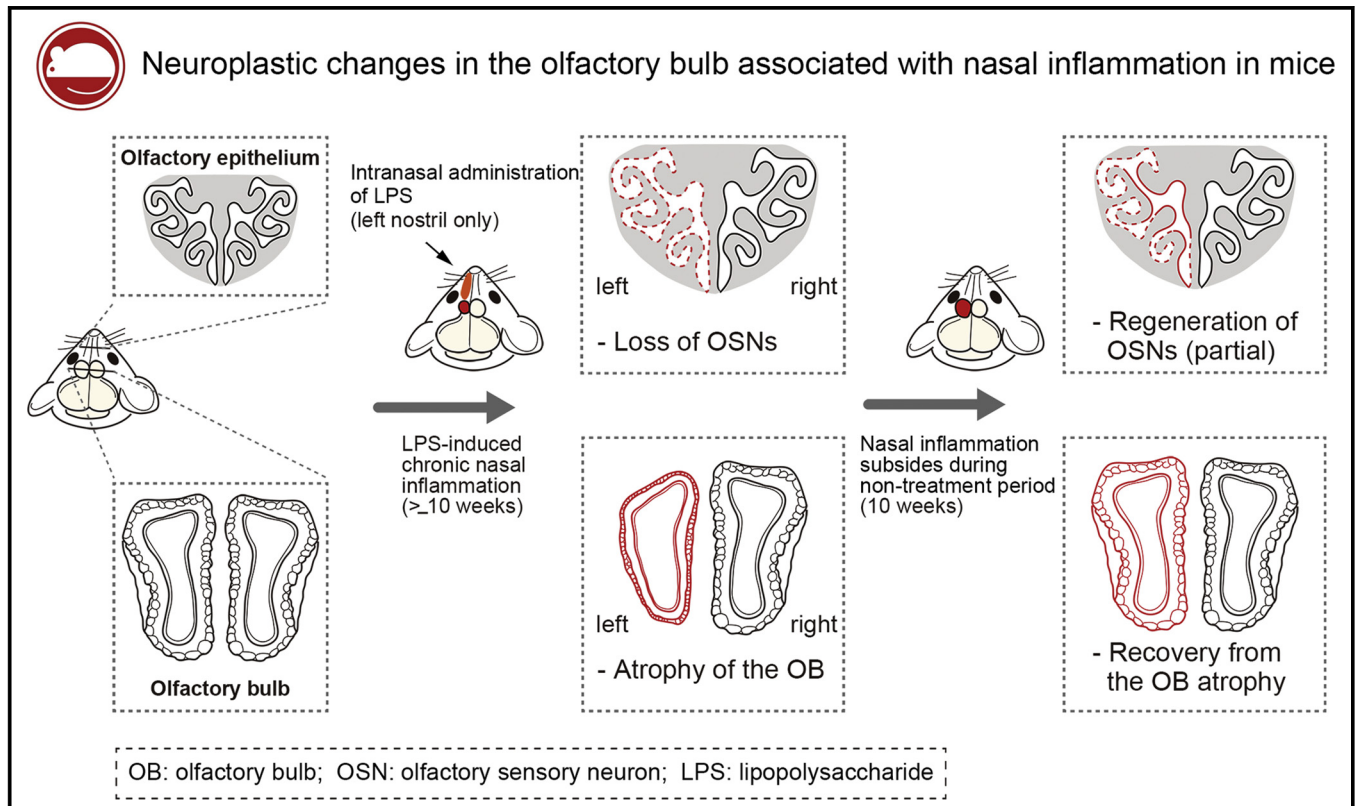


Neuroplastic changes in the olfactory bulb associated with nasal inflammation in mice



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GRAPHICAL ABSTRACT



Background: Rhinitis and rhinosinusitis are olfactory disorders caused by inflammation of the nasal passage and paranasal sinuses. Although patients with chronic rhinosinusitis have smaller olfactory bulbs (OBs), there is limited knowledge regarding the influence of chronic nasal inflammation on OB neurons. **Objective:** Repeated intranasal administration of LPS that induced persistent nasal inflammation in mice caused a loss of

olfactory sensory neurons (OSNs) and gliosis and synaptic loss in the OBs within 3 weeks. The present study aimed to clarify the effects of long-term LPS treatment on the OB neurocircuit. **Methods:** LPS was repeatedly administered into a mouse nostril for up to 24 weeks. For the recovery analyses, the mice received LPS for 10 weeks and were subsequently maintained without additional treatment for another 10 weeks. The effects of these treatments on the OBs were examined histologically. Three or more mice were analyzed per group.

Results: Long-term repeated LPS administration caused OB atrophy, particularly in the layers along which OSN axons travel and in the superficial external plexiform layer, in which tufted cells form synapses with interneurons. Interestingly, the OBs recovered from atrophy after cessation of LPS administration: OB volume and superficial external plexiform layer thickness returned to pretreatment levels after the nontreatment period. In contrast, OSN regeneration was incomplete.

Conclusion: These results suggest that chronic nasal inflammation induces structural changes in a specific OB circuit related to tufted cells, whereas tufted cells retain a high degree of plasticity that enables recovery from structural damages after inflammation subsides. (*J Allergy Clin Immunol* 2019;143:978-89.)

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We are continuously exposed to a variety of potentially harmful environmental agents, such as bacteria, viruses, mold, dust, and pollen. These agents can enter the nasal cavity and become allergens that cause inflammation of the nasal passages, paranasal sinuses (rhinitis, sinusitis, or rhinosinusitis), or both.¹ The symptoms include sneezing, runny, and/or itchy nose and are often associated with an impaired sense of smell.^{2,3} Olfactory loss in patients with acute rhinitis/sinusitis is primarily attributable to blockade of airflow in the nasal cavity; however, damage to the olfactory epithelium (OE) is also considered a potential cause.^{4,5} Intranasal administration of chemical compounds derived from environmental agents, such as LPS, polyinosinic:polycytidylic acid, and mycotoxins, which are derivatives of bacteria, viruses, and mold, respectively, acutely induce inflammatory responses in the OE of rodents.⁶ The inflammatory responses include infiltration of immune cells, activation of macrophages, and secretion of cytokines, such as TNF- α , leukemia inhibitory factor, IL-6, and IL-1 β .⁶

Nasal inflammation further induces the apoptotic death of olfactory sensory neurons (OSNs). A mouse study reported that the number of apoptotic cells in the OE was increased 8 hours after intranasal polyinosinic:polycytidylic acid administration.⁷ However, if the inflammation is transient, the OE can regenerate new OSNs after the inflammatory responses subside.⁷ An animal model using a transgenic mouse line has also indicated the inflammation-induced apoptotic cell death and regeneration of OSNs.⁸⁻¹⁰ Moreover, prolonged nasal inflammation can develop into rhinosinusitis.

According to a multidisciplinary Rhinosinusitis Task Force, chronic rhinosinusitis (CRS) is defined as an inflammatory condition of the olfactory mucosa and the paranasal sinuses that lasts at least 12 weeks.^{11,12} The prevalence of CRS in North America and European countries ranges from 4.5% to 12%.¹³ Epidemiologic studies have shown that approximately 65% of patients with CRS had a decreased sense of smell.¹⁴ Importantly, CRS can cause long-lasting and in some cases permanent olfactory loss.¹ Moreover, histologic analyses of olfactory tissues in patients with CRS showed an abnormal morphology of OSNs. Although impairment of the OE could account for long-term olfactory loss,^{3,15} studies with human patients with CRS have indicated that a smaller olfactory bulb (OB) volume was associated with a greater degree of nasal inflammation.^{16,17} However, the effects of chronic nasal inflammation on the neuronal circuits in OBs have not been systematically investigated with animal models.

Using mice as a model, recently, we reported that repetitive intranasal LPS administration induced persistent inflammation in the olfactory mucosa, which caused not only loss of OSNs but also gliosis and synaptic impairment in the superficial layers of the OBs within 3 weeks.¹⁸ In this study we repetitively administered LPS into the mouse nostril for up to 24 weeks. We report that long-term LPS treatment causes an atrophy of the OBs, which we did not observe at 3 weeks. Moreover, it is noteworthy that the OB volume returns to normal levels after cessation of LPS administration, whereas OSN regeneration was incomplete. These results suggest that the OB shows highly plastic responses to nasal inflammation.

Abbreviations used

ACIII:	Adenylyl cyclase III
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
DAPI:	4'-6-Diamidino-2-phenylindole dihydrochloride
dEPL:	Deep external plexiform layer
EPL:	External plexiform layer
GCL:	Granule cell layer
GL:	Glomerular layer
IPL:	Internal plexiform layer
MCL:	Mitral cell layer
NQO1:	NAD(P)H quinone dehydrogenase 1
OB:	Olfactory bulb
OE:	Olfactory epithelium
ONL:	Olfactory nerve layer
OSN:	Olfactory sensory neuron
sEPL:	Superficial external plexiform layer
TUNEL:	Terminal deoxynucleotidyl transferase dUTP nick end-labeling

METHODS

Animals

The Tbx21Cre $\times$ tdTomato mouse line, in which a red fluorescent protein, tdTomato, is specifically expressed in mitral/tufted cells in the OBs, was used in this study. Because the tdTomato signal is stronger in tufted cells than mitral cells, the superficial external plexiform layer (sEPL) and deep external plexiform layer (dEPL) can be discriminated without further processing. This mouse line was created by crossing B6.CBA-Tg (Tbx21-cre)1Dlc/J mice (stock #024507; Jackson Laboratory, Bar Harbor, Me) with B6.Cg-Gt(ROSA)26Sortm9 (CAG-tdTomato) Hze/J reporter mice (stock #007909; Jackson Laboratory).

After achievement of deep anesthesia with isoflurane, 2-month-old male mice were intranasally administered 10 μ L of physiologic saline or LPS from *Escherichia coli* O55:B5 (1 mg/mL; Sigma, St Louis, Mo) 3 times per week for 3, 10, 18, or 24 weeks. For recovery analyses, the mice were administered LPS for 10 weeks and were subsequently housed without additional treatment for another 10 weeks before death (LPS:10w+NT:10w). LPS or saline was administered only into the left naris of the mice, with the right side maintained intact as an internal control.

For histologic preparation, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with PBS, followed by 4% (wt/vol) paraformaldehyde in PBS. Heads were removed and placed in the same fixative at 4°C overnight. The rostral half of the calvaria (anterior to the bregma) and the nasal bone were then placed *en block* in 0.45 mol/L EDTA in PBS at 4°C for 2 days for decalcification, cryoprotected with 30% sucrose (wt/vol) at 4°C overnight, embedded in OCT compound (Sakura Finetek USA, Torrance, Calif), and maintained at -20°C until use.

In the present study we used only laboratory mice with exactly the same genetic background that were housed in exactly the same environment. We repeated the experiments with at least 3 different animals, which is the same design as in our previous study using similar intranasal LPS administration techniques.¹⁸ Although the sample size might be smaller than that estimated by using a power analysis, the current and previous findings indicated that the data obtained did not vary widely among the mice within the same experimental group. Therefore 3 animals per experimental group were sufficient to be subjected to a statistical analysis. All protocols were approved by and all methods were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Penn State College of Medicine.

Immunohistochemistry

Olfactory tissues were cut on a cryostat into 20- μ m slices, mounted on slides, and stored at -20°C until use. The sections were rehydrated with

TBS-Tween (10 mmol/L Tris-HCl [pH 7.4] and 100 mmol/L NaCl with 0.3% Triton-X100 [vol/vol]), blocked with blocking buffer (5% normal donkey serum [vol/vol] in TBS-Tween) at 20°C to 25°C for 1 hour, and incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Sections were washed with TBS-Tween and incubated with secondary antibodies by using 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific, Waltham, Mass) for nucleus staining for 1 hour. Immunoreacted sections were washed and placed under coverslips with Fluoro-Gel mounting medium (Electron Microscopy Science, Hatfield, Pa).

Primary antibodies used for immunohistochemistry were anti-glial fibrillary acidic protein (rabbit, 1:1000; Agilent Technologies, Santa Clara, Calif), anti-olfactory marker protein (goat, 1:1000; Wako Chemicals, Richmond, Va), anti-adenylyl cyclase III (ACIII; rabbit, 1:200; Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-NAD(P)H quinone dehydrogenase 1 (NQO1; goat, 1:1000; Abcam, Cambridge, United Kingdom). Donkey anti-species IgGs conjugated with Alexa Fluor 488 or Alexa Fluor 647 (Thermo Fisher Scientific) were used as secondary antibodies.

Image analyses and morphometry

All samples were randomly numbered so that the analyses were performed by an experimenter who was blind to sample identities. To measure the size of the OBs, the nasal bones over the OBs were removed after cryoprotection, and the dorsal views of both the ipsilateral and contralateral OBs were imaged by using a digital camera (Canon, Tokyo, Japan) attached to the microscope (Carl Zeiss AG, Oberkochen, Germany). These photo images were used to measure the length and width of the OBs with Photoshop software (Adobe Systems, San Jose, Calif); the length was determined as the length of the rostrocaudal axis between the top and baselines of the OBs, and the width was determined at the middle of the OB length. The proportions of the ipsilateral width and length to those of the contralateral OBs were subsequently calculated (ipsilateral/contralateral) for comparison between the saline- and LPS-treated mice at 3, 10, 18, and 24 weeks ($n = 3, 4, 3$, and 4 for the saline-treated mice and $n = 4, 9, 4$, and 6 for the LPS-treated mice at 3, 10, 18, and 24 weeks, respectively). The same analyses were performed among the saline:10w, LPS:10w, and LPS:10w+NT:10w groups ($n = 4, 9$, and 7 mice, respectively).

The middle OB section was stained with DAPI and divided into each layer to measure the area of the layers in the OB. The area of each layer was measured separately by using Photoshop software. The proportion of each layer was determined by dividing the area of each layer by the total OB area (summation of areas of the glomerular layer [GL], external plexiform layer [EPL], mitral cell layer [MCL] plus internal plexiform layer [IPL], and granule cell layer [GCL]). Values were compared between the saline- and LPS-treated mice at 3, 10, 18, and 24 weeks ($n = 3$ mice per condition).

EPL thickness was quantified by using 3 DAPI-stained OB sections, including rostral, middle, and caudal sections, per mouse. Thicknesses of the total EPL, the length from the top MCL to the bottom GL, and the sEPL, where a strong tdTomato signal was observed, were measured every 200 μm along the tangential axis of the OBs and averaged. The proportion of the sEPL was calculated by dividing the sEPL thickness by the total EPL thickness. The average of the EPL thickness and the proportion of the sEPL were compared between the saline- and LPS-treated mice at 3, 10, 18, and 24 weeks or among the saline:10w, LPS:10w, and LPS:10w+NT:10w groups ($n = 3$ mice per condition).

To quantify the densities of the mitral and tufted cells, we first stained 3 OB sections (ie, rostral, middle, and caudal sections) per mouse and selected an area that spanned approximately 1 mm along the tangential axis of the OBs from each section. Subsequently, we defined the cells that were double positive for DAPI and tdTomato in the MCL and EPL as mitral cells and tufted cells, respectively, and counted their numbers. Densities of the mitral and tufted cells were calculated separately by dividing cell number by MCL length (cells per millimeter) and compared between the saline- and LPS-treated mice at 3, 10, 18, and 24 weeks or among the saline:10w, LPS:10w, and LPS:10w+NT:10w mice ($n = 3$ mice per condition). All numbers shown in the Results section are expressed as means $\pm$ SEMs.

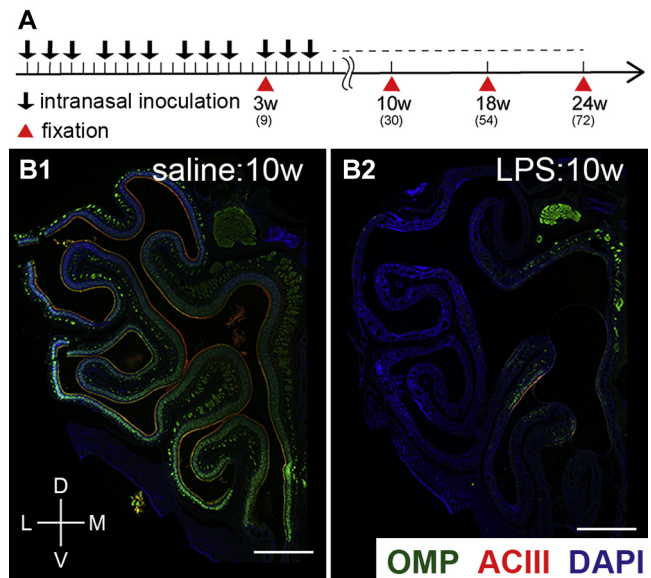


FIG 1. Effects of long-term LPS administration on the OE. **A**, Experimental protocol for intranasal administration of LPS or saline. Mice received repeated intranasal administration of LPS or saline 3 times per week (every other day) and were killed at 3, 10, 18 and 24 weeks after the first administration. Total number of administrations is indicated in parentheses. **B**, Coronal sections of the OE treated with saline (**B1**) or LPS (**B2**) for 10 weeks. OSNs in the saline:10w mice express both olfactory marker protein (OMP; green) and ACIII (red), which are not detected in OSNs of LPS:10w mice. All nuclei are stained with DAPI (blue). Scale bars = 500 μm .

Statistics

Effects of repetitive intranasal LPS administration on the relative OB size, layer area, EPL thickness, proportion of sEPL, and densities of mitral and tufted cells were statistically analyzed by using 2-way ANOVA (2 main effects of treatment and time), followed by a *post hoc* test with Bonferroni correction for multiple comparison. If the *P* value was less than .05, we concluded that a significant difference exists. Recovery analyses (relative OB size, proportion of sEPL, densities of mitral and tufted cells, and proportion of ACIII-positive area) were statistically analyzed by using Bonferroni correction. All statistical analyses were performed by using Statistica software (Dell Software, Round Rock, Tex).

RESULTS

LPS-induced damage in the OE

LPS is an inflammatory endotoxin present in the outer membrane of gram-negative bacteria, such as *E coli* and *Pseudomonas aeruginosa*, which is thought to play a role in the cause of human sinusitis.^{19,20} The left naris of male adult (≥ 2 -month-old) mice was administered LPS (10 μg) or saline 3 times per week for up to 24 weeks (Fig 1, A) to induce chronic inflammation in the nasal cavity. Previously, we reported that repetitive intranasal LPS administration for 3 weeks caused OSN loss predominantly in the lateral region of the OE, which resulted in gliosis and synapse impairment preferentially at the lateral OB.¹⁸ However, LPS administration that lasted for at least 10 weeks caused OSN loss also in the medial region of the OE (Fig 1, B).

Atrophy of the OB

Subsequently, we examined the effects of repetitive intranasal LPS administration on the OB. When killed at 3 weeks, no

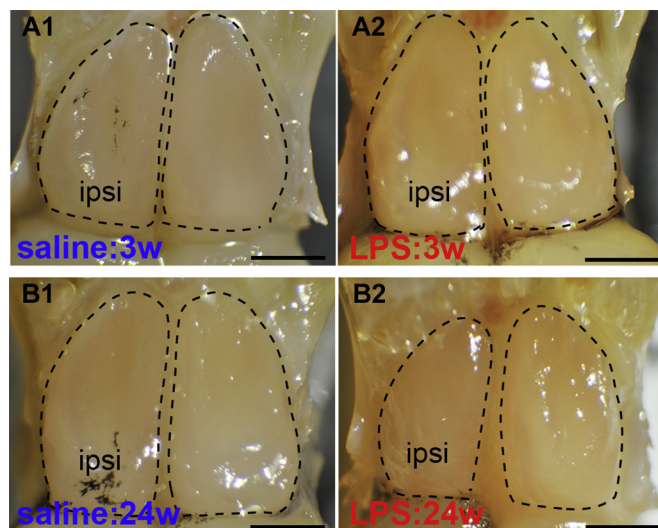
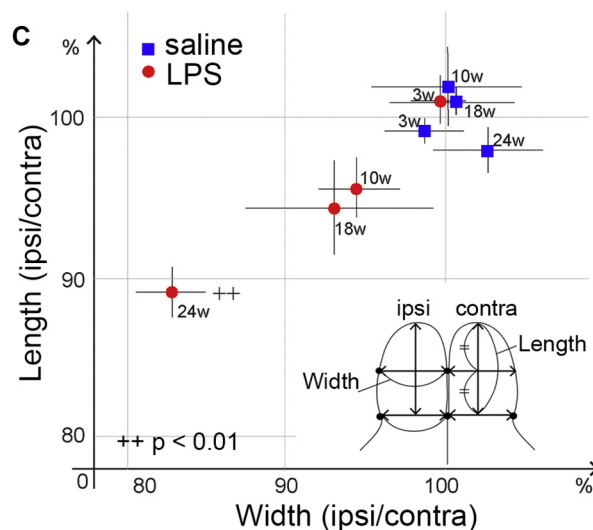


FIG 2. LPS-induced atrophy of the OBs. **A** and **B**, Dorsal view of the OBs of saline:3w (**A1**), LPS:3w (**A2**), saline:24w (**B1**), and LPS:24w (**B2**) mice. There is no difference in OB size between saline:3w and LPS:3w mice. The ipsilateral OBs of LPS:24w mice are prominently smaller than the contralateral OBs and ipsilateral OBs of saline:24w mice. **C**, Repeated LPS administration decreases the relative size of ipsilateral OBs to contralateral OBs (ipsilateral/contralateral). Data are shown as means $\pm$ SEMs and analyzed with a 2-way ANOVA (treatment $\times$ time) with Bonferroni correction. ++ $P < .05$ compared with the width of the saline-treated control at the same time point. Scale bars = 1 mm.



difference was identified in the overall OB size or morphology between the LPS- or saline-treated animals (Fig 2, A). However, in a mouse treated with LPS for 24 weeks (LPS:24w), the OB ipsilateral to the LPS-administered naris was significantly smaller than the contralateral OB of the LPS:24w or the OB treated with saline (saline:24w; Fig 2, B). Because there was no sympathetic atrophy identified in the contralateral OBs and formalin fixation might change the brain size,²¹ we calculated the ratios of the length and width of the ipsilateral OB compared with those of the contralateral OB (Fig 2, C). These results indicated that repetitive intranasal LPS administration caused OB atrophy. In particular, the width was significantly smaller in the LPS:24w group than in the saline:24w group ($P < .01$), whereas saline treatment did not affect the length or width.

Coronal sections of the LPS- or saline-treated OBs show that LPS treatment for 24 weeks resulted in a smaller overall OB size (Fig 3, A). Of note, the olfactory nerve layer (ONL) was thinner and the glomeruli were smaller than those of the saline-treated OBs. A constriction identified at the lateral side of the saline:24w group was not obvious in the LPS:24w group.

To quantify the effects of long-term LPS treatment on the OB layers, we selected a slice from the middle of the OB and measured the area of each layer: the GL, EPL, MCL plus IPL, and GCL (Fig 3, B). The GLs in the LPS-treated OBs for at least 10 weeks were significantly smaller than those in the OBs treated with saline for the same durations ($P < .01$; Fig 3, C). In addition to the GL, the areas of the EPL in the LPS:10w and LPS:18w groups were smaller, although not statistically significant than those in the saline:10w and saline:18w groups, and the EPL area in the LPS:24w group was significantly smaller than in the saline:24w group ($P < .05$; Fig 3, D). No significant differences were identified in the areas of the MCL plus IPL or GCL (Fig 3, E and F).

We also calculated the proportion of each layer in the OBs to eliminate the positional variations of selected slices in the OBs

(see Fig E1 in this article's Online Repository at www.jacionline.org). The mice treated with LPS for 10 or 24 weeks exhibited a significant decrease in the proportion of GL in the OBs and correspondingly increased the GCL proportions. The latter increase was due to a reduction in the denominator (total OB area), reflecting no change in the layers *per se*. The proportion of the EPL in the OBs did not significantly change, with the exception of a slight reduction in the LPS-treated OBs. These results suggest that OB atrophy caused by repetitive intranasal LPS administration is attributed to shrinkage of the superficial layers, namely the ONL, GL, and EPL.

Thinning of the sEPL

Long-term LPS treatment reduced the thickness of the EPL, where OB projection neurons form dendrodendritic synapses with granule cells. To examine the effect of LPS treatment on OB projection neurons, we administered LPS into transgenic mice expressing tdTomato under the control of the Tbx21 promoter. Because Tbx21 is a transcription factor specifically expressed by OB projection neurons in the brain, only mitral and tufted cells express tdTomato in the OB of the Tbx21Cre $\times$ tdTomato mouse.^{22,23} However, for unknown reasons, the tdTomato signal is stronger in tufted cells than mitral cells, which enables us to discriminate the sEPL from the dEPL, where secondary dendrites of tufted and mitral cells make synapses with granule cells, respectively (Fig 4, A). Consistent with area analyses, total EPL thicknesses in the LPS:10w and LPS:24w OBs were significantly reduced compared with those after saline treatment ($P < .05$ for 10 weeks and $P < .01$ for 24 weeks; Fig 4, A-C). In addition, we found that the sEPL was more severely affected than the dEPL (Fig 4, A, B, and D). In the saline:3w, saline:10w, saline:18w, and saline:24w and LPS:3w OBs, the proportions of the sEPL to the total EPL length were between 35% and 37%. In contrast,

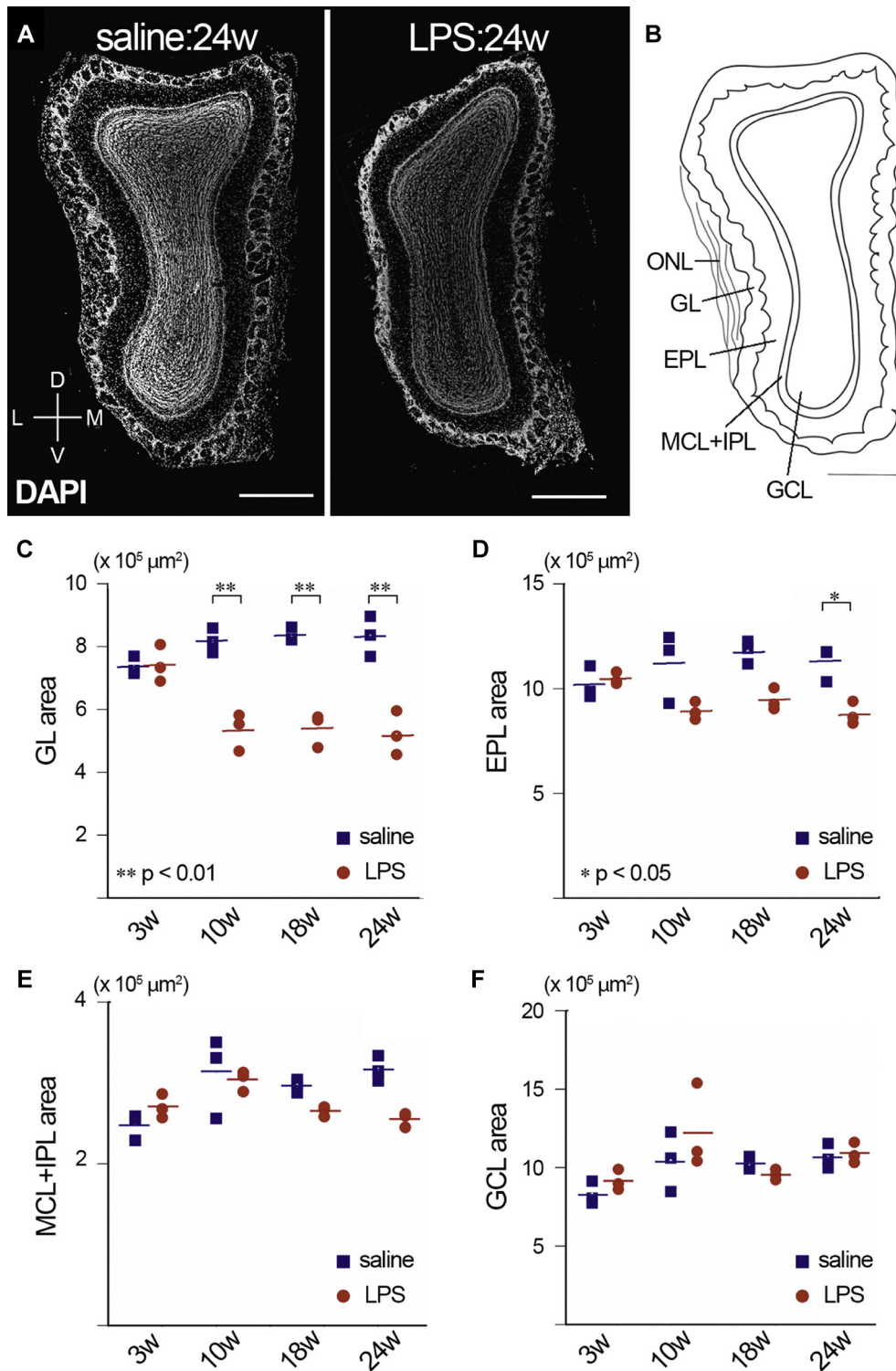


FIG 3. Shrinkage of GL and EPL. **A**, Coronal sections of the OB stained with DAPI. The GL and EPL become smaller in LPS:24w compared with saline:24w mice. **B**, Schematic diagram showing OB layers in a coronal section. **C-F**, Graphs showing areas of GL, EPL, MCL plus IPL, and GCL. GL and EPL areas are significantly reduced in LPS-treated OBs compared with those in saline-treated control OBs at 10, 18, and 24 weeks and at 24 weeks, respectively, whereas the areas of MCL plus IPL and GCL did not change. Individual data are plotted, and means are shown as bars. Data were analyzed with a 2-way ANOVA (treatment $\times$ time) with Bonferroni correction: * $P < .05$ and ** $P < .01$ compared with saline-treated control at the same time point. Scale bars = 500 μm .

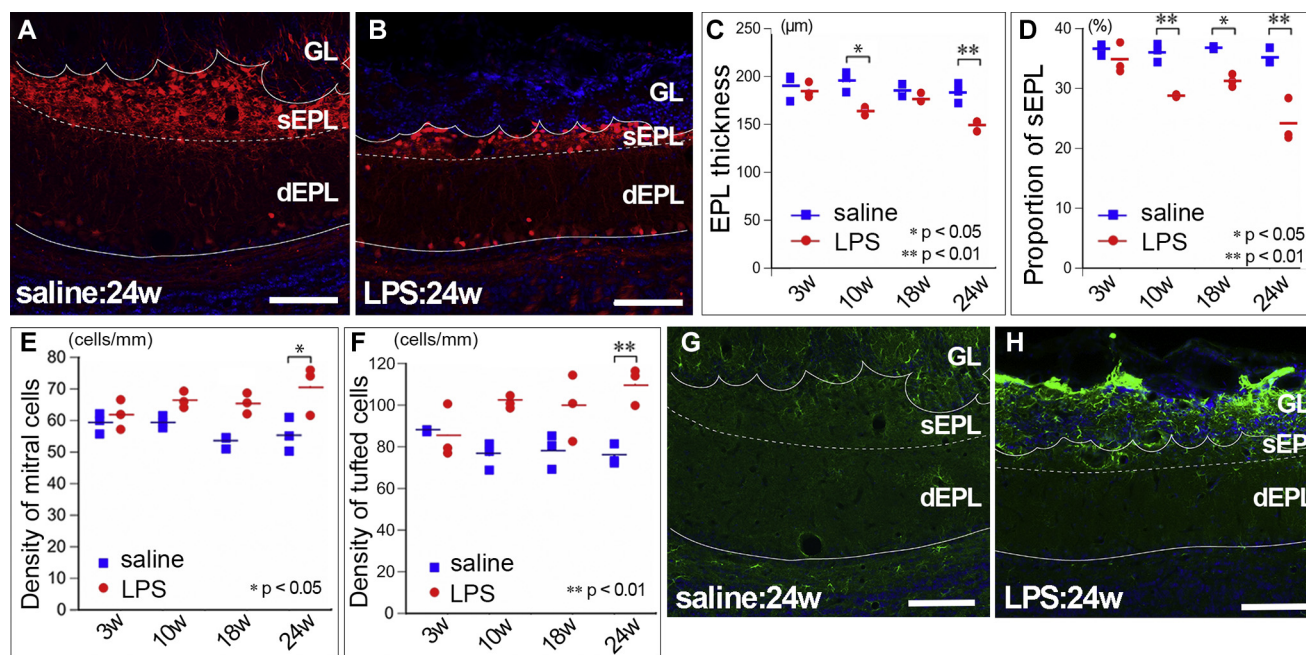


FIG 4. Thinning of the sEPL. **A** and **B**, Coronal sections of OBs derived from saline:24w (Fig 4, **A**) or LPS:24w (Fig 4, **B**) mice. The tdTomato signal (red) is stronger in the sEPL than in the dEPL. Total EPL thickness and the proportion of sEPL decreased in LPS:24w compared with saline:24w mice. All nuclei are stained with DAPI (blue). **C** and **D**, Graphs show decreases in EPL thickness (Fig 4, **C**) and the proportion of sEPL to total EPL length (Fig 4, **D**) in OBs treated with LPS. Data are shown as means $\pm$ SEMs. **E** and **F**, Graphs show the densities of mitral (Fig 4, **E**) and tufted (Fig 4, **F**) cells in saline- or LPS-treated OBs. Densities of both mitral and tufted cells increased in LPS-treated OBs compared with saline-treated control OBs at 24 weeks. Individual data are plotted, and means are shown as bars. Data in Fig 4, **C-F**, were analyzed with 2-way ANOVA (treatment $\times$ time) with Bonferroni correction: * $P < .05$ and ** $P < .01$ compared with saline-treated control at the same time point. **G** and **H**, Coronal sections of OBs derived from saline:24w (Fig 4, **G**) or LPS:24w (Fig 4, **H**) mice were stained with anti-glial fibrillary acidic protein (GFAP) antibodies (green). GFAP-positive astrocytes are hypertrophic in GL and sEPL of LPS:24w mice. There is no robust astrocytic activation in saline:24w mice. All nuclei are stained with DAPI. Scale bars = 100 μ m.

the mice that received repetitive LPS treatment for at least 10 weeks exhibited significant decreases in proportions: 29%, 31%, and 24% for the LPS:10w, LPS:18w, and LPS:24w groups, respectively ($P < .05$ for 18 weeks and $P < .01$ for 10 and 24 weeks; Fig 4, **D**). These results show the preferential thinning of sEPL and suggest that neuronal circuits associated with tufted cells were more severely affected than mitral cells by using long-term repetitive intranasal LPS administration.

Because our previous result suggested that intranasal LPS treatment first damaged the lateral OE,¹⁸ we examined whether there was a difference in the thinning of the sEPL between the lateral and medial OBs. Coronal OB sections were stained with anti-NQO1 antibody to specifically highlight OSNs of the dorsomedial OE that target the glomeruli in the dorsomedial OB.²⁴ Therefore we subdivided the EPL into the dorsomedial and ventrolateral regions based on the NQO1 expression in the GL (see Fig E2, **A**, in this article's Online Repository at www.jacionline.org). In the dorsomedial region the thicknesses of the EPL was not changed with LPS treatment, and a significant reduction in the proportion of sEPL to total EPL was identified only in the LPS:24w group ($P < .01$; see Fig E2, **B** and **C**). In contrast, the ventrolateral EPL was significantly thinner in the OBs of the LPS:10w ($P < .05$) and LPS:24w ($P < .01$) groups, and sEPL proportions were significantly lower in the LPS:10w, LPS:18w, and LPS:24w OBs than those in saline-treated OBs ($P < .01$; see Fig E2, **B** and **C**). This result indicates that sEPL thinning caused

by repetitive LPS exposure occurs first in the ventrolateral region followed by the dorsomedial region.

In the sEPL dendrites of granule cells form synapses predominantly with dendrites of tufted cells.²⁵ Thus sEPL thinning indicates loss of dendrodendritic synapses between tufted cells and granule cells, as well as their dendrites. To examine whether LPS treatment induced cell death in the OB, we first performed a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay on the sections of LPS-treated OBs to detect apoptotic cell death. Although we identified TUNEL-positive cells in the ONL, GL, and GCL, numbers did not significantly increase in the OB ipsilateral to the LPS-treated naris compared with the contralateral OB, and almost no cell death was observed in the EPL and MCL at any time points examined (data not shown). Subsequently, we quantified the densities of mitral cells and tufted cells and found that they were significantly greater in the LPS:24w OBs than in those treated with saline ($P < .05$ for mitral cells and $P < .01$ for tufted cells; Fig 4, **E** and **F**). Given the OB atrophy and generation of OB projection neurons that is restricted to embryonic periods,²⁶ the increase in mitral/tufted cell densities indicates that LPS treatment did not induce mitral/tufted cell death.

Our previous study reported that repetitive LPS treatment for 3 weeks increased OB gliosis.¹⁸ Of note, activation of microglia and astrocytes was prominent in the superficial layers of the OB, including the ONL, GL, and sEPL. In this study we examined

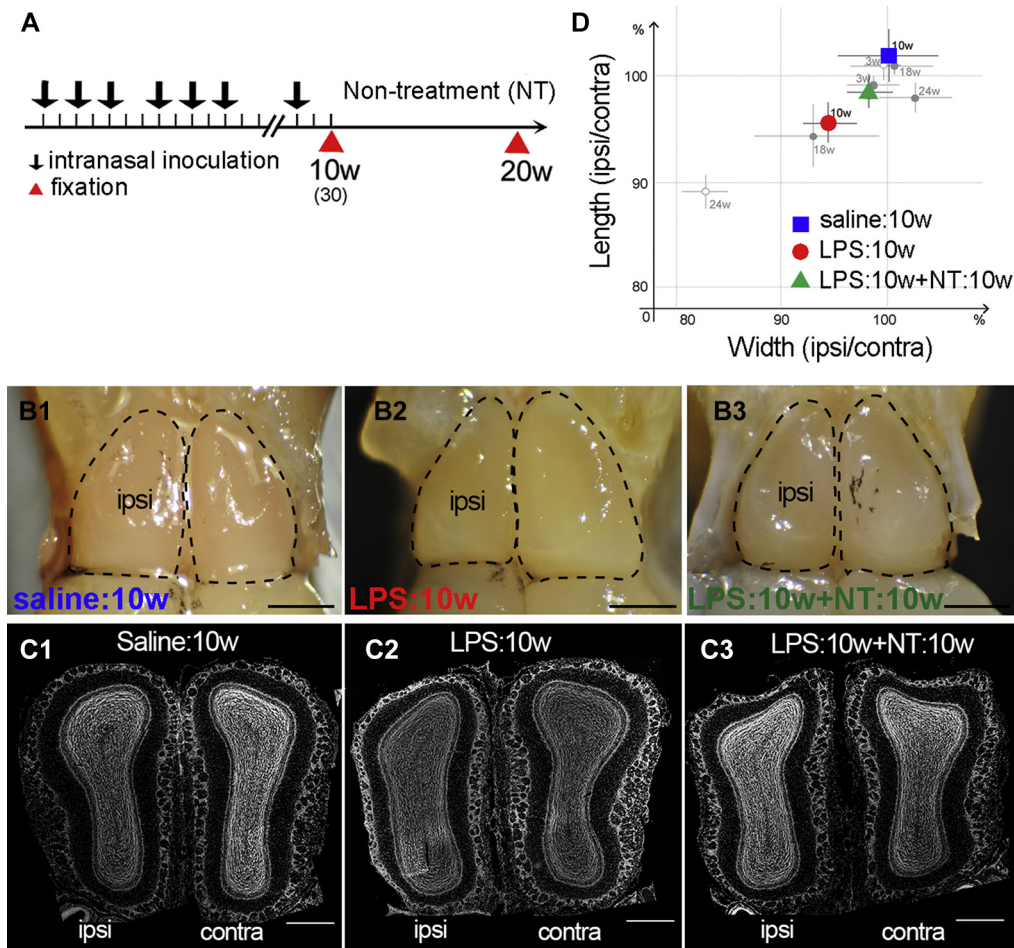


FIG 5. Recovery from OB atrophy. **A**, Experimental protocol for intranasal administration of saline or LPS for 10 weeks (3 times per week) and nontreatment for an additional 10 weeks (LPS:10w+NT:10w mice). The total number of administrations is indicated in parentheses. **B**, Dorsal view of the OBs of saline:10w (**B1**), LPS:10w (**B2**), and LPS:10w+NT:10w (**B3**) mice. Although the ipsilateral OB is atrophied in LPS:10w mice, the size of ipsilateral OBs in LPS:10w+NT:10w is not different from that of the contralateral OB and ipsilateral OB in saline:10w mice. **C**, Coronal sections of OBs derived from saline:10w (**C1**), LPS:10w (**C2**), and LPS:10w+NT:10w (**C3**) mice were stained with DAPI. Although ipsilateral OBs in LPS:10w mice are smaller than contralateral OBs and ipsilateral OBs of saline:10w mice, the ipsilateral OB in LPS:10w+NT:10w mice is not different from contralateral and ipsilateral OBs in saline:10w mice. **D**, Relative size of ipsilateral to contralateral OBs (ipsilateral/contralateral) is smaller in LPS:10w than saline:10w mice; however, it returned to normal in LPS:10w+NT:10w mice. Data are shown as means $\pm$ SEMs. Scale bars = 1 mm in Fig 5, **B**, and 500 μ m in Fig 5, **C**.

the distribution of activated astrocytes in OBs treated with LPS for 24 weeks. Although we did not identify a significant increase in the glial fibrillary acidic protein signal in the saline:24w OBs, robust gliosis occurred in the LPS:24w OBs (Fig 4, *G* and *H*). However, astrocyte activation was localized to the ONL, GL, and EPL and did not expand toward deeper layers. Interestingly, the EPL area, where we observed robust astrocyte activation, corresponded to the sEPL, where a strong tdTomato signal existed.

Recovery from the OB atrophy

To examine whether the OB recovers from atrophy, we ceased LPS administration into mouse nostrils at 10 weeks and waited an additional 10 weeks for recovery (LPS:10w+NT:10w; Fig 5, *A*). As an indication of recovery, the ipsilateral OB was comparable in size to the contralateral OB and the OB treated with saline (Fig 5,

B and *C*). No difference was identified in morphology between the OB sections derived from the saline:10w and LPS:10w+NT:10w animals. The size of the OB section, ONL thickness, and glomeruli size were all comparable, and constriction at the lateral side was also restored (Fig 5, *C*). Quantification of OB size indicated that both the length and width of the atrophied OB returned to levels comparable with those of contralateral nontreated OBs after 10 weeks of the nontreatment period (Fig 5, *D*).

In the EPL the activation of astrocytes subsided after the nontreatment recovery period (Fig 6, *A*). Importantly, we also observed recovery of the sEPL area; the proportions of the sEPL to the total EPL in saline:10w, LPS:10w, and LPS:10w+NT:10w OBs were 36%, 29%, and 36%, respectively (Fig 6, *B*). The percentage of the LPS:10w OBs was significantly lower than that of saline:10w and LPS:10w+NT:10w OBs ($P < .01$), whereas there was no difference between the saline:10w and LPS:10w+NT:10w

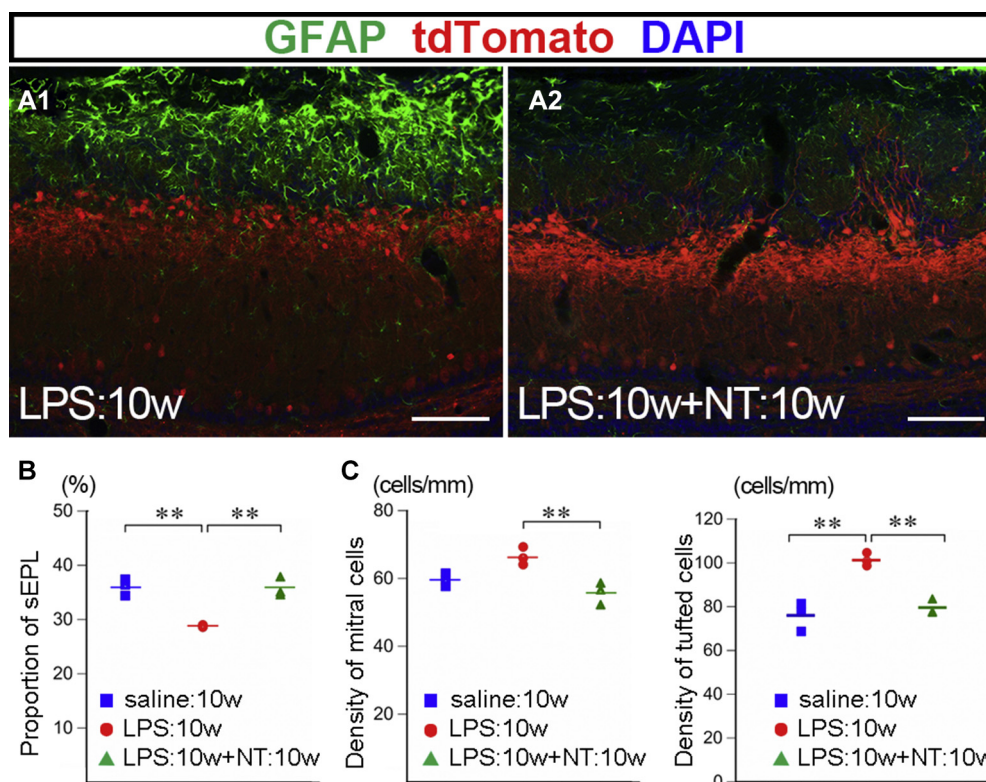


FIG 6. Recovery from sEPL thinning. **A**, Coronal sections of OBs derived from LPS:10w (A1) and LPS:10w+NT:10w (A2) mice were stained with anti-glial fibrillary acidic protein (GFAP) antibody (green). The sEPL of LPS:10w+NT:10w mice becomes thicker than that of LPS:10w mice. GFAP-positive astrocytes are hypertrophic in GL and sEPL of LPS:10w mice but not in LPS:10w+NT:10w mice. All nuclei are stained with DAPI (blue). **B**, Graph shows reduction of the proportion of sEPL to total EPL in the OBs in LPS:10w and recovery in LPS:10w+NT:10w mice. **C**, Graphs show increases in densities of mitral and tufted cells in LPS:10w and recovery in LPS:10w+NT:10w mice. Individual data are plotted, and the means are shown as bars. Data were analyzed by using Bonferroni correction: $^{**}P < .01$. Scale bars = 100 μ m.

groups. Recovery of the sEPL area was observed both at the dorso-medial and ventrolateral OBs. Although the proportion of sEPL to total EPL in the LPS:10w OBs was 29%, which is significantly lower than that of the saline:10w OBs, in the NQO1⁺ dorsomedial and NQO1⁻ ventrolateral regions, the values recovered to 36% in both regions, which is comparable with that of the saline:10w mice (see Fig E3 in this article's Online Repository at www.jacionline.org). In addition, densities of the mitral and tufted cells also returned to normal levels (Fig 6, C).

Incomplete regeneration of OSNs

We also examined OE recovery. OE damage was evaluated by calculating the proportion of the OE surface covered with ACIII that is expressed in OSN cilia, as previously reported.¹⁸ As shown in Fig 1, B, the ACIII signal covered the entire OE in the control animals, such as the saline:10w group, and disappeared from almost all OE regions of the LPS:10w mice. As we expected from the recovery of the OB, we identified a significant recovery of the ACIII signal in the OE of the LPS:10w+NT:10w OBs (Fig 7, A). However, there was a large area that was uncovered with the ACIII signal in the lateral region. It is known that NQO1 is expressed in dorsomedial OSNs but not in ventrolateral OSNs.²⁴ Thus we further subdivided the OE into dorsomedial and ventrolateral regions based on the distribution of OSNs that

expressed NQO1 in the contralateral nontreated OE (Fig 7, A) and found that the ACIII signal was recovered almost completely ($93.8\% \pm 4.0\%$) in the NQO1⁺ dorsomedial OE of the LPS:10w+NT:10w animals (Fig 7, B). However, only $35.6\% \pm 7.0\%$ was covered with ACIII in the NQO1⁻ ventrolateral OE, which is significantly higher than the percentage of ACIII-covered ventrolateral OE in the LPS:10w OBs ($P < .05$) and significantly lower than that of the saline:10w OBs ($P < .01$; Fig 7, B). Of note, there were clear boundaries between the regenerated and nonregenerated regions. We identified olfactory marker protein-positive OSNs broadly within the dorsomedial OE, whereas they were distributed in a patchy fashion in the ventrolateral region, which is consistent with the ACIII-positive area (Fig 7, C). Notably, the OE thickness was not fully recovered, even in the dorsomedial region. These results show incomplete regeneration of OSNs in the ventrolateral OE and indicate that full OSN regeneration is not necessary for the recovery from OB atrophy.

DISCUSSION

Atrophy of the OB caused by chronic nasal inflammation

In this study we found that repetitive intranasal LPS administration caused OB atrophy. The OB atrophy was attributed to the

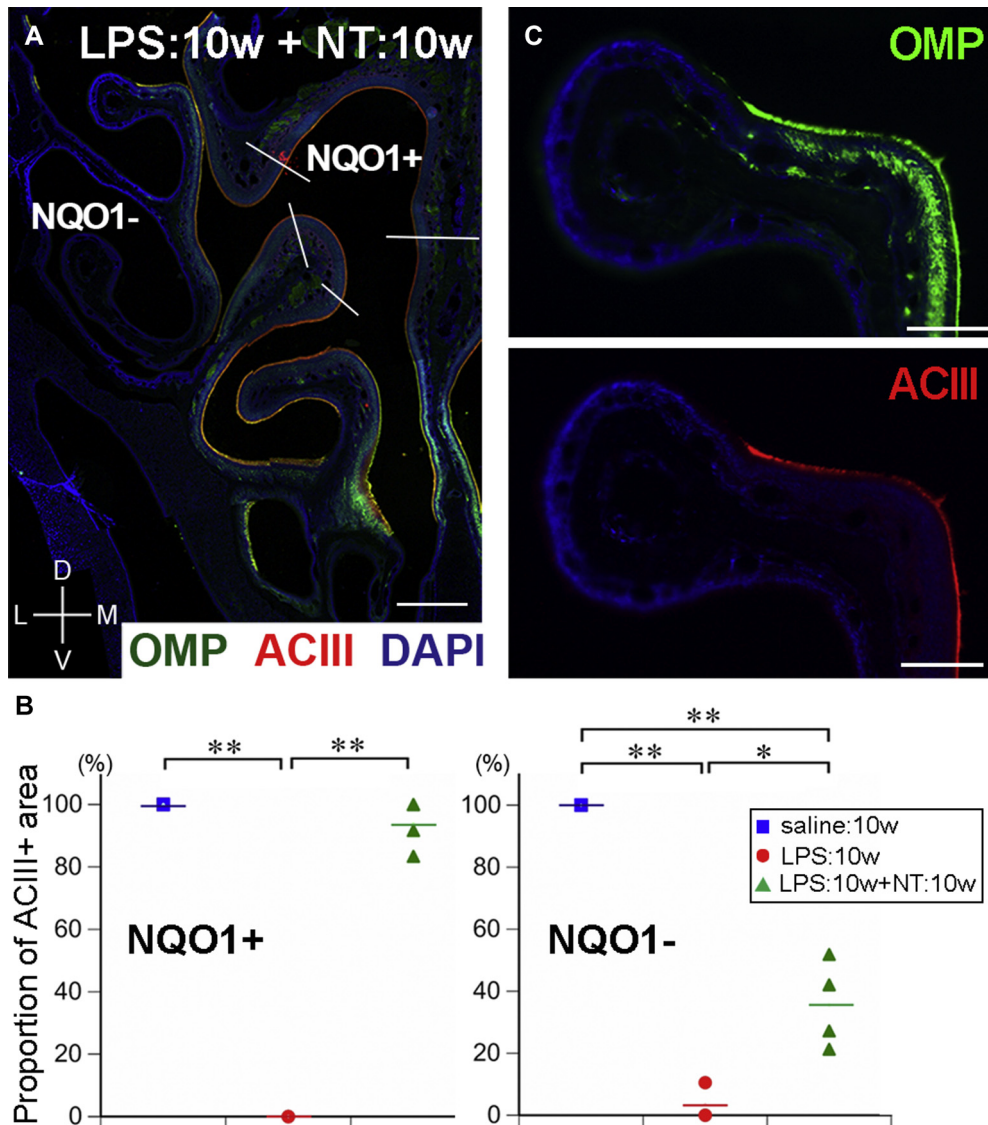


FIG 7. Incomplete regeneration of OSN. **A**, Coronal section of the OE derived from LPS:10w+NT:10w mice was stained with anti-olfactory marker protein (OMP; green) and anti-ACIII (red) antibodies. Expression of ACIII or OMP is not complete, especially in OSNs in the lateral region. White lines indicate boundaries between NQO1⁺ and NQO1⁻ regions determined by NQO1 expression in contralateral OE. All nuclei are stained with DAPI (blue). **B**, Graphs show that the proportion of the ACIII⁺ area robustly decreased in both NQO1⁺ and NQO1⁻ areas in LPS:10w mice. The proportion almost completely recovered in the NQO1⁺ area and partially in the NQO1⁻ area in LPS:10w+NT:10w mice. Individual data are plotted, and means are shown as bars. Data were analyzed with Bonferroni correction: * $P < .05$ and ** $P < .01$. **C**, Magnified views of a turbinate stained with OMP (green) and ACIII (red) indicate a clear boundary between recovered and unrecovered regions. All nuclei are stained with DAPI. Scale bars = 500 μ m in Fig 7, A, and 100 μ m in Fig 7, C.

shrinkage of the ONL, GL, and EPL. Given OSN loss in the OE, shrinkages of the ONL and GL were not surprising. Moreover, it was reported that OSN axotomy destabilized the tufted structures of mitral/tufted cells within the glomeruli.²⁷ OSN loss should cause synaptic loss from the dendritic tufts of mitral/tufted cells and shrinkage of dendritic tufts in the GL. We identified shrinkage of tufted structures within glomeruli when LPS was administered for 10 weeks and longer. In this study we also identified EPL shrinkage in mice treated with LPS for at least 10 weeks compared with mice treated with saline. In contrast, because

OSN axons terminate at the GL and do not penetrate into deeper layers, EPL shrinkage indicates damage to the OB circuit in which OSN axons are not directly involved.

EPL shrinkage suggests a loss of synapses, dendrites, or both from the EPL. One potential cause is a defect in the adult neurogenesis of OB interneurons. It is well known that new granule cells are continuously generated in the subventricular zone of the mouse brain and migrate toward the OB through the rostral migratory stream in the adult mouse brain. Naris closure or intraperitoneal LPS injection reduces the generation/survival of

newly generated granule cells.^{28,29} Our future study includes examination of the effects of chronic nasal inflammation on adult neurogenesis.

Another potential cause of EPL shrinkage is the retraction of dendrites of mitral/tufted cells, granule cells, or both. It is suggested that the loss of spines destabilizes neuronal dendrites.³⁰ In our previous study we showed that nasal inflammation reduced expression of the synaptic proteins vGluT1 and 5T4 in the EPL.¹⁸ Tracing the morphology of a single mitral/tufted cell and granule cell would indicate whether the chronic nasal inflammation causes retraction of their dendrites.

Of particular interest, chronic nasal inflammation caused significant thinning of the sEPL. Although mitral and tufted cells share many common features, they differ in the location of the cell body and projection patterns of their dendrites and axons; in particular, mitral and tufted cells extend their secondary dendrites predominantly in the dEPL and sEPL, respectively.³¹⁻³⁴ Thus our results suggest that the neuronal circuits in which tufted cells are involved are preferentially affected by nasal inflammation. Why tufted cells? One reason might be associated with the differences in odor-evoked activities between mitral and tufted cells.³⁵⁻³⁸ In brief, tufted cells are more excitable than mitral cells: tufted cells are typically activated by lower concentrations of odors and fire with a greater frequency than mitral cells.^{32,35,36,39} Of note, Gire et al⁴⁰ have demonstrated that tufted cells receive stronger monosynaptic connections from OSNs than mitral cells. This finding indicates that tufted cells are more influenced by the activity of OSNs than that of mitral cells. The clarification of whether the loss of olfactory input, OSN axons, or both has different effects on mitral and tufted cells would be a critical issue in our future study.

Glial activation in the OBs is another potential cause of EPL shrinkage. We showed that gliosis occurred in the superficial layers of the LPS-treated OBs. Interestingly, robust activation of astrocytes was restricted to the superficial region in the EPL even after 24 weeks of repetitive LPS treatment, which suggests chronic neuroinflammation. Although neuroinflammation in the central nervous system can produce both protective and toxic effects on neurons, chronic neuroinflammation is typically more toxic than protective.⁴¹ A link between neuroinflammation and synaptic loss is also suggested.⁴² Our previous study reported the activation of microglia and astrocytes and the synaptic impairment in the sEPL of LPS:3w OBs,¹⁸ thus suggesting that glial activation in the sEPL preceded its shrinkage. This finding supports the hypothesis that glial activation triggers synaptic loss and dendritic retraction of tufted cells, granule cells, or both from the sEPL. However, the mechanisms for restricting neuroinflammation to the sEPL remain an enigma.

Recovery from OB atrophy

An important finding of our present study is the recovery from OB atrophy after nasal inflammation ceased. However, to our surprise, the OE did not completely recover from the damage. Short-term, (ie, <3 weeks) intranasal LPS administration caused OSN loss preferentially in the lateral OE,¹⁸ whereas longer LPS treatment caused OSN loss in the entire OE. After 10 weeks of the nontreatment period, newly generated mature OSNs were identified almost entirely within the region of the dorsomedial OE. In contrast, regeneration of OSNs in the ventrolateral OE occurred only in a patchy fashion. Of particular note is the

existence of clear boundaries between regenerated and nonregenerated regions. Although we suggested a possibility that the restricted access of the LPS solution to the lateral OE is an underlying mechanism of regional specificity of the OSN damage,¹⁸ different cellular properties of OSNs, basal cells, and sustentacular cells between the dorsomedial and ventrolateral OEs might determine resilience from the damage caused by nasal inflammation.⁴³

Partial recovery of the OE indicates that OB recovery from atrophy might be an event independent of the regeneration and reinnervation of OSNs. Given that gliosis in the OBs subsided after cessation of LPS treatment, chronic glial activation might have prevented reconstruction of functional neuronal circuits. It is known that chronic stress induces dendritic retraction of neurons in the medial prefrontal cortex and hippocampus, and these neurons re-extend their dendritic branches after the cessation of stress.^{44,45} It is noteworthy that stress accumulates oxidative/nitrosative mediators in many brain regions, including the prefrontal cortex and hippocampus, which leads to activation of microglia.⁴⁶ Destruction and reconstruction of OB neuronal circuits might share some mechanisms with those induced by stressors. Nitric oxide and/or cyclooxygenases derived from activated microglia can also be involved in destruction of neuronal circuits in the sEPL.

Chronic nasal inflammation model

This study indicated a high plasticity of the OB circuit that is manifested in atrophy, followed by recovery in association with the inflammatory state of the nasal cavity. Human patients with CRS with a higher degree of nasal inflammation had smaller OB volumes.^{16,17} Moreover, increases in OB volume and olfactory function were identified in patients who had undergone functional endoscopic sinus surgery.⁴⁷ Our mouse model is a useful tool to investigate the mechanisms that underlie OB changes similar to changes in patients with CRS. Rhinosinusitis is inflammation of the nasal cavity and paranasal sinuses, such as the maxillary, frontal, sphenoidal, and ethmoidal sinuses. CRS is broadly divided into CRS with nasal polyps and chronic rhinosinusitis without nasal polyps (CRSsNP). CRS with nasal polyps is characterized by the presence of polyps and allergic eosinophilic inflammatory infiltrates, whereas CRSsNP is characterized by noneosinophilic inflammation associated with neutrophil accumulation. We previously reported that administration of LPS to the mouse nasal cavity caused olfactory mucosal inflammation infiltrated by neutrophils, macrophages, and T cells.¹⁸

However, we did not observe inflammatory responses in the paranasal sinuses or polyp formation in the nasal cavity. These findings might be due to differences in sinonasal anatomic structures between human subjects and rodent. First, mice have only a single paranasal sinus, the maxillary sinuses.⁴⁸ Second, rodents do not have large sinus cavities that pneumatize into their skull and facial bone.⁴⁹ Moreover, there are few mouse models in which inflammatory responses are induced in the maxillary sinuses, whereas some models induced nasal polyposis.^{10,50,51} Apart from rodents, a sheep CRS model has been created by inoculating bacteria, such as *Staphylococcus aureus*, into the frontal sinuses.⁵² At least in mice the anatomic connectivity between the nasal cavity and the OBs is substantially stronger than that between the paranasal sinus and the OBs; thus changes in the OBs are very likely attributed to the inflammatory responses in the

olfactory mucosa. We believe that our mouse model has advantages for investigating the mechanisms that underlie the OB changes observed in patients with CRS, particularly CRSsNP.

There are also several differences between human and rodent OBs. First, numbers of functional odorant receptors and glomeruli are different. Mice and human subjects have 1000 and 350 functional odorant receptor genes in the genome,^{53,54} respectively, whereas there are approximately 4000 and 5000 glomeruli in mouse and human OBs, respectively.^{55,56} Second, there are differences in OB size and the proportion of the OB to the brain between mice and human subjects. Although human subjects have a larger OB (approximately 80-190 mm³) than mice (approximately 23-29 mm³), the mouse OB constitutes a significantly larger proportion of the brain volume (approximately 5%) than that of human OBs (approximately 0.01%).⁵⁷⁻⁶⁰ Third, there are differences in expression of transcription factors in OB interneurons between mice and human subjects.⁶¹ Fourth, there might be little or no adult neurogenesis in the human subventricular zone.^{62,63} These differences should be carefully taken into account when we apply our research results to human CRS.

In addition to CRS, loss of OB volume is a feature of neurodegenerative diseases that reflects olfactory dysfunction.⁶⁴⁻⁶⁶ Moreover, OB volume decreases with advancing age.⁶⁷ Our animal model can provide a platform for investigating the molecular and cellular mechanisms that regulate the reversible changes of the OB circuit. Identification of the mechanisms that underlie destruction/reconstruction of the OB circuit will provide significant insight into not only olfactory dysfunctions but also neurodegenerative diseases, mental disorders, and aging.

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Key messages

- Chronic nasal inflammation causes atrophy of the OB and damages a specific olfactory circuit.
- OBs recovered from damages after nasal inflammation had subsided; nevertheless, OSN regeneration was incomplete.

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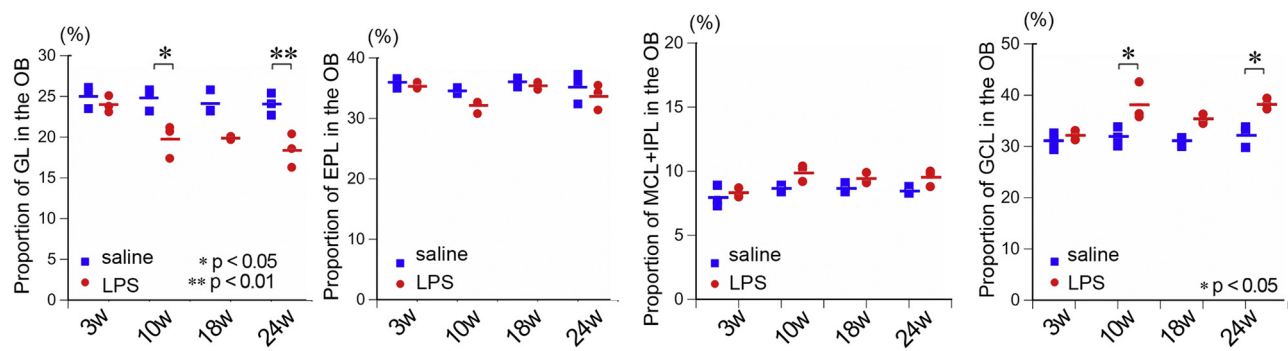


FIG E1. Proportion of each layer in OBs. Graphs show the proportion of the area of GL, EPL, MCL plus IPL, or GCL to total OB area. In LPS:10w and LPS:24w mice, proportions of GL and GCL reduced and increased, respectively. Data are shown as means $\pm$ SEMs and were analyzed with a 2-way ANOVA (treatment $\times$ time) with Bonferroni correction: * P < .05 and ** P < .01 compared with the saline-treated control at the same time point.

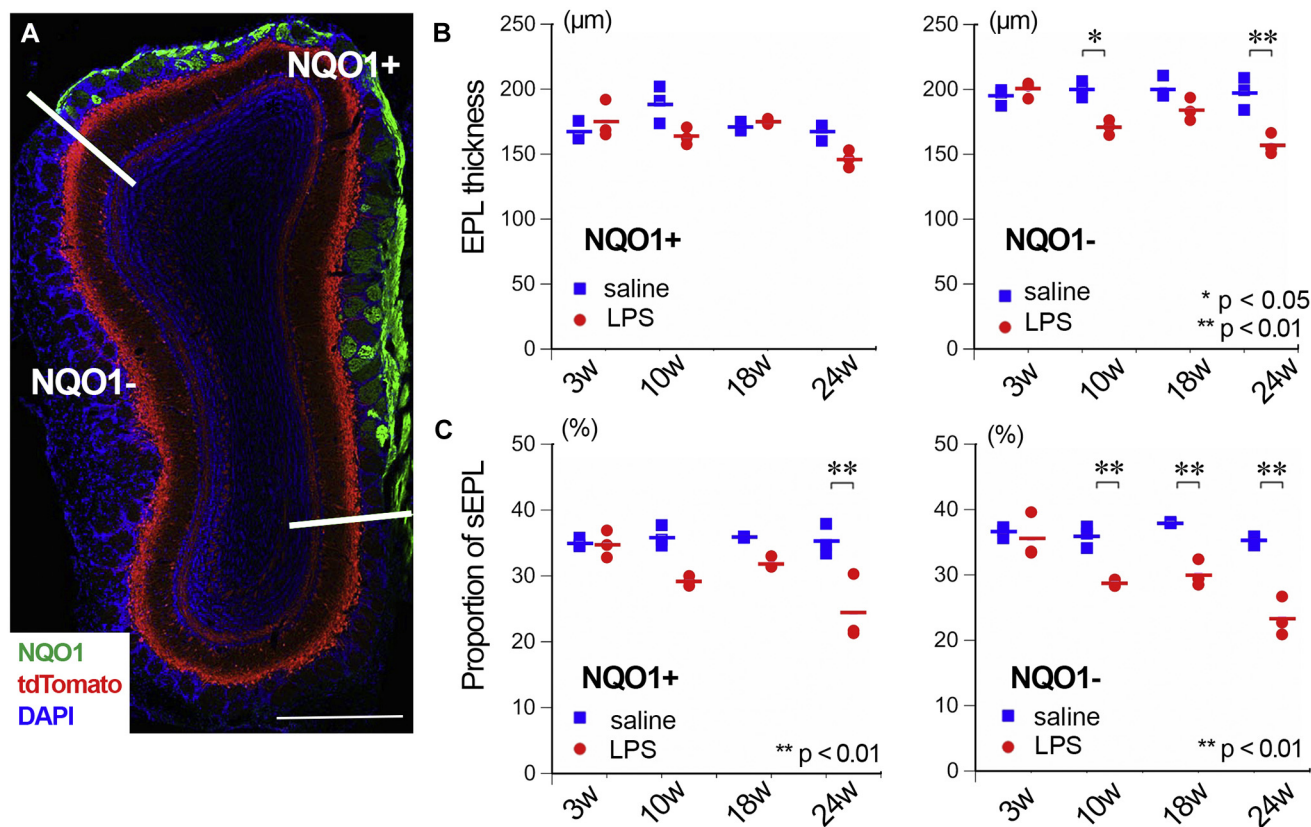


FIG E2. Effects of repeated LPS administration on EPL thickness. **A**, Coronal section of OBs derived from Tbx21Cre $\times$ tdTomato mouse was stained with anti-NQO1 antibody (green). Boundaries between dorsomedial NQO1⁺ and ventrolateral NQO1⁻ regions are determined based on NQO1 expression in GL and indicated with white lines. tdTomato (red) is expressed by mitral and tufted cells, and the signal is stronger in the sEPL. All nuclei are stained with DAPI (blue). Scale bar = 500 μ m. **B**, Graphs showing EPL thickness in NQO1⁺ and NQO1⁻ regions separately. EPL thickness decreased in LPS-treated mice at 10 and 24 weeks in the NQO1⁻ region compared with saline-treated control at the same time points, whereas the EPL thickness did not change in the NQO1⁺ region. Data are shown as means $\pm$ SEMs and were analyzed with a 2-way ANOVA with Bonferroni correction: ** $P < .01$ compared with saline-treated control at the same time points. **C**, Graphs showing the proportion of sEPL to total EPL in NQO1⁺ and NQO1⁻ regions separately. The proportion of sEPL decreased in LPS-treated mice at 24 weeks in the NQO1⁺ region and at 10, 18, and 24 weeks in the NQO1⁻ region compared with saline-treated control values at the same time points. Data are shown as means $\pm$ SEMs and analyzed with a 2-way ANOVA with Bonferroni correction: * $P < .05$ and ** $P < .01$ compared with saline-treated control at the same time points.

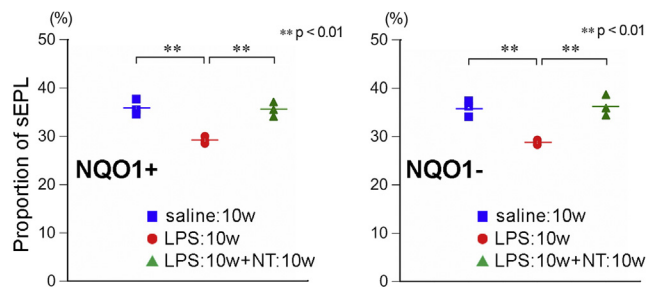


FIG E3. Recovery from sEPL thinning. Graphs showing proportion of sEPL in NQO1⁺ and NQO1⁻ regions. In both regions proportion of sEPL to total EPL is significantly reduced in LPS:10w mice and recovered to the normal level in LPS:10w and NT:10w mice. Individual data are plotted, and means are shown as bars. Data are analyzed with Bonferroni correction: ***P* < .01.