate toward distinct layers with an inside-out manner, and possess distinct dendritic morphologies and axonal target regions (Molyneaux *et al.*, 2007; Rakic *et al.*, 2009). In the dorsal spinal cord, the extensor and flexor premotor interneurons are spatially segregated in medial and lateral portions in a birthdate-dependent manner, although they are derived from common progenitor domains (Tripodi *et al.*, 2011). At the circuit level, in the hippocampus it was suggested that synapses preferentially form between neurons generated within narrow time windows (Deguchi *et al.*, 2011). We previously reported using thymidine analogs that early- and late-generated mitral cells were differentially localized in the dorsomedial and ventrolateral MCL, respectively, in the MOB (Imamura *et al.*, 2011). Those data suggested the possibility that MOB projection neurons generated at different time points are differentially integrated into distinct neuronal or synaptic circuits. Here we provide new data that supports that notion and moreover, introduces a hypothesis that these two distinct subpopulations of mitral cells can be distinguished based on the sublamina organization of their secondary dendrites. We have now shown the segregated dendritic extension of early- and late-born projection neurons in the deep and superficial EPL, respectively. Though a similar segregation has been suggested between mitral and tufted cells (Mori, 1987), the cohort of mechanisms underlying the differential topography or the involvement of tufted and mitral cells in olfactory circuitry remain elusive. We showed here that there are mitral cells projecting secondary dendrites toward the superficial EPL. Since such mitral cells were often observed in the olfactory bulb electroporated with GFP at E12. Our preliminary observation of P23 OB slices indicates that at least 30% of the mitral cells labeled with E12 electroporation have secondary dendrites projecting to the superficial EPL (22 among 68 mitral cells). However, this is a conservative estimate because the dendrites of labeled cells were often cut during the slice preparation. The percentage of mitral cells whose secondary dendrites distribute preferentially in the superficial EPL may be larger if we could fully reconstruct the entire dendritic arbor. Our observation indicates that even among mitral cells dendritic extension in the EPL was regulated, at least in part, with the timing of neurogenesis.

The criterion that differentiates mitral cells and tufted cells is the location of cell bodies. However, the boundary between the MCL and EPL is not clear, and the cells located close to the boundary were sometimes classified as displaced mitral cells and sometimes as internal tufted cells (Shepherd *et al.*, 2004). Many mitral cells labeled with E12 electroporation were included in these populations. One possibility is that mitral and tufted cells are not totally different cell types. Rather, their properties and the locations of their circuitry within the MOB shift along a continuum during the course of development. Previous reports suggested another sublamina in between deep and superficial EPL that was innervated with mitral cell dendrites and labeled with cytochrome oxidase (Mouradian & Scott, 1988; Ezeh *et al.*, 1993). According to our hypothesis, if there is a subpopulation of mitral cells whose dendrites are restricted to this intermediate sublamina, they should be generated before E12, but in a very narrow time window. As we work toward narrowing the time window of *in utero* electroporation, we may be able to detect those mitral cells falling within this narrow range.

There is a continuing interest in adult neurogenesis in the OB and the contributions made by newly generated granule cells that establish reciprocal dendrodendritic synapses with the mitral cell secondary dendrites (Whitman & Greer, 2007; 2009; Gheusi & Lledo, 2014). There has been a suggestion that superficial granule cells, whose dendrites preferentially innervate the superficial EPL, may be longer surviving than granule cells innervating the deep EPL and whose cell bodies are found in the deeper granule cell layer where newborn neurons are most abundant in the adult (Mandairon *et al.*, 2006). This raises the very interesting possibility that the segregation of mitral cell secondary dendrites may contribute to a selective formation of EPL dendrodendritic circuits that differentiates between longer surviving granule cells in the superficial EPL and new formed granule cells, thought to be particularly important for learning new tasks, in the deeper EPL.

Parallel pathways derived from OB projection neurons to higher order centers have been of much interest, in part due to the possibility that they regulate quite specific odor-related behaviors (Nagayama *et al.*, 2004; Fukunaga *et al.*, 2012; Payton *et al.*, 2012). The posteromedial cortical amygdala receives input only from AOB mitral cells (von Campenhausen & Mori, 2000), whereas both the AOB and MOB mitral cells send axons to the medial amygdala, although to different medial amygdala sublamina (Kang *et al.*, 2009). Piriform cortex receives massive input from mitral cells and much less from tufted cells (Haberly & Price, 1977; Scott, 1981), while the olfactory tubercle is innervated heavily by tufted cells and by small subsets of mitral cells (Scott, 1981; Nagayama *et al.*, 2010; Igarashi *et al.*, 2012). Moreover, recent studies revealed that the cortical amygdala receives heavier axonal input from mitral cells in the dorsomedial MCL, while more ventrolateral mitral cells send their predominant innervation to the olfactory tubercle (Miyamichi *et al.*, 2010; Imamura *et al.*, 2011). These differential projection patterns are also reflected in mouse behavioral studies. Innate aversive behavior toward predator odors is strongly mediated by projection neurons in the dorsomedial olfactory bulb, consistent with their projections to the amygdala (Kobayakawa *et al.*, 2007; Cho *et al.*, 2011; Dewan *et al.*, 2013). However, mechanisms regulating different axonal projection patterns have not been well studied. Here, we showed that we could trace the axons of electroporated cells, and that early- and late-

generated OB projection neurons pass through the different sublamina within the LOT. Therefore, the target of the projection neuron axons may also be regulated by birthdates. Unfortunately, it was difficult to analyze the innervation pattern of projection neurons in detail due to the GFP expression in the cells outside of the OB, including olfactory cortex, following electroporation. Transgenic mouse lines that express Cre recombinase exclusively in the OB projection neurons can be used to overcome the problem. (Nagai *et al.*, 2005; Feng *et al.*, 2009; Haddad *et al.*, 2013). Combination of our *in utero* electroporation technique and the Cre/loxP recombination system can drive the GFP expression exclusively in the OB projection neurons generated within a specific time window. Development of the technique that would enable us to separately label the AOB mitral cells, MOB mitral and tufted cells is prerequisite to test our hypothesis that the targeting of OB projection neuron axons is temporally regulated, which is our next research goal. The results will provide us with further insights into the parallel pathways of the olfactory information processing in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

OB	olfactory bulb
MOB	main olfactory bulb
AOB	accessory olfactory bulb
EPL	external plexiform layer
MCL	mitral cell layer
LOT	lateral olfactory tract
BrdU	5-bromo-2'-deoxyuridine
GFP	green fluorescent protein

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Figure 1. Differential labeling of OB projection neurons based on the birthdates (A–D) OB coronal sections in which *in utero* electroporation was performed at E10 (A, B) or E12 (C, D). Cells electroporated with pGFP are visualized with GFP signals enhanced with anti-GFP antibody (green). BrdU that was injected into pregnant dams at E10 (A, C) or E12 (B, D) was immunohistochemically detected with anti-BrdU antibody (red). Projection neurons in the OB are labeled with anti-Tbx21 antibody (blue). Many cells double-labeled with GFP and BrdU, which are seen in yellow (arrowheads), are found when the electroporation and BrdU injection were performed at the same day (A, D). (E) Percentages of BrdU-labeled cells (BrdU+/GFP+/Tbx21+) among electroporated projection neurons (GFP+/Tbx21+) are shown in different combinations of electroporation and BrdU injection timings. The largest percentages are seen when both the electroporation and BrdU injection were performed at the same day. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA followed by Tukey HSD test ($n = 3$ animals/condition). (F–H) Coronal sections of AOB in which *in utero* electroporation was performed at E10 (F), E11 (G), or E12 (H). While many Tbx21+ (blue) AOB mitral cells were labeled with GFP (green) by E10 electroporation, electroporation at later time points labeled less or no AOB mitral cells. Scale bars, 50 μm in (A–D), and 200 μm in (F–H).

Figure 2. Distribution of electroporated projection neurons in the MOB
 (A–D) Coronal section of P7 OBs electroporated pGFP at E10 (A) or E12 (B). Projection neurons expressing GFP were detected with immunostaining with antibodies against Tbx21 (blue) and GFP (green). (C, D) Positions of mitral cells in the MCL (red dots) and tufted cells in the EPL (green dots) are separately traced in the OB electroporated at E10 (C) or E12 (D). (E) Graph shows the change in proportion of GFP expressing mitral and tufted cell in the MOB electroporated at different time points. (F, G) The MOB electroporated at E10, E11, or E12 were divided into four regions, dorsomedial (DM), dorsolateral (DL), ventrolateral (VL), and ventromedial (VM), and the proportion of GFP-expressing mitral cells (F) and tufted cells (G) located in each region is shown. When electroporation was

performed at E12, GFP+ mitral cells were preferentially distributed in VL region. ** $p < 0.01$; one-way ANOVA followed by Tukey HSD test ($n = 9$ animals/condition). Scale bars, $200\mu\text{m}$.

Figure 3. Sublaminar organization of projection neuron dendrites in the EPL

(A, B) To analyze the distribution of the secondary dendrites of projection neuron, the EPL where Tbx21+ processes (blue) are seen is equally subdivided into superficial middle, and deep. (C) The proportion of GFP+ processes observed in each EPL sublamina. Percentages of GFP+ area observed in each sublamina among total GFP+ area in the EPL are shown and compared between different electroporation timing (n=9 animals/condition). (D, E) Coronal section of the OBs from 3-week old mice in which pGFP electroporation was performed at E10 (D) or E12 (E). When the electroporation was performed at E10, GFP+ processes (green) is preferentially observed in PGP9.5+ deeper sublamina of the EPL (red). While

GFP⁺ processes is predominantly distributed the superficial sublamina that is negative to PGP9.5 with E12 electroporation. **(F, G)** Reconstructed morphology of a tufted cell whose soma is in the EPL (F) and a mitral cell whose soma is in the MCL (G) in the OB electroporated pGFP at E12. Both the tufted and mitral cells extend secondary dendrites preferentially in the superficial sublamina of the EPL. Scale bars, 20μm in (A, B); and 100μm in (D–G).

Figure 4. Segregation of axonal path in the LOT

(A, B) Coronal sections of anterior part of the mouse brain where the LOT is observed. The LOT is defined as NP1+ area (red). When electroporation was performed at E10, GFP signal is seen throughout the LOT (A). However, strong GFP signal is observed only at the most superficial sublamina of the LOT with E12 electroporation (B). Scale bars, 200 μ m.

Table 1**Antibodies: Sources, Specificities, Dilutions**

Primary antibody	Source, catalog #, Immunogen, Specificity	Dilution
1. anti-BrdU antibody (rat, monoclonal)	Source: Accurate Chemical & Scientific Corporation (Westbury, NY), OBT0030 Clone: BU1/75 (ICR1) Preparation: Purified IgG prepared by affinity chromatography on Protein G Specificity: Recognizes BrdU incorporated into single stranded DNA, attached to a protein carrier and free BrdU. Cross reacts with chlorodeoxyuridine (CldU) but does not cross react with thymidine or iododeoxyuridine (IdU) (Imamura et al., 2011).	1:100
2. anti-GFP antibody (chicken, monoclonal)	Source: abcam (Cambridge, MA), ab13970 Immunogen: Recombinant full length GFP Specificity: Recognizes a single band on western blot of around 27–30 kDa, whereas no band was apparent in a wild-type control (manufacturer's information).	1:1000
4. anti-PGP9.5 antibody (rabbit, polyclonal)	Source: CEDARLANE (Ontario, Canada); CL95101 Immunogen: Human PGP9.5 protein purified from pathogen-free human brain Specificity: Recognizes PGP9.5 (~23 kDa) by immunoblot assay (Kitamura et al., 2001). Immunohistochemical staining pattern of cellular morphology and distribution in the olfactory bulb was identical to previous report (Imamura et al., 2006).	1:2000
5. anti-Neuropilin-1 antibody (goat, polyclonal)	Source: R&D Systems (Minneapolis, MN); AF566 Immunogen: mouse myeloma cell line NS0-derived recombinant rat Neuropilin 1 Specificity: Recognizes Neuropilin-1 (~120 kDa) by immunoblot assay (Giacobini et al., 2014). Immunohistochemical staining pattern of cellular morphology and distribution in the lateral olfactory tract was identical to previous report (Inaki et al., 2004).	1:500
6. anti-Tbx21 antibody (rabbit, polyclonal)	Source: Dr. Y. Yoshihara (Saitama, Japan) Immunogen: Synthetic peptide, 20 aa (GAPSPFDKETEGQFYNYFPN) from C-terminal of mouse (Yoshihara et al., 2005) Specificity: Recognizes Tbx21 (58 kDa) by immunoblot assay (Mizuguchi et al., 2012). Immunohistochemical staining pattern of cellular morphology and distribution in the olfactory bulb was identical to previous report (Imamura et al., 2011).	1:10000