Region Specific Amyloid-β Accumulation in Olfactory Sensory Neurons Influences Ecto-Ventral Olfactory Dysfunction in 5xFAD Mice

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Research

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Abstract

Background: Hyposmia in Alzheimer's disease (AD) is a typical early symptom according to numerous previous clinical studies. Although the causes of damage have been proposed in every olfactory system including olfactory epithelium, olfactory bulb and olfactory cortex, the main causes of AD-related hyposmia are largely unknown.

Methods: We here focused on peripheral olfactory sensory neurons (OSNs) and delved deeper into the direct relationship between pathophysiological and behavioral results using odorants. We also histologically confirmed the pathological changes in three-month-old 5xFAD mouse models which recapitulates AD pathology. We introduced a numeric scale histologically to compare physiological phenomenon and local tissue lesions regardless of anatomical plane.

Results: We observed the odorant group, which 5xFAD mouse could not detect, also neither did physiologically activate the OSNs that propagate to the ventral olfactory bulb. Interestingly, the amount of accumulated amyloid-β (Aβ) was high in the ecto-ventrally located OSNs that showed reduced responses to odorants. We also observed irreversible damage to the ecto-region of the olfactory epithelium by measuring impaired neuronal turnover ratio from the basal cells to the matured OSNs.

Conclusions: Our results showed that partial and asymmetrical accumulation of Aβ coincided with physiologically and structurally damaged areas in the peripheral olfactory system, which evoked hyporeactivity to some odorants. Taken together, partial olfactory dysfunction closely-associated with peripheral OSN's loss could be a leading cause of the AD-related hyposmia, a characteristic of early AD.

Background

Olfactory dysfunction affects approximately 90% of patients with Alzheimer's disease (AD) (1, 2). Abnormal olfaction has been recognized as one of the earliest clinical manifestations of AD over the past few decades (3, 4). Numerous attempts have been made to comprehend early AD by using olfaction-based assessments (5–7). Recent research has shown that patients with AD who have difficulty with olfaction are not completely anosmic (8). According to the results of clinical studies, patients have difficulty identifying only a few odorants presented, and not all odorants (7, 9–11). Besides, the temporal issue corresponds with the fact that hyposmia has been observed in the early stage of AD. Interestingly, amyloid-β (Aβ), which is one of the toxic factors upregulated early in AD, has been identified in many studies, even in the peripheral area (12–14). However, the pathology involving olfactory sensory neurons (OSNs) remains poorly understood.

Odor detection via OSNs is the first step in smell processing. The signals converge in the glomerulus in the olfactory bulb (OB), where synapses form between axon terminal of OSNs and the dendrites of mitral, periglomerular and tufted cells (15, 16). Each OSN expresses one type of odorant receptor, and its axon propagates to the OB. The axons of OSNs which express the same odorant receptor form two or three glomerulus per each bulb, which follows a topological map or axis (17, 18). The OSNs targeted to ventral glomeruli are located in the ecto-turbinate of the olfactory epithelium (OE), whereas the OSNs targeted to the dorsal glomeruli are located in the endo-turbinate of the OE. We also previously demonstrated a distinctive accumulation pattern of Aβ oligomers and suggested that the uneven partial spatial damage leads to abnormal AD symptoms in the olfactory system (12). In this study, we delve deeper into the functional impairment related to the spatial distribution of accumulated Aβ oligomers.

We observed a distinctive pattern of hyposmia using an olfactory detection test and electrophysiological changes in OSNs in the early stage of oligomerized Aβ accumulation. In addition, we revealed the spatial correlation between the functional changes and immunohistologic signals using a heat map analysis. In view of the characterization of the olfactory topographical axis with intrinsic turnover, we identified an interrelation between Aβ oligomer deposits and breakdown of OSNs-turnover. To explain the role of Aβ in hyposmia, we used transgenic mice with early-onset familial forms of AD (FAD). These mice have elevated Aβ levels caused by the co-expression of five FAD (5xFAD) mutations (three in amyloid precursor protein (APP) and two in presenilin 1 (PS1)). This model develops a large Aβ12 load (known to generate toxic oligomerized species of Aβs) starting at two months of age (19). It is also well known that these mice develop central nervous system (CNS) deficits after five months. Thus, we used three-month-old 5xFAD mice as a means of identifying a potential causative role for Aβ-derived neurotoxicity in early AD (19). Taken together, we show that region specific damage of OSNs induces partial olfactory dysfunction, and this may be a feature of AD-specific olfactory dysfunction.

Methods

Experimental design

To investigate a potential causative role for Aβ-derived neurotoxicity in the olfactory dysfunction in early AD, we designed behavioral tests, two-photon calcium imaging, immunoreactivity measurement, and histologic analysis using mouse model. All details are described in the following chapters.

Key Resources Table
### Experimental model and subject details

#### Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committees of DGIST (DGIST-IACUC_0104). All applicable guidelines for the care and use of laboratory animals from the National Institutes of Health Guide were followed. Only male animals were used in this study. Adult mice (C57/BL6, 7–8 weeks old) were obtained from KOATECH (Daegu, Korea). 5xFAD transgenic mice harboring the mutated human APP (695 amino acids) and human PS1 genes (Tg6799, 3–4 months old) were obtained from Prof. K.A. Chang (Gachon Medical School, Incheon, Korea).

#### Behaviors

All behavioral and histological analyses were blinded.

1) **Y-maze test**

The test was performed to evaluate the ability of the mice to act in a sequence and to measure short-term memory. Each branch (A, B, and C) of the maze was 40 cm long, 5 cm wide, and 10 cm high at an angle of 120°. The maze was constructed of white polyvinyl plastic. The animal was placed in the maze for 8 min, and the frequency with which the tail entered each branch was counted for each branch. The number of times the animal entered the branches (in the A, B, C sequence) was also counted and awarded 1 point (real change, actual alternation). Ability to take action to change (%) = Actual change (actual alternation)/maximum change (maximum alternation) × 100 (maximum change: the total entrance number – 2)

2) **Morris water maze (MWM) test**

The test was conducted in a circular pool (diameter 90 cm, height 45 cm, outer height [from the ground] 61.5 cm) using the EthoVision Maze task system (Noldus Information Technology, Wageningen, the Netherlands). The time required to find the platform and the latency to escape (escape latency) were
measured. The animals underwent four training trials per day (one time per quadrant) over 4 consecutive days with a 30 min interval. If the animal could not find the platform within 60 s, they were placed on the platform for 20 s. The platform was removed on the last day, the animals were placed in the water to swim for 60 s, and memory was compared by measuring swimming around the area where the platform was installed.

3) Food-seeking test

The test was performed in three-month-old 5xFAD mice (WT, n=6; 5xFAD, n=6) and as described previously (12). Prior to the food-seeking tests, food restriction was applied for over 35 h to motivate animals to search for food, either hidden underneath a layer of bedding or not. Therefore, this test was used to assess latency in finding food as the buried pellet-seeking test. Mice were habituated in a clean home cage for one day prior to testing. A food pellet was buried 5 cm under the bedding in a middle region of the edge of the cage and a mouse was placed at the opposite edge. The time to first bite of the food pellet was measured using an installed digital camera (maximum recording time was 10 min based on the assumption that food-restricted mice that fail to use odor cues to locate the food within a 10-min period are likely to have deficits in olfactory abilities).

4) Odor detection (nose poke) tests

The test was performed at three months of age for the Tg6799 and wild-type (WT) groups (n = 6 per group) and by modifying the odor-preference test described previously (20, 21). Instead of filter paper, a cotton tip scented with odorant was used to allow for the nose poke of mice to more accurately direct them to the odor (Fig. 1c). The following commonly used odor preference test odorants were used and presented for 2 min: acetophenone (8.6 M), allyl phenylacetate (5.9 M), eugenol (6.5 M), geraniol (5.7 M), heptanal (7.2 M), heptanoic acid (7.1 M), and linalool (4.7 M). Mineral oil (MO) was used as a control odorant. After 5 min habituation, mice were transferred to a new cage and the tip scented with a test odorant carefully set so that the mouse could not directly reach it. Investigation times were measured for 2 min. The performance index (PI) was determined based on a previously described method (22). PI is the percentage of time to detection of the experimental odorant minus the percentage of time in the control. A PI close to 0 indicates difficulty in detecting an odor and a PI of 100 indicates that a mouse could definitively detect an odor. The mouse behavior was recorded with a digital video camera (rate of 30 frames/s). Four points of interest (POIs) that we tracked in each frame were the nose, ears and tail (Movie S1). In each group, we randomly selected 180 frames and manually labeled POIs in those frames and used them to train and test a neural network model implemented in DeepLabCut (23). Evaluation of labeling accuracy was achieved by comparing the labels acquired from the convolutional neural network on the test set with manual labels. The model was then used to evaluate all frames in each group of the 20 videos used for training. The resulting x and y coordinates corresponding to the middle position of four POIs within each frame were used to determine location. The test cage was divided into equally sized three compartments, and the duration that odorant part of mice stayed inside the third that contain a cotton tip was analyzed for odor attraction.

Method details

Calgary imaging

Imaging was performed at three months of age for the Tg6799, age matched WT, and C57BL/6 mice (n = 5–6 per group). Prior to operations and administration of a calcium indicator (Fura-2-AM), rats were anaesthetized with a mixture of ketamine (90 mg kg−1) and xylazine (10 mg kg−1). Thirty minutes after the operation, the experiments were initiated by nasal administration of a calcium indicator (using PE-90 tubes attached to a micropipette). A volume of 5–6 μl (8 μl mixture of 8%/0.2% calcium dye/Triton-X) was administered to each nostril. A simple olfactometer was built by using manually controlled valves, silicon tubing, and three glass syringes (10 cc) with shortened G18 injection needles as an odor nozzle, placed 10 mm away from the OE (the turbinate). The carrier air stream was water moisturized. Odorants, odorants diluted in mineral oil, or mineral oil only (control) were used to generate approximate vapor saturation in the syringes. The syringes and directly connected silicon tubing were cleaned with ethanol and water and were incubated for at least 12 h in ethanol before every experiment to remove trace odor contamination. The final odor concentration in an odor stimulus was estimated to be 0.0001% in the syringe. For wide-field fluorescence imaging, mice were sacrificed using CO2. Next, the skin overlying the skull was removed and the head was sagittally bisected along the midline. One half of the head was mounted, medial side up, on the mounting dish with melted 0.5% agarose in modified Ringer's solution. The septum was carefully removed to expose the whole olfactory epithelium and bulb area. The dish with the mounted tissue was placed onto the recording stage and the stage was aligned so that the recording location on the turbinate was centered under the microscope. The sample was covered with modified Ringer's solution. The olfactometer was turned on to deliver humidified air to the turbinate surface and the air delivery tube was placed approximately 10 mm away from the turbinate. The flow rate was ~600 μl/min. A typical stimulation protocol may be a 10-s duration repeated 30 times separated by one min between stimulations. The bulb surface of wide-field optical signals was measured using a Nikon 10×, 0.5 NA (2.3 x 2.3 mm field of view), Nikon 16× 0.8 NA (1.4 x 1.4 mm field of view), objectives with a 200 mm focal length lens for single bulb measurements. 4x 0.16 NA (3.5 x 3.5 mm field of view) was used for dual bulb measurements. In addition, to confirm our experimental scheme of the odor activity map in the OB, two-photon wide-field imaging was conducted with the anesthetized mice. The bone broadside of the olfactory bulbs was removed, and the exposure was covered with a glass coverslip and sealed with dental cement. To perform the correlation, a heat map analysis was also performed on only the subsets of pixels overlaying the glomerular ROIs identified in each preparation's activity map using unfiltered activity maps. All experiments were performed and evaluated by five independent tests.

Tissue preparation

Animals were anesthetized by intraperitoneal injection of 65 mg/kg ketamine with 5 mg/kg xylazine. The mice were then transcardially perfused with prechilled phosphate-buffered saline (PBS, pH 7.6). Heads were removed, skinned, and post-fixed overnight in 4% paraformaldehyde in PBS at 4 °C. The mandibles were discarded, and the trimmed heads were skinned and fixed by immersion in the same fixative for one day at 4°C. The heads were decalcified in Calci-Clear Rapid solution (National diagnostics, GA, USA) for 20 min at room temperature. After decalcification, the specimens were washed, dehydrated in increasing concentrations of ethanol, and transferred into xylene to clear the tissue. The specimens were infiltrated with paraffin and embedded. For cryosectioning, tissue was soaked in sucrose and transferred into Tissue-Tek OCT compound (Sakura Finetek Europe BV, Zoeterwoude, the Netherlands) after
post-fixation in 4% paraformaldehyde. Frontal sections (coronal, 5 μm) were cut serially from the tip of the nose to the posterior extension of the OE and OB, and each section was preserved on Matsunami coating slide glass (Matsunami Glass Co., Tokyo, Japan).

**Immunohistochemistry (IHC)**

For IHC, the tissue was permeabilized in PBS-T (0.1% Triton X-100 in PBS) for 15 min. The endogenous peroxidase in the samples was quenched using 3% hydrogen peroxide in 10% methanol for 30 min. To retrieve antigenicity, the samples were boiled in 0.1 M citrate buffered saline (pH 6.0) for 5 min. The sections were cooled for 30 min, and then washed twice in PBS (5 min each). After washing in PBS-T for 30 min, the sections were blocked for 1 h in blocking solution (4% normal donkey serum in PBS-T) and incubated with primary antibodies overnight at 4 °C. Anti-Aβ oligomer (1:100) and anti-bromodeoxyuridine (BrdU, 1:250) antibodies were used. After washing in PBS-T, the sections were incubated with a biotinylated secondary antibody for 1 h at room temperature. Sections were subsequently treated with the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit) for 1 h at room temperature. The sections were developed for 5 min in a 0.05% DAB solution, and counter-stained with hematoxylin. Images were captured with a Nikon digital camera (DS-Ri1) attached to a Nikon-Eclipse 90i microscope (Nikon Corp., Tokyo, Japan).

**Immunofluorescence (IF)**

For immunofluorescence, tissues were permeabilized in 0.1% PBS-T for 15 min. To retrieve antigens, the samples were boiled in 0.1 M citrate buffered saline (pH 6.0) for 5 min. The sections were cooled for 30 min, and then washed twice in PBS (5 min each). After washing in PBS-T for 30 min, the sections were blocked for 1 h in blocking solution (4% normal donkey serum in PBS-T). The sections were incubated with primary antibody overnight at 4 °C. Anti-oligomer A11 (Invitrogen, CA, USA) (1:100), anti-synaptophysin (Agilent Dako, CA, USA) (1:250), anti-TH (Santa Cruz Biotech, TX, USA) (1:250), and anti-Ki67 (Cell Signaling, MA, USA) (1:250) antibodies were used. Alexa 488 and Cy3-conjugated secondary antibodies (Jackson Laboratory, Bar Harbor, ME, USA) were used. The sections were counter-stained and mounted using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector laboratories, CA, USA). The images were visualized and photographed by confocal fluorescence microscopy (Carl Zeiss, Thomwood, NY, USA).

**Western blot (WB)**

OE was harvested in a prechilled lysis buffer (Sigma, St. Louis, MO, USA) containing a protease inhibitor cocktail (1X, Roche, Branchburg, NJ, USA). Extracts were thawed, homogenized by sonication, and centrifuged at 10,000 rpm to remove cellular debris. Protein (100 μg) from each sample was loaded for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline buffer with 0.1% Tween 20 and then incubated with primary antibodies. The primary antibodies used were anti-BACE1 (1:100), anti-PS1 (1:1000), anti-PS2 (1:1000), and GAPDH (1:1000). Immunoblots were visualized using a commercial development kit (Pierce, Rockford, IL, USA) and quantification was performed using the ImageJ program (NIH, Bethesda, MD, USA).

**TUNEL staining assay**

For TUNEL staining, deparaffinized and rehydrated sections were washed in PBS for 5 min and treated with Proteinase K (10 μg/mL) in PBS at room temperature for 30 min. After they were washed with distilled water for 5 min, the TUNEL incubation solution (Roche, Switzerland), containing TdT enzyme solution and label solution, was prepared in accordance with the manufacturer's protocol. The sections were incubated in TdT enzyme and label mixture for 1 h at 37 °C and then washed twice with PBS (5 min each). Fragmented DNA was visualized as green fluorescence inside the nuclei.

**BrdU assay**

BrdU (Sigma, St. Louis, MO) was injected and detected with an antibody recognizing BrdU. For acute labeling experiments, 100 mg/kg BrdU was injected 1, 3, and 7 day(s) prior to sacrifice.

**Image processing**

All images were acquired using a Nikon ECLIPSE 90i microscope and a Nikon DS-Ri1 digital camera (Nikon Inc., Japan) and LSM700 (+ Zeiss slide scanner). Digital images were processed adjusting only brightness, contrast, and color balance. The numbers of immunoreactive cells were counted manually by two independent investigators blinded to the experimental conditions. Three slides were analyzed for each animal and observed under a microscope (x100–400). To quantify the reciprocal intensity, the intensity per unit area was measured using Image J and the color deconvolution plug-in (http://wiki.imagej.net/Colour_Deconvolution). The target unit area of images was processed using the color deconvolution tool in Image J to separate brown from other colors. The area of brown staining was then quantified and divided by the total area to yield a percentage of staining area. Stereological analyses were conducted using Prism software (GraphPad software, USA).

**Spatial analysis**

To rate olfactory synapse positions within the sectioned OB (sagittal view), we divided equally among 10 frames from the anterior to posterior within a sagittal olfactory view. The top of the glomeruli in each frame was considered as “degree of zero” and used to set the relative angle from zero (dorsal to ventral of OB) (Fig. 2c). Spatial correlations of the calcium signal heat maps (Fig. 3d, Fig. 4c) were calculated using the function “cor” in the R software package (version 3.4.2; http://www.r-project.org/), and the correlation coefficient of each calcium signal map evoked by an odor was displayed as a heat map using the function “leverageplot” in R (Fig. 2e, g). To rate olfactory synapse positions, the sectioned OB with coronal and rostral migratory stream (RMS) was used as the standard (center of the dorsal-ventral axis). The most pointed/top of the glomeruli are located along the upper RMS track and regarded as “degree of zero” (360° at the same time due to coronal view), and the ROI was measured along with glomeruli; relative angles were then based on this zero point (Fig. 3a). Spatial heat maps (Fig. 3b) representing the Aβ oligomer expression were constructed using the function “leverageplot” in R. A heat map matrix represents the intensity
distribution of the deconvolution of the DAB signal by the protein expression along the angle. The intensity is presented as a scale bar (0–200 \(\Delta F/F\)) at the left side of the maps.

### Statistical analysis

Statistical analyses for histological ROI evaluation were conducted using Prism software (GraphPad software, USA). Comparisons between WT and experimental mice were conducted using a t-test. Results are presented as mean ± standard error of the mean (SEM). Differences with P-values of \(\leq 0.05\) were considered to be statistically significant. Correlations were assessed with a non-parametric Spearman's rank correlation test. Graphs (Fig. 4) show regression lines with a 95% confidence interval.

### Results

**Atypical olfactory dysfunction is observed in 5xFAD mice overexpressing Aβ**

We performed food-seeking tests to examine whether olfactory dysfunction was present (Fig. 1a). Our results show that three-month-old 5xFAD mice exhibited a significant increase in the latency time of seeking the buried food compared with wild-type (WT) mice (Fig. 1b) suggesting severe defects in olfactory function, as opposed to learning and memory dysfunction. To better understand this abnormal odor detection in the early stages of AD progression, we carried out the odor detection test in three-month-old 5xFAD with several different odorants by modifying the odor preference test (Fig. 1c). We systematically compared seven different odorants for which odor detection has been associated within the main olfactory system; lyral [L], acetophenone [A], eugenol [E], geraniol [G], allyl phenylacetate [AP], heptanoic acid [HA], and heptanal [H] (0.001%). WT mice detected all odors well and stayed near cotton tips scented with odorants. 5xFAD mice were able to detect lyral, acetophenone, and eugenol (odorant group A). However, geraniol, allyl phenylacetate, heptanoic acid, or heptanal (odorant group B) were not detected (Fig. 1d, e). The 5xFAD mice showed partial impairment of odor detection and not entire loss of function. Based on this behavioral pattern, we concluded that the olfactory abnormality in 5xFAD mice has an uneven particular pattern.

In order to monitor olfactory dysfunction in the early stages of AD, we assessed cognitive and locomotor abilities that could be influenced by olfactory behavior. We performed the spontaneous alternation test and the Morris water maze task in two-, four-, and six-month-old mice (Fig. S1a). We noted that total mobility was not significantly different between WT and 5xFAD mice in the Y-maze test (Fig. S1b). The results are shown as a ratio of the results from the two cognitive tests representing the number of new arm entries in the Y-maze test and the latency time to escape the platform in the Morris water maze test, respectively. Six-month-old mice had a decreased ability to move to a new arm (Fig. S1c) and required more time to find the platform in the water maze (Fig. S1d). However, no statistically significant difference in memory impairment was found between two- and four-month-old mice (5xFAD compared with WT; Fig. S1e, f, Table S1). Based on these collective results, we confirmed that 5xFAD mice display behaviors that mimic the early stages of AD progression between two and four months (Fig. S1e).

**OSNs-derived Ca\(^{2+}\) signals by odorants are decreased in the ventral glomeruli of 5xFAD mice**

Due to the previous results showing that olfactory dysfunction may appear in the peripheral nervous system (PNS) prior to defects in the CNS, we analyzed sensory input signals by specific odorant stimulation in the OB. It is well known that OSNs expressing a specific odorant receptor project their axons to a specific set of glomeruli in the OB. We refer to the regional projection of OSNs as the endo-dorsal axis and ecto-ventral axis in the peripheral olfactory system (Fig. 2a). Using these characteristics of the olfactory system, we have introduced a system that could isolate the detection function by introducing the calcium indicator (fura-2 AM) only in the OSNs (Fig. 2b). Specifically, we used a heat map model to represent the input activity from the OSNs terminal of the entire OB to digitize and visualize the local information. When the OB was sagittal sectioned, the highest point of olfactory synapses (anatomically glomeruli) was noted as “degree of zero” (dorsal side). On the contrary, the lowest point of olfactory synapses was noted as “degree of 180” (ventral side) (Fig. 2c). To make a common odor map in the OB, we analyzed general OSNs-input patterns using C57 mice, a widely used inbred strain (Fig. S2a). The intensity of calcium signals by specific odorants was represented and compartmentalized with specific localization on the glomerulus layer by superimposing them over the repeated calcium image (Fig. S2b). Based on our results, the odor map grouping could be viewed from two different groups using a spatial correlation matrix: group A (lyral, acetophenone, and eugenol) or group B (geraniol, allyl phenylacetate, heptanoic acid, and heptanal) (Fig. S2c). Each odorant was classified according to a spatially correlated value. Interestingly, each of the two groups showed the same odor component in both the spatial matrix and odor detection pattern (Fig. 1e, 2d, S2c). Similar to the results obtained in C57 mice, the intensity of calcium signals by specific odorants in WT/5xFAD mice localized to the glomerulus layer (Fig. S2d). Using the localized activity map, the intensity in WT/5xFAD mice was quantified as a heat map with the spatial information (Fig. 2e, g). It was clustered in two distinct groups, the dorsally located group (group A odorants) and the ventrally located group (group B odorants) (Fig. 2d). The group A odorants presented similar patterns of calcium intensity between WT and 5xFAD mice (Fig. 2e, f, Table 1). On the other hand, group B odorants resulted in a significant decrease in the intensity of calcium on the glomerulus layer in 5xFAD mice (Fig. 2g, Table 1). Through both behavioral and physiological tests using specific odorants, we suggest that spatial abnormality of OSNs calcium signaling is related to the pattern of olfactory dysfunction in 5xFAD mice.
Table 1

| Unpaired t-test | L  | A  | E  | G  | AP | HA | H  |
|-----------------|----|----|----|----|----|----|----|
| P value (two-tailed) | 0.2271 | 0.8298 | 0.9171 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| P value summary | ns | ns | ns | *** | *** | *** | *** |
| T               | 1.287 | 0.2207 | 0.1067 | 8.422 | 9.916 | 12.53 | 9.448 |
| Df              | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Are means significantly different? | No | No | No | Yes | Yes | Yes | Yes |

Data are represented as a numerical value representing the odor-derived Ca$^{2+}$ activity between WT and 5xFAD mice (Fig. 2f, h). The experimental odorants included lyral [L], eugenol [E], acetophenone [A], geraniol [G], allyl phenylacetate [AP], heptanoic acid [HA], and heptanal [H]. Alzheimer’s disease (AD), five familial AD mutations (5xFAD). For the statistical analysis, a two-tailed unpaired t-test was performed. Statistical significances are denoted as follows: non-significant (ns); ***, P < 0.001.

Ecto-ventral OSNs accumulate oligomeric Aβ in 5xFAD mice

Next, we examined whether neurological pathology, including the olfactory defect of 5xFAD mice, is the result of Aβ oligomer formation. We examined the expression pattern of oligomeric Aβ by using A11-immunoreactivity in the OSNs. Basically, OSNs penetrate their axons into the glomerulus of the OB, which is considered as a first olfactory synapse. A11 represents a marker of toxic soluble oligomeric Aβ. By using coronal-sectioned 5xFAD olfactory tissue, A11-immunoreactivity was assessed histologically in both the OSNs layer and its synapses. We introduced a novel stereological analysis on Aβ oligomers in 5xFAD mice. First, the two-dimensional position of olfactory glomeruli digitized as an angle (detailed criteria provided in the materials and methods section) (Fig. 3a). A heat map matrix represents the distribution of the A11-immunoreactivity in olfactory glomeruli along a specific angle (Fig. 3b, c). The A11-positive region of interest (ROI) signal was significantly enriched in 5xFAD mice, especially the ventral layer of the glomeruli (WT dorsal: 5xFAD dorsal = 1.00: 1.13, WT ventral: 5xFAD ventral = 1.54:2.30) (Fig. 3d, e). The Aβ oligomers accumulated along the topographical axis. Specifically, they were within the OSNs layer of the ecto-turbinate compared with the endo-turbinate in 5xFAD mice (WT endo: 5xFAD endo = 1.00: 1.13, WT ecto: 5xFAD ecto = 1.13:2.10) (Fig. 3f, g). As shown above, the uneven Aβ distribution in 5xFAD mice implies that increasing Aβ oligomers correlates with partial dysfunction of OSNs.

Ca$^{2+}$ signals and Aβ accumulation are negatively correlated in the ventral glomeruli in 5xFAD mice

We conducted Spearman's correlation analysis to examine the relationship between A11-immunoreactivity and odorant-dependent calcium activity (Fig. 4). According to angle measurements, the ROI of A11-immunoreactivity displayed a strong correlation in the ventral glomeruli (180°±80°) and a weak correlation in the dorsal glomeruli (0°±80°) of the olfactory bulb (Fig. 3c). The changes in Ca$^{2+}$ fluorescence in the glomeruli by group A odorants (lyral, acetophenone, and eugenol) were higher in the dorsal glomeruli, and group B odorants showed higher Ca$^{2+}$ changes in the ventral glomeruli (Fig. 4a). Interestingly, the changes in Ca$^{2+}$ fluorescence in the glomeruli by group B odorants (geraniol, allyl phenylacetate, heptanoic acid, and heptanal) reduced in line with the strong A11-immunoreactivity in the glomeruli of 5xFAD mice (Fig. 4b), and each group B odorant showed a negative correlation with the A11 intensity, which was not seen for group A (Fig. S3). The most noticeable result is that the stronger the correlation with A11 intensity, the lower the odor detection and OSNs activation in 5xFAD mice (Table 2, Fig. 1d, 2h). The results indicate that decreased OSNs-derived activity and odor detection in 5xFAD mice are influenced by Aβ oligomers.
Table 2

Correlation analysis between levels of A11 immunoreactivity and odor-induced Ca$^{2+}$ activity at the angle-matched olfactory synapse.

| Parameter                  | group A | L     | A     | E     | group B | G     | AP    | HA    |
|----------------------------|---------|-------|-------|-------|---------|-------|-------|-------|
| Number of XY Pairs         | 180     | 180   | 180   | 180   | 180     | 180   | 180   | 180   |
| Spearman’s rho             | 0.5951  | 0.3453| 0.5132| 0.4722| -0.4434 | -0.1664| -0.2983| -0.4641|
| 95% confidence interval    | -0.6849| -0.4710| -0.6159| -0.5811| 0.3141| 0.01672| 0.1551| 0.3375| 0.5743|
| P value (two-tailed)       | < 0.0001| < 0.0001| < 0.0001| < 0.0001| < 0.0001| 0.0251| < 0.0001| < 0.0001|
| P value summary            | ***     | ***   | ***   | ***   | ***    | *     | ***   | ***   |
| Exact or approximate P value? | Gaussian Approximation | Gaussian Approximation | Gaussian Approximation | Gaussian Approximation | Gaussian Approximation | Gaussian Approximation | Gaussian Approximation | Gaussian Approximation |
| Is the correlation significant? (alpha = 0.05) | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

Data represent the relationship between A11 immunoreactivity and odor-induced Ca$^{2+}$ activity at the angle-matched olfactory synapse (Fig. S3). The experiment included lyral [L], eugenol [E], acetophenone [A], geraniol [G], ally phenylacetate [AP], heptanoic acid [HA], and heptanal [H]. For the statistical analysis, a non-parametric correlation was performed, followed by linear regression with a 95% confidence interval. Statistical significances are denoted as follows: non-significant (ns); p < 0.001.

**Ventral periglomerular cells decrease the expression of tyrosine hydroxylase induced by input activity from ecto-OSNs**

We measured tyrosine hydroxylase (TH) immunoreactivity in glomeruli to identify the negative effect of Aβ oligomers on the synaptic function of OSNs. This represents a marker of active OSNs. The number of periglomerular neurons expressing TH was determined as a marker of sensory input in the OSNs. The number of TH-positive neurons was significantly decreased (on average two-fold lower in 5xFAD mice than in WT mice) in the ventral glomeruli (WT dorsal: 5xFAD dorsal = 1.00:1.13, WT ventral: 5xFAD ventral = 1.67: 0.61) (Fig. 5a, b). These results show that the spatially one-sided accumulation of Aβ oligomers may topographically induce partial dysfunction of the OSNs in parallel.

**Decreased maintenance of structure induced by OSNs turnover disruption in the ecto-ventral olfactory region of 5xFAD mice**

Neuronal synaptic dysfunction evokes interaction deficits of each neuron and leads to neural connection problems. The OB is the meeting place of the PNS and CNS, enabling a comparative study relative to an altered condition. Thus, we compared the proportion of each OB layer (Fig. 6a). The total volume of the OB did not differ between WT and 5xFAD mice (Fig. 6b). The dorsal GL area was not significantly changed (Fig. 6c), however, the ventral glomerular layer (GL) area was decreased (WT: 5xFAD = 0.17:0.14) in 5xFAD mice (Fig. 6d). We measured synaptophysin immunoreactivity in the glomeruli to monitor populations of presynaptic vesicles. The ROI of synaptophysin decreased in the ventral olfactory glomeruli of 5xFAD mice (WT dorsal: 5xFAD dorsal = 1.00:0.84, WT ventral: 5xFAD ventral = 0.99:0.63) (Fig. 6e, f).

Next, we examined the turnover cycle of OSNs maintaining the structure of the olfactory system. OSNs proliferation and death were considered as a “turn” and “over,” respectively. The proliferating cells were evaluated by Ki67 positivity in basal cells. The number of Ki67-positive cells in the endo-turbinate had a similar ratio between WT and 5xFAD mice, but the ecto-turbinate in 5xFAD mice showed a significantly decreased number of Ki67-cells that was two-fold lower than in WT mice (Fig. 6g, h). We further measured the ratio of apoptotic cell death using a TUNEL assay. The ratio of apoptotic cells was similar between WT and 5xFAD mice. However, in the ecto-turbinate, the TUNEL-positive cells increased two-fold in 5xFAD mice compared to WT mice (Fig. 6i, j). Moreover, the mature OSNs marker, the olfactory marker protein (OMP) (+) OSNs numbers were decreased in 5xFAD mice, especially in the ecto-turbinate (Table 3), suggesting that these changes are caused by disruption of OSNs turnover.
Discussion

Previous studies have confirmed that olfactory abnormalities in AD may occur in its early stages (12, 24). In particular, AD-related olfactory abnormalities may be specific to the peripheral olfactory system and occur prior to the increased Aβ pathology in the CNS (13, 14). Based on the time frame we established, we confirmed the characteristics of potential AD-related olfactory problems using various odors. In order to exclude the CNS-derived effect on olfactory behavior, we examined functions that mainly involve central nerves including cognition, mood, and locomotion. These tests showed that 5xFAD mice were not affected by any abnormal behavior that can affect olfaction, such as anxiety and/or stress (Fig. S1). Although the mice were mutated so that every neuron overexpresses Aβ, the olfactory behavior was significantly impaired before severe dysfunction of CNS-behavior occurred. According to a previous study, a profound olfactory impairment has been demonstrated to precede the severe memory decline and AD pathology in the brain (12). With regard to AD progression, our experimental time point suggests that hyposmia appears at an early stage of the disease.

Each odorant has a variety of chemical features, and it is well known that the mouse may show preference when detecting odors (25, 26). Olfactory signals are initiated in the periphery and propagate to the central olfactory system. This proceeds with three-step sequences. First, detection and identification of the odor occur via the peripheral olfactory system. Second, odor discrimination occurs, and third, odor cognition occurs via the central olfactory system. As the functional symptoms of AD are primarily related to the brain, studies of abnormal olfaction have pointed towards the dysfunction of the brain. Studies on the peripheral pathology have been overlooked, and we are the first to thoroughly illustrate the relationship between disturbances of OSNs and AD hallmarks. According to our result, Aβ overexpressing AD model mice show particular smell deficits that are processed in the ecto-ventral OSNs. Clinically patients with AD with olfactory dysfunction have been reported to have an atypical dysfunctional phenotype. They are not able to sense particular odors but are not completely anosmic. They recognize the presence of odor; however, this phenomenon does not mean that they can detect all odorant information correctly. In terms of the first-step in the processing of odor information, study of OSNs of the peripheral olfactory system is required to clarify mechanisms involved in hyposmia.

Odors could be divided into at least two groups according to the behavior and physiological response in this paper. One odor group was found to activate ecto-OSNs targeting the dorsal glomeruli, and the other group activated endo-OSNs targeting the ventral glomeruli. Considering the “zone” theory (i.e., topographical axis), which suggests an important role for the projection from the OE to OB, it can be hypothesized that partial damage in the OE caused by AD may lead to differences in odor-specific responses. To better understand these mechanisms, we aimed to uncover the “region” involved by using various physiological analyses. It is thought that odor identity is determined by a combination of odorant receptors activated by an odorant. Activity maps are presented by each odorants’ input, and the spatial patterns of input activity were created for glomeruli. Despite the OB receiving complex odor information, humans and other animals can identify a specific odorant owing to spatial patterns across glomeruli. We attempted to quantify the relationship between the odor map by kinetics and the local information of AD pathology of the glomerular layer in the olfactory system. The measurement applied to the degree of glomeruli suggests advanced topographical analysis in the peripheral olfactory system. This digitized measurement enables us to compare among various factors from anatomy to physiology and to confirm a correlation based on the virtual region-coordination. Our results show that odor grouping according to the odor map information (Fig. 2c) was consistent with odor grouping in the behavioral study (Fig. 1e). Only odors from group B showed a larger difference in kinetics of 5xFAD than WT mice. Interestingly, the olfactory behavior corresponded with the level of calcium kinetics (Fig. 1e, Table 2). The results revealed a strong correlation between the partial behavioral defect and the pattern of low calcium kinetics. Additionally, the regional characteristics of the olfactory system in AD are associated with topographical organization formed by regeneration of OSNs. Further investigation on mapping OSNs-activity by various odorants needs to be carried out in the future to permit clearer visualization and quantification of global glomerular patterns. Although we focused only on the glomerular layer, the odor map data contains all the responses from the entire OB between WT/5xFAD mice. Thus, these methods can be easily applied to AD diagnosis by evaluating the patterns in distinct OB layers and correlating them with the glomerular patterns.

We identified the accumulation pattern of Aβ oligomers to define the relationship between abnormal behavior and AD pathology. Previous studies using transgenic mice suggested that both AD brain-derived and synthetically prepared Aβ oligomers could influence the neuronal network. The results imply that an increased threshold caused by Aβ oligomers may alter the behavioral and physiological thresholds for the detection of certain groups of odors, although more studies using various stimuli are necessary. Furthermore, Aβ could induce early synaptic toxicity (27, 28), long-term potentiation (LTP) deficits (13), and/or trigger cellular toxicity by involving tau phosphorylation and neurofibrillary tangles (29). Moreover, we pointed previously that Aβ oligomer toxicity in the OE may induce direct impairment of the olfactory system in early AD as OSNs express an APP-cleavage enzyme and can generate Aβ autonomously (12, 30). Besides, oligomers are formed early in Aβ accumulation and can be harmful to the neural function and structure of olfaction in this paper. In addition to widely-known pathological effect of Aβ, recent studies have noted interesting feature of Aβ. Aβ species suggested as pathological seeds and spreading process deteriorating proteinopathy in neurodegenerative disease (31). Indeed, co-proteinopathy like α-synuclein could be associated with toxic aggregation of proteins by being associated with endogenous Aβ expression in 5xFAD (32), α-synuclein measured together with Aβ expression in the olfactory

| % of OMP (+) cells (mm²) | WT    | 5xFAD |
|--------------------------|-------|-------|
| endo                     | 65.29 ± 5.10 | 62.70 ± 4.04 (ns) |
| ecto                     | 71.11 ± 3.95 | 52.74 ± 5.25 (***)) |

Data are represented as percentages of OMP (+) cells in the OE (mm²) (means ± SDs). For statistical analysis, a two-way ANOVA was performed, followed by Bonferroni post hoc test. Olfactory marker protein (OMP), olfactory sensory neurons (OSNs), Alzheimer’s disease (AD), five familial AD mutations (5xFAD), endo: endo-turbinate; ecto: ecto-turbinate. Statistical significances are denoted as follows: non-significant (ns); ***, P < 0.001.
epithelium of Human APP-overexpressing transgenic mice (N5 TgCRND8) (33). Hence, Aβ present in OSNs not only can generate and accumulate misfolding protein itself, but also is enough to establish systemic vulnerability in the early stage of AD.

We confirmed the relationship between oligomerized Aβ proteins and odor dysfunction using 5xFAD mice. We determined the synaptic activity of our target glomeruli and confirmed direct toxicity of Aβ. Sensory deprivation between OSNs and mitral cells induces decreasing TH expression. TH-immunoreactivity in periglomerular neurons reflects neuronal activation (34–36). In the current study, a significant loss of TH-positive neurons was observed in the ventral OB of 5xFAD mice. This indicates double-confirmed data of Ca^{2+} activity representing OSNs-derived input signals. Most strikingly, damaged regions were found in the ventral region, our focused region, which matched well with the diminished amounts of OMP, mature olfactory sensory receptor neurons (37). This was also confirmed by the results of our TUNEL analyses and the reduced proliferation rate within this region. It is well known a reduction in neurogenesis is induced by oligomerized Aβ proteins, but this is the first report showing that this event also occurs in the peripheral olfactory system. Since the OE maintains the number of OSNs by continuous proliferation and differentiation of basal cells, a reduction of this reconstitution may be a direct cause of the reduction in OSNs numbers. This phenomenon is intrinsically faster in the ecto-turbinate than the endo-turbinate (Fig. S4). The OE has topographical zones, which have a different environment during their regeneration from both the external environment and intrinsic molecular mechanisms (38, 39). We can infer that the region could be the cross point between regeneration and degeneration because APP and its machineries give rise to not only Aβ generation but also neural development and axon guidance related with synaptic formation and neural reconstitution (40, 41). Taken together, our results suggest that the regional differences identified may play a role in one-sided Aβ accumulation and partial olfactory dysfunction.

**Limitations**

The main objective was to identify hyposmia accompanying in the early phase of AD using the 5xFAD mouse. This mouse line has limitation in reflecting complete human symptoms of AD. Nevertheless, the experiment using 5xFAD can recapitulate Aβ expression and accumulation in the neuron system, leading causes in AD. Furthermore, the experimental design was specifically employed to capture the pathologic Aβ effect on the olfactory dysfunction, one of the cardinal AD symptoms. We have chosen the mouse model by rational hypothesis (Fig S1), but we also clarify the limitations in this selection.

We delve deeper into the patho-physiologic and anatomic effects in the peripheral olfactory sensory neurons by Aβ. The 5xFAD begins to exhibit decline in cognition, neuronal loss and changes in LTP/LTD in four to six months of age. Although the 5xFAD is conventional transgenic line that has not been specifically mutated in the olfactory sensory neuron, our data showed neuronal and functional loss in three-month-old mouse. In addition, we tested that the CNS-derived effects were excluded on olfactory function, and therefore, we hypothesized that the stage mimics the early stage of AD. However, the study design that was hypothesized the early stages of AD progression should not be overlooked when interpreting the results because the early stage of AD may be relative and not always feasible for manifestation.

Seven odorants were tested in the experiment based on the preference test in the previous research. Regarding the limited numbers of odorants, it could be argued that these odorants cannot fully create categories for future therapeutic application such as diagnosis and clinical trials. Hence, further experiments should classify odor types for AD-associated olfactory dysfunction.

**Conclusions**

The present study characterized hyposmia in 5xFAD mice. We found that the olfactory abnormality in 5xFAD mice was determined by the relationship between Aβ oligomers and the regions of the nervous system that are responsible for odor detection. We have also shown, through advanced topographical analysis, that the specific patterns of Aβ oligomer accumulation can attenuate the activity in our target synapses. We confirmed a negative correlation between aggregated Aβ and both OSNs-derived Ca^{2+} signals and corrupted structural stability. Moreover, these effects were identified by focusing on the spatially ecto-ventral halves formed by OSNs turnover, representing a feature of the peripheral olfactory system. Thus, the collapse of the peripheral system could be the greatest feature of AD-related olfactory abnormalities.

**Abbreviations**

AD: Alzheimer's disease
OSNs: olfactory sensory neurons
FAD: familial forms of AD
APP: amyloid precursor protein
PS1: presenilin 1
5xFAD mouse: human APP and PS1 transgenic mouse with a total of five AD-linked mutations
Aβ: amyloid-β
OB: olfactory bulb
OE: olfactory epithelium
CNS: central nervous system
Declarations

Ethics Approval and Consent to Participate

All applicable guidelines for the care and use of laboratory animals from the National Institutes of Health Guide were ethically followed. All experimental protocols were approved by the Institutional Animal Care and Use Committees of DGIST (The details were referred in Method-Animal section). Provided appropriate consents for experiments using 5xFAD transgenic mice were given by Prof. K.A. Chang.

Consent for Publication

Not applicable. We do not contain data from any individual person.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
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Author Contributions

G.S., S.-J.Y., and C.M. contributed to the conception or design of the work; G.S. and S.-J.Y. contributed to data collection, data analysis, data interpretation, drafting the article, and figure drawing; S.W.K., A.R., H.P., B.C., and K.-A.C. contributed to the data collection and analysis; D.H.J., Y.-H.S., H.W.M.S. and C.M. contributed to the critical revision of the article; C.M. supervised all experiments and analyses.

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Not applicable.

Competing Interests

The authors have declared that no conflicts of interest exist.

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Figure 1

Atypical, AD-like olfactory behavior in 5xFAD transgenic mice. Experimental subjects are three-month WT or 5xFAD mice unless otherwise noted. (a–b) Food seeking test (WT, n = 6; 5xFAD, n = 6). (a) Scheme illustration. (b) The latency was measured as mean ± SEM. For statistical analysis, an unpaired t-test was performed. (c–e) Odor detection test was performed (WT, n = 6; 5xFAD, n = 6). (c) Scheme illustration. For PI analysis, areas A and B were defined to distinguish the location of the experimental odorant in the cage. (d) PI value represented as mean ± SEM. The experimental odorants refer to lyral [L], eugenol [E], acetophenone [A], geraniol [G], ally phenylacetate [AP], heptanoic acid [HA], and heptanal [H]. For statistical analysis, an unpaired t-test was performed. (e) Representative heat map showing duration (H; high, L; low). Statistical significances are denoted as follows: ns, non-significant; *P < 0.05, **P < 0.01, ***P < 0.001). Wild-type (WT), five familial AD mutations (5xFAD), Alzheimer's disease (AD).
Figure 2

Odor-derived Ca2+ signal in peripheral olfactory glomeruli divided into two groups. (a) Illustration of topographical axis. (b) Illustration of wide-field fluorescence imaging to measure OSNs-derived input signal. (c) The imaging location with high resolution stereoscopic fluorescence image of fura-2 by OSNs calcium concentration (left) and averaged activity sample map along histologic angle (D; dorsal, V; ventral, A; anterior, P; posterior) (right). (d) Heat map clustering showing the Spearman's correlation coefficient between each active calcium signal pattern with the angle of glomeruli located. (WT, n = 5–6). Each odorant was divided into two groups; lyral [L], acetophenone [A], and eugenol [E] (group A), and geraniol [G], allyl phenylacetate [AP], heptanoic acid [HA], and heptanal [H] (group B). (e,g) The merged intensity (cumulated signal) (f,h) of olfactory synaptic activity (ΔF/F) induced by each group odorant (WT, n = 5–6; 5xFAD, n = 5–6). (e–f) Odor-group A. (g–h) Odor-group B. ΔF/F represented as mean ± SEM from five independent experiments. For statistical analysis, an unpaired t-test was performed using Prism software (GraphPad software). Statistical significances are noted (***P < 0.001). Olfactory sensory neurons (OSNs), wild-type (WT), five familial AD mutations (5xFAD), Alzheimer's disease (AD).
Figure 3

Aβ oligomer accumulation in the ecto-ventral halves of the peripheral olfactory system of 5xFAD mice. (a) Illustration of criteria to indicate the relative angle position of olfactory synapses. (b) Representative A11-positive ROI heatmap in WT (left) and 5xFAD (right) mice. (c) Radial chart represents A11-positive ROI along the angle of olfactory synapses. (d-e) A11 immunoreactivity was determined in the olfactory glomeruli of WT versus 5xFAD mice (WT, n = 7; 5xFAD, n = 9). (d) The local intensity of A11 (+) (green), OMP (+) (red), and DAPI (blue). (e) A11 immunoreactivity in the glomerulus (top) and topographical illustration (bottom). (f–g) A11 immunoreactivity was determined in the OSNs-layers of WT versus 5xFAD mice (WT, n = 7; 5xFAD, n = 9). (f) The local intensity of A11 (+), OMP (+), and DAPI (blue). (g) A11 immunoreactivity in OSNs-layers (top) and topographical illustration (bottom). All data are presented as mean ± SEM from three independent experiments. For statistical analysis, a two-way ANOVA was performed, followed by Bonferroni post hoc test. Statistical significances are noted (ns, non-significant; ***P < 0.001). Olfactory marker protein (OMP), Olfactory sensory neurons (OSNs), wild-type (WT), five familial AD mutations (5xFAD), Alzheimer's disease (AD).
Figure 4

(a) WT

(b) 5xFAD

Increased A11 immunoreactivity and decreased odor Ca2+ activity in the ventral glomeruli of 5xFAD. (a–b) Scatter plots with Spearman’s correlations among the degree of glomeruli (°), A11 immunoreactivity (intensity), and odor Ca2+ activity in the olfactory glomeruli in WT (a) compared with 5xFAD (b) mice. Scatter diagrams displaying correlations between each variation, a linear regression analysis with 95% confidence intervals was used (gray dashed line). Dot and line showing odor Ca2+ activity indicated in orange (group A) and purple (group B), and A11 immunoreactivity data indicated in red dots and line. Wild-type (WT), five familial AD mutations (5xFAD), Alzheimer’s disease (AD).
Figure 5

Reduction of TH expression in the ventral olfactory periglomerular cells of 5xFAD mice. (a–b) TH (+) (green) PG cells were identified in the olfactory glomeruli of WT versus 5xFAD mice (WT, n = 4; 5xFAD, n = 3). (a) The local intensity of TH (+) PG cells. (b) Quantitative analysis of TH (+) PG cells/5 GL cells. Data are represented as means ± SEMs from three independent experiments. For statistical analysis, a two-way ANOVA was performed, followed by Bonferroni post hoc test. Statistical significances are denoted (ns, non-significant; **P < 0.01). Wild-type (WT), five familial AD mutations (5xFAD), Alzheimer's disease (AD).
Figure 6

Disrupted OSNs structure in the ecto-ventral region of the peripheral olfactory system. (a–d) Analysis of the volume of the olfactory glomerular layer (WT, n = 3; 5xFAD, n = 3). (a) Depiction of coronal sections of the olfactory bulb with laminar structure. glomerular layer (GL), external plexiform layer (EPL), granule cell layer (GCL). (b) Total OB volume. GL, EPL, and GCL volume was measured in the dorsal olfactory synapses (c) and ventral olfactory synapses (d). (e–f) Synaptophysin (+) (green) glomeruli in WT versus 5xFAD mice (WT, n = 4; 5xFAD, n = 3). (e) The local immunoreactivity of synaptophysin (+) glomeruli. (f) Synaptophysin immunoreactivity. (g) Representative KI67 (+) data in the local OSNs layer (WT, n = 3; 5xFAD, n = 3). (h) Quantitative analysis of KI67 (+) cells. (i) Representative TUNEL-positive data in local OSNs layers and (j) comparative quantification (WT, n = 3; 5xFAD, n = 3). Data are represented as mean ± SEM from three independent experiments. For statistical analysis, a two-way ANOVA was performed, followed by Bonferroni post hoc test. Statistical significances are noted (ns, non-significant; **P < 0.01, ***P < 0.001). Olfactory sensory neurons (OSNs), wild-type (WT), five familial AD mutations (5xFAD).

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