New granule cells in the olfactory bulb are associated with high respiratory input in an enriched odor environment

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ABSTRACT

New neurons are constantly generated in the olfactory bulb and the dentate gyrus of the hippocampus. The number of new cells depends on sensory experiences; an enriched odor environment increases neurogenesis and neural survival. The aim of this study was to investigate whether enriched olfactory stimuli affect neurogenesis of mitral and granule cells of the olfactory bulb and dentate gyrus, and whether respiratory activity accompanied by olfactory stimuli is associated with new cells in these regions. To this end, respiratory activity during enriched odor stimuli was continuously measured in mice and new cells were stained with 5-bromo-2'-deoxyuridine, which selectively labels proliferating cells. An enriched olfactory environment significantly increased neurogenesis of mitral and granule cells in the olfactory bulb, but not in the dentate gyrus. Additionally, an increase of new granule cells under the enriched odor condition was correlated to sniffing frequency power, which had a significantly different pattern from the no-odor condition. A high respiratory frequency with frequent odor stimuli may be associated with activation of granule cells to form inhibitory neurons and this active state might increase granule cell neurogenesis.

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1. Introduction

Neurogenesis occurs in the olfactory bulb and the dentate gyrus (DG) throughout life (Altman and Das, 1965). New neurons that appear in the olfactory bulb originate in the subventricular zone of the lateral ventricles and migrate to the olfactory bulb (Luskin, 1993; Alvarez-Buylla and Garcia-Verdugo, 2002; Lledo et al., 2006). Neurogenesis is dependent on experience and is enhanced by multiple olfactory stimuli rather than a single olfactory stimulus (Rochefort et al., 2002; Lazarini and Lledo, 2011). For example, an enriched odor environment increases neurogenesis and neural survival (Rochefort et al., 2002).

Olfaction involves a unique sensory projection to the olfactory center of the brain. Olfactory perception largely depends on respiratory activity; olfactory molecules reach the olfactory nerve by inspiration. Respiration plays an important role in initiating sensory transduction in olfactory sensory neurons, and respiratory rhythmic activity synchronizes with the bulbar neural circuit (Spors et al., 2006).

Animal studies have shown that sniffing multiple odorants rather than a single odorant enhances the neurogenic capacity of the olfactory bulb (Rochefort and Liedo, 2005; Lazarini and Lledo, 2011; Politi et al., 2020). Inspiration with air intake activates olfactory neurons in the bulb (Grosmaire et al., 2007). In humans, exposure to olfactory stimuli improves olfactory ability, and olfactory training or ‘sniffing’ improves olfactory function in patients with olfactory abnormalities (Hummel et al., 2009; Damm et al., 2014). Such sniffing results in a significant improvement in olfactory function using multiple types of odorants (Altundag et al., 2015). Based on these studies, our hypothesis was that sniffing activity as a part of respiratory activity might be important for
neurogenesis, and may be associated with improved olfactory ability.

In the present study, our first aim was to investigate whether enriched olfactory stimuli affect neurogenesis of the mitral cell layer (MCL) and granule cell layer (GCL) of the olfactory bulb and the DG in the hippocampus, which are regions that continuously generate new neurons.

Our second aim was to assess whether sniffing activity accompanied by olfactory stimuli is associated with new cells in the olfactory bulb and DG. To this end, respiratory activity was continuously measured in mice during exposure to enriched odor stimuli and new cells were stained with 5-bromo-2'-deoxyuridine (BrdU), which selectively labels proliferating cells.

2. Materials and methods

2.1. Experimental animals

Eighteen wildtype male C57BL/6J mice at 8 weeks of age were purchased from Sankyo Labo Service (Tokyo, Japan). The animals were bred in our animal care facility under conventional holding conditions, housed in cages with a 12-h light/dark cycle, and provided with water and food ad libitum. Eighteen mice were randomly divided into two groups: the enriched odor condition (weighing 23.3 ± 0.5 g) and no-odor condition (weighing 23.1 ± 0.3 g). No significant difference in body weights was found between the two groups (t-test, P > 0.05).

All experiments were performed in accordance with protocols approved by the Showa University Animal Experiment Committee based on the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Measurement of respiration

The apparatus used to measure respiratory activity in mice under enriched odor and no-odor conditions is shown in Fig. 1A. This setup has been described by Hegoburu et al. (2011). A plethysmograph is composed of two chambers: a subject chamber and a reference chamber. A differential pressure transducer (model dpt, emka TECHNOLOGIES, Osaka, Japan) was connected to both chambers to measure pressure differences; this signal reflects the respiratory activity of each mouse.

Two whole-body plethysmographs (diameter: 30 cm, height: 30 cm) (emka TECHNOLOGIES) were placed in a sound-attenuating box (Bio Research Center Co., Ltd., Tokyo, Japan; width: 60 cm, length: 41 cm, height: 48 cm) equipped with a fan to allow continuous evacuation of the odorant (LE26A, Bio Research Center Co., Ltd.). One chamber was used for the enriched odor condition and the other was used for the no-odor condition.

To maintain a constant flow through the plethysmograph, a ventilation pump (VENT2; emka TECHNOLOGIES) was connected to the whole-body plethysmograph to vacuum out the equivalent of the air pushed into the chamber by the air pump at 1000 mL/min (Linicon, LV-125A, Nitto Kohki Co. LTD). Before each session, the plethysmograph was calibrated by pushing 1 mL of air into the chamber. The pressure variation was recorded to measure the respiratory volume. The air-pushed side of the ventilation pump was connected to a 50-mL glass bottle with odorants to deliver odors into the chamber (enriched odor condition). The other chamber was arranged in the same manner and the pump was connected to another 50-mL glass bottle to deliver air (no-odor condition).

The measured data from the differential pressure transducer were acquired as the flow signal, which was 1 kHz of sampling frequency, and stored in an IOX software (emka TECHNOLOGIES). The plethysmograph...
measures natural breathing, and the signal appears as a periodic phenomenon showing alternating negative (inspiration) and positive (expiration) deflections (Fig. 2A). The algorithm that was used included signal smoothing for noise reduction and detected zero-crossing points to define the inspiration and expiration phases. The detection of respiratory cycles was based on an algorithm described in Roux et al. (2006).

Animal behavior and respiratory cycles were simultaneously monitored using an infrared camera (SQ-11, Safetyride, Tokyo, Japan) placed in the corner of the sound-attenuating cage to confirm the following specific behaviors for offline analysis: sniffing, exploration, freezing, and sleeping.

To calculate the power of the sniffing frequency, raw data of flow signals in the enriched odor and no-odor condition phases were extracted as text data, which were used for spectral analysis using MATLAB (R2015B; MathWorks Inc., Natick, MA, USA). The flow pressure signal was band-pass filtered (0.1–100 Hz) and Welch’s method was adopted for spectral density estimation with an analysis at a 1-s window length and 50% window size. The extract range was defined as 0–40 Hz and we analyzed 3–5 Hz as resting respiration and 9–11 Hz as sniffing (Wesson et al., 2008). We also analyzed the 6–8 Hz frequency in the same manner for an exploratory analysis.

2.3. Delivery of olfactory stimulation

Before olfactory stimulation, we assessed the olfactory function of all mice to ensure that they had normal olfactory function. The evaluation of olfactory function was conducted using cycloheximide, which is commonly used as a repellent of rodents (Ozaki et al., 2010; Shig et al., 2008). For 48 h before the first and second tests, the mice were not provided with access to water. Avoidance behavior of the cycloheximide solution was examined by testing the ability to distinguish a 0.01% cycloheximide solution from tap water. Water bottles with or without cycloheximide were positioned randomly (Supplemental Fig. 1A). Ten trials were repeated each time to evaluate the avoidance behavior (% success rate) (Supplemental Fig. 1B).

For the enriched odor condition, five kinds of odorant (lemon, lavender, orange, bergamot, and lemon grass; scientific names: *Citrus limon, Lavandula angustifolia, Citrus sinensis, Bergamia*, and *Cymbopogon citratus*, respectively) (Pranarom & Kensoigakusha Co. Ltd., Yamanashi, Japan) were selected because they had been used in a previous report (Rochefort et al., 2002). Enriched odor mice were exposed to two types of odors for 30 min daily for 5 days a week over 4 weeks. For the enriched odor condition, filter paper was placed in another 50-mL glass bottle to deliver air to the chamber of the no-odor condition. Mice from both groups were placed in the chamber for 15 min as a resting period and then continuously received odorant or non-odorant stimulation for 30 min.

2.4. BrdU labeling

Animals were injected intraperitoneally with a single dose of the S-phase marker BrdU (50 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) for 5 consecutive days after starting the olfactory stimulation (Fig. 1B). For each coronal section, cells with BrdU incorporated into DNA were detected by immunohistochemistry.

2.5. Tissue processing

Mice were intraperitoneally anesthetized with medetomidine/midazolam/butorphanol (0.3/4.0/5.0 mg/10 mL/kg body weight). The animals were transcaldurally perfused with 0.9% saline followed by 4% paraformaldehyde in 50 mM phosphate buffer (PB; pH 7.2) and the brain, including the olfactory bulb, was then removed rapidly and carefully.

Tissues of the olfactory bulb and hippocampus were post-fixed overnight, immersed in 20% sucrose in 0.1 M PB for 2 days, and then embedded in liquid nitrogen-cooled isopentane using an embedding solution (20% sucrose in 0.1 M PB; (Optimal Cutting Temperature) O.C. T. compound [Sakura Finetek, Tokyo, Japan]: 2:1). Coronal brain sections (10 μm thick) were prepared using a cryostat (Leica CM1520; Leica Biosystems, Nussloch, Germany). Sections were frozen at −30 °C until use.

2.6. Immunohistochemistry

For multiple staining, sections were washed with phosphate-buffered saline (PBS) containing 0.05% Triton X-100 (PBST), incubated for 30 min with 2 N HCl at 37 °C for DNA denaturation, and then washed with PBST. The sections were then immersed for 2 h in 2% normal horse serum (Vector, Burlingame, CA, USA) in PBST to block non-specific reactions. Next, the sections were incubated overnight with primary antibodies (Table 1). The sections were rinsed with PBST and incubated with appropriate fluorescent dye-labeled secondary antibodies for 2 h. Subsequently, nuclei were stained with 4′,6-diamidine-2-phenylindole (DAPI) and sections were air-dried and mounted under coverslips for imaging. Immunohistochemical analysis was performed using images from the Leica M1630 and M1630 (Leica Microsystems, Wetzlar, Germany). Diluted antibodies were used as follows: goat anti-BrdU (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-NeuN (1:500; Chemicon International, Temecula, CA, USA), mouse anti-GFAP (1:200; Sigma-Aldrich, St. Louis, MO, USA), and rabbit anti-NF200 (1:500; Millipore, Billerica, MA, USA). The same antibody was used in the same dilution for each section. Immunohistochemistry was performed on sections from three different animals in each group (enriched odor and no-odor conditions) for staining with BrdU, NeuN, GFAP, and NF200.

**Fig. 2.** Respiratory waveform of mice in enriched and no-odor conditions. A: The plethysmograph measures natural breathing signals, which appear as a periodic phenomenon showing alternating negative (inspiration) and positive (expiration) deflections. An algorithm performed signal smoothing for noise reduction and detected the zero-crossing points to define the inspiration and expiration phases. B: Examples of raw sniffing signals recorded by the plethysmograph during the enriched odor (left) and no-odor (right) conditions. The representation of these raw signals on time-frequency maps are included beneath the plethysmograph recordings.
Table 1  Primary antibodies used for immunohistochemistry.

| Primary antibodies (Clone#) | Host | Companies | Catalog# | Dilution | Target |
|-----------------------------|------|-----------|----------|----------|--------|
| BrDU | rat | Abcam (Cambridge, UK) | ab6326 | 200 | Proliferating cell |
| NeuN | rabbit | Cell signaling (DANVERS, MA) | 243075 | 1000 | Mature neuron |
| GFAP | rabbit | Bioss (Boston, MA) | bs-0199R | 500 | Astrocyte |
| Iba1 | rabbit | Wako (Osaka, Japan) | 019–19741 | 1000 | Microglia |
| DCX | rabbit | Cell signaling (DANVERS, MA) | 4604S | 500 | Neural progenitor cell |

BrdU: 5-Bromo-2′-deoxyuridine, NeuN: neuronal nuclei, GFAP: glial fibrillary acidic protein, Iba1: ionized calcium-binding adapter molecule 1, DCX: doublecortin, OMP: olfactory marker protein.

dihydrochloride (DAPI, 1:5000; Roche, Manheim, Germany) for 5 min. The anatomical position of each layer in the olfactory bulb and hippocampus was confirmed by coronal sections stained with Nissl. In the anatomical structures, immunopositive cells were observed under an AX70 DP2-BSW fluorescence microscope with image analysis software (Olympus, Tokyo, Japan) or a BZ-700 fluorescence microscope (Keyence, Osaka, Japan). All primary and secondary antibodies used for immunostaining are listed in Tables 1 and 2.

2.7. Cell counting

To quantify the numbers of BrdU-positive cells and double-labeled cells, we used combined tiled images (MCL in olfactory bulb: 1 × 10³ μm², GCL: 1 × 10⁵ μm², and DG in hippocampus: 4 × 10⁵ μm²) after each image was acquired by microscopy with ∼20 or ∼10 objective lenses. For counting, we used one of the 10 prepared series of coronal sections. The interval between the counted sections was 160 μm to avoid multiple counts of individual neurons. Cell counts of BrdU-, BrdU/Neuronal Nuclei( NeuN-), BrdU/Doublecortin(DCX-), BrdU/Gliai Fibrilary Acidic Protein(GFAP-), and BrdU/ Ionized calcium binding adaptor molecule 1 (Iba1)-positive cells were performed blindly by a researcher who was not involved in the staining or imaging.

2.8. Statistical analyses

Sniff frequency was analyzed by power spectra analysis. Each mouse respiratory raw waveform was extracted as a text file using MATLAB and the power of sniff frequency was analyzed in the range from 1 to 40 Hz in offline mode. Power of the respiration frequency in the range from 3 to 5 Hz (resting) and 9–11 Hz (sniffing) between the enriched and no-odor conditions was compared using a distribution-free test, the Mann–Whitney U-test. For the exploratory analysis, the comparison of 6–8 Hz between the enriched and no-odor conditions was performed in the same manner. The number of labeled neurons is presented as the mean ± standard error of mean(SEM). Cell counts were compared between odor and control groups using the Mann–Whitney U-test.

More than two groups of comparisons for the sniffing power and number of cells analyses were tested using the non-parametric Mann–Whitney U-test. Because a general linear model is a parametric test, all data were first assessed using the Shapiro–Wilk test to test the normality of distribution. After the confirmation of normal distribution (P > 0.05), a general linear model was constructed to determine whether a differential relationship existed between the number of new cells and sniff frequency. The number of newborn cells was set as the dependent variable and the condition (enriched or no-odor condition) was set as the fixed variable, with the power of the sniff frequency included as a covariate. Each model included the main effects of the condition and sniff and condition × sniff interactions. The main effects of condition × sniff interactions were the outcomes of interest.

3. Results

3.1. Sniffing signals

Examples of sniffing signals measured by the plethysmograph are presented in Fig. 2B. A representation of these raw signals on a time-frequency map is presented. Sniffing behavior was confirmed by visual inspection of the video recordings.

The distribution of the averaged sniff frequency from nine mice in the enriched odor condition (red) and nine mice in the no-odor condition (black) is presented in Fig. 3A. No difference in resting respiration frequency and sniffing frequency was found between the enriched and no-odor conditions (3–5 Hz, z = −1.1, P = 0.29, 9–11 Hz, z = −0.39, P = 0.73) (Fig. 3B). Additionally, there was no difference in the power of respiration frequency from 6 to 8 Hz between the two conditions (z = −0.12, P = 0.22). Overall, similar resting respiration and sniffing was observed in both the enriched and no-odor conditions.

3.2. Increase in BrdU-positive cells

Structural images with Nissl staining of the olfactory bulb and hippocampus are presented in Fig. 4A. All BrdU-positive cell counts were conducted in a blinded manner with regard to mouse status. BrdU-positive cells were counted on a series of sections. BrdU-positive and double-positive (NeuN/BrdU) MCL cells were significantly increased during the enriched odor condition (BrdU-positive cells, t = 3.01, P = 0.008, NeuN/BrdU-positive cells, t = 3.82, P = 0.001) (Fig. 4B). BrdU-positive cells and NeuN/BrdU-positive GCL cells were significantly increased during the enriched odor condition (BrdU-positive cells, t = 2.18, P = 0.04, NeuN/BrdU-positive cells, t = 3.49, P = 0.003) (Fig. 4C). There was no significant difference in BrdU-positive and NeuN/BrdU-positive cells in the DG between the two conditions (BrdU-positive cells, t = 1.57, P = 0.13, NeuN/BrdU-positive cells, t = 0.6, P = 0.55) (Fig. 4D).

In the MCL, GCL, and DG, DCX was used as a marker of neuronal progenitor cells (as shown in Extended Data Fig. 2), GFAP was used as a marker of astrocytes (Supplemental material Fig. 3), and Iba1 was used as a marker of microglia (Extended Data Fig. 4). There were no significant differences in cell numbers between the two conditions (all P > 0.05, statistical details in Supplemental material Table 1).

3.3. Sniffing and the increase in BrdU-positive cells

There was no interaction between the enriched and no-odor conditions in terms of the relationship between the resting respiration frequency power of 3–5 Hz and the number of BrdU-positive cells in the GCL (Fig. 5A). There was a significant interaction between the enriched and no-odor conditions in terms of the relationship between the sniffing frequency power of 9–11 Hz and both the number of BrdU-positive cells in the GCL (F(1,14) = 11.17, P = 0.005, partial eta-squared = 0.44) and the number of NeuN/BrdU-positive cells in the GCL (F(1,14) = 6.9, P = 0.02, partial eta-squared = 0.33) (Fig. 5B). Thus, individuals with a

Table 2  Secondary antibodies used for immunohistochemistry.

| Secondary antibodies (Clone#) | Host | Companies | Catalog# | Dilution |
|-------------------------------|------|-----------|----------|----------|
| Rat IgG(Alexa 546) | Goat | Termo Fisher Scientific (Waltham, MA) | A11081 | 400 |
| Rabbit IgG(Alexa 488) | Goat | Termo Fisher Scientific (Waltham, MA) | A11034 | 400 |
high frequency power had more BrdU-positive cells.

There was no interaction between the enriched and no-odor conditions in terms of the relationship between the sniffing frequency power of 9–11 Hz and the number of new MCL and DG cells, and between the resting respiration frequency power of 3–11 Hz and the number of new MCL and DG cells (statistical results shown in Supplemental material Table 2).

4. Discussion

In this study, we confirmed and extended prior findings that an enriched olfactory environment significantly increases neurogenesis in the GCL and MCL of the olfactory bulb, but not in the DG of the hippocampus. The main finding of this study was that the increase of new cells in the GCL under the enriched odor condition correlated to the sniffing frequency power, and had a significantly different pattern from the relationship of the no-odor condition. Specifically, although there was no difference in the sniffing frequency power of 9–11 Hz between the two conditions, an enriched odor environment with higher sniffing power was associated with more new granule cells. This pattern was not observed in the no-odor condition.

4.1. Neurogenesis in the olfactory bulb and hippocampus

Numerous studies indicate that enriched odor exposure increases new neurons in the olfactory bulb (Rosselli-Austin and Williams, 1990; Petreanu and Alvarez-Buylla, 2002; Rochefort, 2002; Yamaguchi and Mori, 2005; Alonso et al., 2006; Mouret et al., 2009). Our results were consistent with previous studies showing that an enriched odor environment of continuous sensory stimuli increases newly formed mitral and granule neurons.

Why exposure to an enriched odor environment increases neurogenesis remains unsolved. It has been suggested that precursors of granule cells require trophic factors to affect neurogenesis (Kichernbaum and Goldman, 1995). Mutation and retention of new neurons are affected by BDNF in animals (Zigova et al., 1998). Another possibility is that expression of tenascin-R, which is dependent on the level of sensory input, accelerates the formation of new neurons in the olfactory bulb (Saghatelyan et al., 2004). How these mediators are associated with new neurons in an enriched olfactory environment remains unknown and requires further research.

In the enriched odor environment, our results showed new neurons in the GCL and MCL of the olfactory bulb, but not in the DG of the hippocampus. This result was in line with the study of Rochefort et al. (2002), who showed that an enriched odor environment increased newly generated neurons in olfactory bulb granule cells, but not in the DG. New neurons are continuously generated in the hippocampus and stem cells located in the subgranular zone of the DG give rise to new granule neurons (for review, see Ming and Song, 2011). The MCL of the olfactory bulb projects olfactory information via the entorhinal cortex, the gateway to the DG of the hippocampus, which might play an important role in olfactory memory (for review, see Eichenbaum, 2017).

Our study showed that simple odor exposure does not affect hippocampus neurogenesis. Our experimental design might not involve odor-associated memory tasks that cause retrieval of odor-associated memory. Furthermore, neurogenesis of the DG might be associated with olfactory-associated memory tasks and spatial memory. Indeed, a study found that a spatially enriched environment led to new cells in the DG, changes in hippocampal thickness and dendritic arborization, and an increase in the number of glial cells (Kempermann et al., 1997). Kempermann et al. (1997) suggested that not only an external source of rich stimuli but also individual plasticity and function might be involved in the complex interaction between hippocampal neurogenesis and individual behavior. However, a number of factors were lacking in our study, including memory retrieval, motivation, and intention toward the olfactory stimulus; thus, further studies of hippocampal neurogenesis are needed.

4.2. A possible relationship between respiratory activity and neurogenesis of granule cells in the olfactory bulb

The most interesting finding in this study was that the increase of GCL neurons in the enriched odor environment was correlated to the individual respiratory response. Thus, odor associated with a high sniffing rate may be linked to neurogenesis of the GCL in the olfactory bulb. How olfactory neurons of the MCL and GCL interact with respiration might be important to explain our results because only new GCL cells were positively correlated to respiration responses.

Synaptic inputs from olfactory sensory neurons process to the glomerular layer, which is the gateway to the olfactory bulb (for review, see Mori et al., 1999). From the glomerular layer, olfactory information ascends to the olfactory cortex through two types of neurons—mitral and tufted cells—in the olfactory bulb (Scott, 1981; Nagayama et al., 2004). The deep layers of the olfactory bulb have GCL interneurons that receive synaptic inputs from apical dendrites of mitral and tufted cells, and GCL inhibits both cell types. Burst actions of these mitral/tufted cells occur at the timing of inspiration and expiration during the spontaneous rhythm of respiration (Phillips et al., 2012). The GCL surrounding the MCL is composed of inhibitory networks that respond to inputs from respiration alone and odor stimulation (Pukunaga et al., 2012). Short et al. (2016) reported that MCL activity coupled with the sniffing respiratory rate activates a large population of inhibitory granule cells during odor stimulation. Furthermore, for odor discrimination, Abraham et al. (2010) showed that the manipulation of...
glutamatergic signaling in granule cells alters the synaptic inhibition of mitral cells during odor discrimination. According to Short et al. (2016), odor discrimination might imply that granule cell inhibition modulates respiratory activity without eliminating the gating sensory-evoked response, which may increase the odor-associated signal-to-noise ratio by separating sensory and spontaneous respiratory inputs.

In the present study, GCL under the enriched odor condition was correlated with sniffing frequency power; this active respiratory state toward olfactory sensory inputs likely increases the inhibition of granule cells to increase MCL activity. Thus, a high respiratory frequency under the odor-enriched condition may be associated with the strong activation of GCL inhibitory neurons.

On the basis of these observations, there are two possibilities to interpret our results. The first is that the high respiratory response with odor enrichment accelerates neurogenesis in the GCL. Even in the absence of odor stimuli, inspiration with air intake activates olfactory neurons in the olfactory bulb (Grosmaitre et al., 2007). Breathing oscillation might be a factor that increases neurogenesis. However, in our study, as observed in the group comparisons of respiration, there was no difference in sniffing state between the no-odor and enriched odor groups. In addition, within the no-odor group there was no correlation between sniffing power and GCL neurogenesis. Thus, a high frequency of sniffing might not be the only factor that increases new GCL cells.

Fig. 4. Neurogenesis of mitral and granule cells in the olfactory bulb and hippocampus. A: Layers in the olfactory bulb and hippocampus after Nissl staining in the enriched odor condition (left) and no-odor condition (right). B: Mature neurons in the MCL were stained for NeuN. The bar graph shows the number of cells with BrdU single staining and double staining with BrdU. C: Mature neurons in the GCL were stained for NeuN. The bar graph shows the number of cells with BrdU single staining and double staining with BrdU. Arrows indicate co-labeling of BrdU and NeuN. D: Mature neurons in the DG were stained for NeuN. The bar graph shows the number of cells with BrdU single staining and double staining with BrdU. Arrows indicate co-labeling of BrdU and NeuN. The red bars represent the enriched odor condition mice and the black bars represent the no-odor condition mice. The olfactory bulb has a six-layered structure. ONL: olfactory nerve layer, GL: glomerular layer, EPL: external plexiform layer, MCL: mitral cell layer, IPL: internal plexiform layer, GCL: granule cell layer. Scale bar is 50 µm. The results of cell counts are shown as the mean ± SEM. Statistical analysis was conducted using the Mann–Whitney U-test (*P < 0.05, **P < 0.01). DG: Dentate gyrus. Scale bar is 100 µm. The results of cell counts are shown as the mean ± SEM. Statistical analysis was conducted using the Mann–Whitney U-test (*P < 0.05, **P < 0.01).
The second is the relationship between the amount of odor information and the respiratory response. A high respiratory frequency processes more olfactory information than a low respiratory frequency, and stimulates GCL neurons to undergo activation of inhibition. This assumption is similar to the observation that strong odor information causes high activation of GCL neurons to inhibit the MCL and balance the respiratory cycle and odor processing (Short et al., 2016). A high respiratory response with enriched odor might be involved in increasing new cells.

Together with two possible interpretations, we assume that not only respiratory rhythmic activity but also rhythmic olfactory information might enhance GCL cell neurogenesis.

Although the same species, age, and sex were used in this study, the respiratory response to odor was individual in the odor-enriched environment. Which factors generate such a difference remains unknown, but individual differences might be involved for neurogenesis. For example, it may be argued that the higher sniffing power in some animals was not caused by odor stimuli, but was rather a trait of the individual animals. It is also possible that the observed enhancement of neurogenesis was merely the result of individual animals with higher sniffing power. We had an experimental limitation for the BrdU tracing; we were unable to count the number of cells in each individual animal before the experiment. Because of this limitation, we were only able to demonstrate that there was no difference in the overall sniffing power of 9–11 Hz between the two conditions, while within the enriched odor environment the level of neurogenesis in the GCL was associated with higher sniffing power (9–11 Hz). These findings suggest that sniffing power with constant olfactory stimuli might be a key factor for neurogenesis in the GCL. However, it may also be worth investigating whether individuals with a higher sniffing power at rest (i.e., not during olfaction) or during anxious/fearful states affects neurogenesis.

### 4.3. Limitations and further research

In this study, there was no difference in sniffing between the enriched and no-odor conditions. Two investigators observed animal behavior using offline video recordings, and found that mice in the no-odor condition exhibited sniffing behavior during the delivery of air into the chamber. Although the same sniffing power was observed in both conditions in the present study, this indicated that not only a high sniffing power but also enriched odor stimuli were able to increase neurogenesis of the GCL.

On the basis of this study, there are four future research directions. The first is how new neurons in the DG are associated with respiratory activity in an enriched odor environment, which not only includes an odor-enriched environment but also a rich spatial environment or odors associated with a memory task; for example, by repeatedly applying the same odor. Such experiments may need to include behavioral cognitive tests in young animals and older animals or in animal models of Alzheimer’s disease. The second is how long new cells of the GCL survive after 4 weeks in animals with a high respiratory input. Rochefort and Ledo et al. (2005) hypothesized that decreased cell death of new cells after odor enrichment is counterbalanced by an increase of cell death in older neurons to maintain the total population of granule cells. It may be worthwhile to investigate the differences in long-lasting new cells between animals with high or low respiratory responses in a rich odor environment. However, a careful experimental design must be used for BrdU tracing because a study has indicated that a loss of newborn neurons in the olfactory bulb occurs with a standard dose of BrdU (Platel et al., 2019). Thus, the effects of different doses of BrdU on the survival of newborn neurons might be considered for future studies.

The third is whether an increase of granule cells in animals with a high respiratory response increases olfactory discrimination or memory tasks in behavioral experiments. Further study will be necessary to resolve these issues. However, our study showed that a high sniffing frequency or high respiratory input in addition to odorant stimulation promoted neurogenesis in the GCL in the olfactory bulb.

It should be noted that neurons in the GCL comprise the largest population of neurons in the olfactory bulb, and can be divided into three types—intermediate, deep, and superficial granule cells—according to their dendritic morphologies (Shepherd et al., 2004). In the present study, we did not investigate the cell types of the observed new cells in the GCL; this will be clarified in the fourth possible future research using horseradish peroxidase injection and Golgi staining.

These findings may contribute to the elucidation of therapeutic mechanisms and the establishment of therapeutic methods for olfactory rehabilitation. Olfactory rehabilitation, by providing odor stimulation, is often used as a treatment to restore the sense of smell and improve quality of life in patients with olfactory abnormalities. The mechanism...
underlying this improvement in olfactory ability from the viewpoint of newborn neurons in the olfactory bulb needs to be clarified in future studies.

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Conflict of Interest

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

YM, Sawa K, and AY proposed the study design. Sawa K and AY conducted all experiments. Sawa K, AY, Shotaro K, and KS performed the olfactory stimulation experiments. Sawa K, AY, and HO performed immunohistochemistry. YM, Sawa K, AY, NK, and MH performed the statistical analysis. YM, Sawa K, AY, HK, and MI were responsible for drafting the manuscript. All authors revised the manuscript and provided approval for publication of the content, and agree to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neures.2022.05.007.

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