Age-induced disruption of selective olfactory bulb synaptic circuits

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Little is known about how normal aging affects the brain. Recent evidence suggests that neuronal loss is not ubiquitous in aging neocortex. Instead, subtle and still controversial, region- and layer-specific alterations of neuron morphology and synapses are reported during aging, leading to the notion that discrete changes in neural circuitry may underlie age-related cognitive deficits. Although deficits in sensory function suggest that primary sensory cortices are affected by aging, our understanding of the age-related cellular and molecular changes is sparse. To assess the effect of aging on the organization of olfactory bulb (OB) circuitry, we carried out quantitative morphometric analyses in the mouse OB at 2, 6, 12, 18, and 24 mo. Our data establish that the volumes of the major OB layers do not change during aging. Parallel to this, we are unique in demonstrating that the stereotypic glomerular convergence of M72-GFP OSN axons in the OB is preserved during aging. We then provide unique evidence of the stability of projection neurons and interneurons subpopulations in the aging mouse OB, arguing against the notion of an age-dependent widespread loss of neurons. Finally, we show ultrastructurally a significant layer-specific loss of synapses; synaptic density is reduced in the glomerular layer but not the external plexiform layer, leading to an imbalance in OB circuitry. These results suggest that reduction of afferent synaptic input and local modulatory circuit synapses in OB glomeruli may contribute to specific age-related alterations of the olfactory function.

aging | axodendritic synapses | dendrodendritic synapses | mitral cells

Age-related neurodegenerative diseases, such as Parkinson and Alzheimer's, involve localized or widespread neuronal loss, but little is known about the changes occurring in the brain during normal aging. A growing consensus argues against widespread neuronal loss and atrophy during aging (1, 2). Rather, subtle region- and layer-specific alterations of neuronal morphology and synaptic connections are reported in the neocortex and hippocampus, where they may contribute to age-related cognitive deficits.

The laminar organization of olfactory bulb (OB) neurons and synapses provides a simplified cortical model in which we can probe principles of neuronal and synaptic organization during aging. Sensory functions are affected by aging, including alterations in olfactory acuity, discrimination, and memory (3–12). The OB is the first central relay in the pathway processing odor information: it receives afferent input from olfactory sensory neurons (OSNs) located in the olfactory epithelium (OE) and is responsible for detecting odors in the nasal cavity. OSN axons synapse on mitral/tufted cell dendrites in OB glomerular neuropils. OB local interneurons modulate the activity of mitral/tufted cells, contributing to odor signal integration, before propagation to the piriform cortex. Projections from the OE to the OB are organized in an odor receptor (OR) map; each subpopulation of OSNs expressing the same OR converge into approximately two stereotypically located glomeruli per OB (13).

A cohesive understanding of the cellular and molecular organization of the aging mouse olfactory system is lacking. Some age-related changes in the OE and OB were reported, mainly in rats (4, 14–20), but none document the kinetics of the defects. Here, we report quantitative morphometric analyses of the aging mouse

OB at 2, 6, 12, 18, and 24 mo, focusing on changes in layer morphology, neuronal populations, synaptic circuitry, and OR map. We show that the OB laminar and cellular organization remains stable during aging, but that synaptic circuits are significantly altered in a layer-specific manner. Our findings support the notion that discrete alterations in synaptic circuitry occur systematically during aging, potentially leading to imbalances in sequentially organized cortical networks.

Results

Laminar Organization of the Aging OB. The OB is organized into concentric laminae (Fig. 1A). The superficial olfactory nerve layer contains afferent axons from OSNs. In the glomerular layer, OSN axons synapse with dendrites of OB projection neurons, mitral/tufted cells, and subpopulations of local periglomerular cells. Within the external plexiform layer (EPL), local circuit synapses occur between the mitral/tufted cell lateral dendrites and granule cell apical dendrites. Mitral cell somata are found in the mitral cell layer. The granule cell layer (GCL) contains the somata and basal dendrites of granule cells. The total OB volume was stable across ages (Fig. 1B and Table S1), ranging from 5.69 ± 0.31 to 5.56 ± 0.17 mm³ at 2 and 24 mo. Similarly, independent volumetric analysis of each OB layer (glomerular, EPL, and GCL) showed stability during aging (Fig. 1C–E). Finally, the proportion represented by each layer did not change (Fig. 1F). Our results thus show a high stability of the total OB volume and its constituent layers during aging.

Broad OSN Axon Projections on the Aging OB. OSN axons innervate OB glomeruli providing afferent input. Therefore, the glomerular diameter and number of glomeruli are useful as broad indicators of the integrity of OSN axon projections. The vesicular glutamate transporter 2 (VGluT2) is selectively expressed in OSN axon terminals (21) and we used this neuropillar staining to measure the diameter of individual glomeruli (Fig. 2A). Glomerular diameter did not change during aging, ranging from 55.4 ± 2.9 μ m to 53.9 ± 1.2 μ m at 2 and 24 mo (Fig. 2B, Fig. S1, and Table S1). The number of glomeruli per section was also stable (Fig. 2C and Table S1), averaging 111.5 ± 3.7 and 120.4 ± 2.8 at 2 and 24 mo. The estimated total glomeruli per OB did not change significantly, ranging from $3,599 \pm 433$ to $3,947 \pm 275$ at 2 and 24 mo (Fig. 2D and Table S1). These results strongly suggest that OSN axon projections to the OB are preserved during aging.

M72-GFP Olfactory Axon Convergence. OSN axons expressing the same OR begin to coalesce in the nerve layer before their final convergence into only two or three glomeruli per OB (13, 22,

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Fig. 1. Mouse OB volume is stable during aging. (A) OB layers delineation. (Scale bar, 500 μm .) (B) Total OB volume. (C) Glomerular layer volume. (D) EPL volume. (E) GCL volume. (F) Contribution of each layer. ($n = 3$). GL, glomerular layer; ONL, olfactory nerve layer.

23). To assess the trajectory of a molecularly defined subset of OSN axons, we used M72-GFP mice that express GFP under the M72 OR promoter (23). Two M72-GFP glomeruli were detected in each OB analyzed, one on the dorsolateral surface and the other on the posterior ventromedial region (Fig. 3A), indicating that neither loss nor addition of M72-GFP glomeruli occurred during aging. We used two criteria to map glomerular position: (i) the angle from the medial line between OBs, and (ii) the distance to an origin point located at the border of the cortex (Fig. S2A). The angle was stable during aging ($55.7 \pm 0.9^\circ$ and $53.0 \pm 2.6^\circ$ at 2 and 24 mo) (Fig. 3C and Table S1). However, the distance was significantly affected by aging ($F_{4, 26} = 7.900$, $P = 0.0003$) and shorter at 6 than at 12, 18, and 24 mo (Fig. 3D). The change in position was significant on the x and y axes, with both distances shorter at 6 than at 24 mo (Fig. S2A–C). The position of the dorsolateral M72-GFP glomerulus shifted $\approx 200 \mu\text{m}$ with aging. This result is most likely because of an OB size increase as, unlike C57Bl6J mice, the total OB volume of M72-GFP mice increased significantly from 6 to 24 mo ($+12\%$; 7.5 ± 0.3 to $8.4 \pm 0.2 \text{ mm}^3$ at 2 and 24 mo) (Fig. S2D).

Confocal imaging of M72-GFP axon convergence showed single axons and multiple fascicles of diverse diameters entering the dorsolateral glomerulus (Fig. 3B). Most axons and fascicles

innervated the glomerulus from the anterior side, although some followed tortuous courses and entered posteriorly. Throughout aging, the diversity in shape, size, and convergence of M72-GFP axons was retained; no age-dependent alterations were evident. Our results show that the targeting and convergence of M72-GFP axons are preserved from 2 to 24 mo.

Projection Neurons and Interneurons of the Aging OB. Mitral/tufted cells are the OB projection neurons. Although both express Tbx21 (24), mitral cell somata are easily distinguished by their location in the mitral cell layer. Mitral cell density was stable during aging from 56.4 ± 1.5 to 61.9 ± 5.4 cells/ mm^2 at 2 and 24 mo (Fig. 4A and Table S1). Similarly, the number of mitral cells per OB (33,000) did not change during aging (Fig. S3A). Mitral/tufted cell activity is modulated by two local interneurons, granule and periglomerular cells (PG). Granule cells are GABAergic inhibitory interneurons, and PG cells are a heterogeneous population, containing GABAergic interneurons, glutamatergic short axon cells, and dopaminergic (TH⁺) cells (25, 26). All granule cells express NeuN, as does a subpopulation of PG cells, which constitutes $\approx 60\%$ of the GAD67-GFP⁺ PG cells (Fig. 4D). Interestingly, mitral/tufted cells do not express NeuN (27), allowing us to use NeuN to selectively identify OB interneurons. Granule cell density was stable during aging, from $4.04 \pm 0.3 \times 10^5$ to $3.88 \pm 0.6 \times 10^5$ cells/ mm^3 at 2 and 24 mo (Fig. 4B). The total number of granule cells was also maintained, ranging from $1.03 \pm 0.8 \times 10^6$ to $1.00 \pm 0.2 \times 10^6$ cells at 2 and 24 mo (Fig. S3B). Newborn granule cells continuously integrate into the adult OB and preferentially settle in the deeper part of the GCL (28, 29). Because neurogenesis decreases with aging (17, 30), the deep GCL seemed more likely to be affected. However, the density and total number of granule cells in the deep GCL were stable during aging, as was the volume of this region (Fig. S3C–F). In the glomerular layer, densities of NeuN⁺ and TH⁺ PG cells were both constant during aging (Fig. 4C and E), averaging $1,077 \pm 18$ and $1,085 \pm 135$ cells/ mm^2 for NeuN⁺ PG cells and 318 ± 60 and 301 ± 28 cells/ mm^2 for TH⁺ PG cells at 2 and 24 mo. Collectively, our results show that OB projection neurons and interneurons are not lost during aging.

Synaptic Organization of the Aging OB. OB synapses are located principally in the glomerular layer and EPL. The glomerular layer contains axodendritic (A:D) synapses from OSN axons onto mitral cell dendrites, as well as dendrodendritic (D:D) synapses between mitral and PG cell dendrites (Fig. 5A–B'). The EPL contains reciprocal D:D synapses between mitral/tufted cell lateral dendrites and granule cell dendritic spines (31). These synapses can be classified as asymmetrical (mitral to granule), symmetrical (granule to mitral), or reciprocal (Fig. 6A–C'). The criteria used to identify each type of synapses are detailed in SI Methods. Synapses were identifiable at all ages, indicating no major changes in synaptology (Fig. S4).

Total synapse density in glomeruli was significantly reduced during aging ($F_{3, 8} = 4.467$, $P = 0.0402$) (Fig. 5C). Post hoc tests

Fig. 2. Broad OSN axon projections on the aging OB are stable. (A) Example of VGluT2 immunostaining. (Scale bar, 500 μm .) (B) Mean glomerular diameter. (C) Number of glomeruli per section. (D) Total number of glomeruli per OB. ($n = 3$).

In striking contrast, EPL total synapse density was stable across ages, ranging between 18.7 ± 2.1 and 18.6 ± 1.0 synapses per $100 \mu\text{m}^2$ from 2 to 24 mo (Fig. 6D). Furthermore, individual analyses of asymmetrical, symmetrical, and reciprocal synapse densities (Fig. 6E–G) did not show any significant effect of age (Table S1). Our results thus show that synapse densities are stable during aging in the EPL although they are reduced in glomeruli.

Changes in Volume and Cell Numbers in the Aging Brain. Contrary to earlier reports of extensive volume and cell losses in the aging brain, our data suggest more subtle region- and layer-specific changes (1, 2). In the OB, there are reports of both strain- and species-dependent effects of aging (14–17). Our results, which included more intermediate time points, establish that OB volume is stable from 2 mo onward in C57BL/6J mice. Unexpectedly, the OB volume of M72-GFP mice increased from 6 to 24 mo. The difference in C57BL/6J vs. M72-GFP may reflect strain-specific susceptibility to aging or the strategy used in measuring OB volumes: we included the accessory OB and olfactory nucleus to account for the shift of the M72-GFP dorsolateral glomerulus, although C57BL/6J measurements focused on the main OB.

Using cell-specific markers, we report a total of $\approx 33,000$ mitral cells and $\approx 10^6$ granule cells in the adult mouse OB. Our finding that OB neuronal populations are stable across aging contributes to the emerging notion that age-related cell loss is not a ubiquitous event in the brain. PG cell subpopulations are molecularly diverse

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Fig. 5. Synaptic density decreases with aging in mouse glomeruli. (A–D) A:D and D:D synapses in the glomerular layer. Arrow designates the polarity of the synapse. (Scale bar in A, 100 nm.) (C) Total synapse density. (D) D:D synapse density. (E) A:D synapse density. (F) Ratio of A:D to D:D synapses. ($n = 3$; $P < 0.05$). Ax, OSN axon; Md, mitral cell dendrite; PGd, PG cell dendrite.

(26); they all establish synaptic contacts with mitral/tufted cell dendrites, but only some of them contact OSN axons (25). We focused our analysis on the TH^+ [12.6% of total PG cells (26)] and NeuN^+ subpopulations ($\approx 60\%$ of GAD67 PG cells). Conversely, the NeuN^+ subpopulation is predominately composed of GABAergic interneurons (90% of NeuN^+ cells are GAD67 $^+$). Our results are unique in providing evidence that NeuN^+ GABAergic and TH^+ dopaminergic PG cells, which both receive direct input from OSN axons (25), are maintained during aging. Together with our data, reports on calretinin $^+$ and dopaminergic PG cells (17, 30) converge on a relative stability of PG subpopulations during aging.

Changes in Synapses in the Aging Brain. Increasingly, age-related cognitive defects are considered to result from synaptic and molecular changes, leading to alterations of neuronal communication (1, 2). We provide a unique demonstration that age-induced changes in OB synaptic density are dependent on the layer; the synaptic density is reduced in glomeruli but stable in the EPL. Layer-specific changes of the synaptic density seem to be a general feature of the aging brain: reduction occurs in layer II but not in layer IV of the sensorimotor cortex; in the parietal cortex, synapse loss is more pronounced in the deeper layers V and VI than in the superficial layers I to IV (32, 33). Mitral cells have D:D synapses on their apical dendritic tuft in glomeruli and on their lateral dendrites in the EPL. The age-dependent loss of D:D synapses in glomeruli, but not in the EPL, suggests that the dynamic maintenance of synapses is location-dependent (apical vs. lateral dendrites). Further studies would be necessary to investigate whether common mechanisms are responsible for location-dependent susceptibilities of synapses in the aging brain.

Fig. 6. Synaptic density is stable during aging in the mouse EPL. (A–C) Asymmetrical, symmetrical, and reciprocal D:D synapses in the EPL. Arrow indicates the polarity of the synapse. (Scale bars: A–B', 100 nm; C, 300 nm; C', 200 nm.) (D) Total synapse density. (E) Asymmetrical synapse density. (F) Symmetrical synapse density. (G) Reciprocal synapse density. ($n = 3$). Grd, Granule cell dendrite; Md: mitral cell dendrite.

Both A:D and D:D glomerular synapses are lost during aging, ruling out a reduction in density affecting preferentially one type of synapse in the OB. Despite the decreases, the ratio between A:D and D:D synapses is stable, suggesting an equilibrium between glomerular OSN input and local circuit modulatory synapses. In the EPL, the ratio between symmetrical and asymmetrical synapses is also preserved, as well as the density of reciprocal synapses, supporting the hypothesis that D:D symmetrical and asymmetrical synapses are interdependent (31). Such balance between synapse types has also been reported in frontal and parietal cortices, where the ratio of excitatory to inhibitory synapses is maintained during the parallel decrease of both presynaptic boutons (34). However, maintenance of this balance is not a ubiquitous phenomenon, because inhibitory synapses are preferentially affected by the synapse reduction in the sensorimotor cortex (32).

Decreases in synapse numbers may result from reductions in: (i) number of pre- or postsynaptic neurons; (ii) branching of pre- or postsynaptic processes; (iii) number of synaptic contacts established by a single process; or (iv) any combination of the preceding. The significant loss of glomerular A:D synapses we report here is likely related to global reductions in OSN density and OR-specific OSN subpopulations (4, 14, 18–20). In contrast, D:D synapses are also lost despite stability of the number of PG and mitral/tufted cells. In addition, synapse loss in the glomeruli is not caused by shortening of axonal and dendritic processes because the glomerular diameter is stable. Moreover, there was no evidence of glial hypertrophy that could compensate for a reduction in the glomerular space occupied by axons and dendrites. However, stability of the glomerular diameter, despite OSN loss, suggests

that remaining OSN axons are likely arborizing, but without making new compensatory A:D synapses. All these data converge to suggest that mechanisms involved in synapse maintenance and establishment are down-regulated with aging (e.g., synaptic adhesion molecules, cytoskeleton proteins, trophic factors, neurotransmitters, and so forth) and may provide a fruitful focus for future studies in OB as in neocortex.

The OB Is Stable During Aging Despite Ongoing Neurogenesis in the Olfactory System. Adult neurogenesis occurs in both the OE and OB (28, 29, 35). In the OE, progenitors give rise to new OSNs that send an axon to the OB. Neuroblasts born in the subventricular zone migrate to the OB, where they differentiate into granule and PG cells and integrate into the synaptic circuitry.

Neurogenesis persists in the aging OE but at a reduced rate (18, 19). As noted above, stability of the glomerular diameter demonstrates that axons of new OSNs grow appropriately toward the OB during aging. Our data on M72-GFP axons provide a unique report of convergence and targeting of an OSN subpopulation in aged animals beyond 3 mo postnatal (22, 23). This result provides unique evidence that mechanisms and molecular substrates ensuring axon growth and targeting persists throughout life in the olfactory system, in contrast to most areas of the nervous system. The dorsolateral M72-GFP glomerulus position shifts $\approx 200\ \mu\text{m}$ between 6 and 12 mo, probably because of the 12% increase in total OB volume of these mice. Such shifts might also result from loss or shrinkage of neighboring glomeruli corresponding to other ORs. Indeed, OR-specific reductions in OSN number occur during aging, although zonal organization is retained (20), in accordance with our data showing the maintenance of the M72-GFP glomerulus position.

Approximately 95% of adult-born cells in the OB differentiate into granule cells; the remaining 5% become PG cells (28, 29). It is not clear if newborn neurons replace old interneurons or if they are added and preexisting interneurons are maintained. Because subventricular zone stem cell proliferation decreases with age (30, 36, 37), we hypothesized that the total number of granule cells would decrease proportionately. Here, we provide evidence that granule cells (total and deep populations) are stable during aging. This finding may suggest that the number of new granule cells added is negligible compared with the number of preexisting granule cells. Alternatively, the population stability may reflect a balance between addition and depletion of granule cells. Mechanisms compensating for the reduced number of newborn neurons in aged animals could include a reduced cell death of old granule cells or an increased survival of newborn cells. Such aging-compensatory mechanisms are also likely to be at play at the level of EPL D:D synapses, allowing a balance between the number of synapses made by newborn granule cells and lost by old cells.

Are Morphological Changes in the OB Related to Alterations in Olfactory Function? Olfactory deficits reported in aging rodents include alterations of acuity, discrimination, and memory, although changes are variable depending on the paradigm (3–12). Olfactory information processing involves successively the OE, OB, and piriform cortex. How may cellular alterations localized at each level of the olfactory pathway contribute to age-related changes in olfactory function?

Alterations in olfactory acuity are often thought to rely on changes in the OE. Although reductions of specific OSN subpopulations (20) may contribute to detection threshold deterioration, odor-induced activity patterns are maintained in the aged OE (18), as are the sensitivity and response of MOR23-GFP OSNs (20). The data suggest that morphological changes in the OE are a minor component of aging-related olfactory deficits. We propose rather that the loss of glomerular A:D synapses is leading to higher detection thresholds. It might be surprising that the decrease in

A:D synapses is not accompanied by a reduction in TH expression and activity (17, 38). TH expression in the glomerular layer is decreased when odorant-induced activity is eliminated with naris occlusion (39) or OSN elimination (40). However, the age-related decrease in glomerular synapses may result in more subtle changes in activity that would be unlikely to affect TH expression. Notably, the precise mechanism by which OSN activity influences TH expression is unknown.

Odor discrimination relies on the mapping of the identity of an odor on the OB glomerular surface as a combination of glomeruli displaying various levels of activation (41). A precise OR map is established by the convergence of homogeneous populations of OSN axons in a restricted number of OB glomeruli (13). Therefore, odor discrimination could be altered if the OE to the OB projections were modified. However, our results show that these projections are stable during aging and that OR map perturbation is unlikely to underlie olfactory discrimination alterations. Instead, current models suggest that odor discrimination relies on local circuit D:D synapses in the EPL, as well as intra- and interglomerular inhibition mediated via PG cells (41–43). Because D:D synapse density is decreased in glomeruli and not the EPL, reduced glomerular inhibition is a likely candidate for contributing to alterations in olfactory discrimination during aging. Interestingly, within glomeruli, A:D synapse density progressively decreases with age, but D:D synapse density drops abruptly between 2 and 6 mo. This difference in the time-course of synapse loss may be related to the kinetics of olfactory deficits; it would be interesting in future studies to test if discrimination deficits occur before threshold alterations.

Olfactory discrimination and memory also involves activation of the piriform cortex, but age-related morphological changes have not been thoroughly investigated in this area. Pyramidal cell dendritic arborization regresses but afferent synaptic input from the OB is preserved (44, 45), consistent with our data showing stability of the number of mitral cells. However, changes in the density of associative synapses have not yet been investigated and their loss might contribute to olfactory deficits.

In summary, we show that age-related changes in the OB are restricted to glomerular synaptic circuits, where the density of local D:D and primary afferent A:D synapses are decreased. These changes may disrupt the linear nature of odor information integration in the OB, by creating an imbalance between glomerular and EPL processing. Although earlier studies had speculated on neuronal losses, our findings now strongly suggest that disruptions in OB synaptic circuitry predominate during aging. These results have broad implications for understanding age-related sensory system perturbations throughout the brain, and emphasize the importance of stable synaptic circuits during normal aging.

Methods

Animals. Animal care and use was approved by the Yale Animal Care and Use Committee (#2009–11014). C57Bl6/J females (Jackson Laboratory) and homozygous M72-IRES-tauGFP mice (M72-GFP, male and female) (23) (*SI Methods*) were analyzed at 2, 6, 12, 18, and 24 mo.

Immunostaining. Tissue preparation and immunostaining protocols are detailed in *SI Methods*.

Quantification Procedures. OB sections were immunostained for NeuN/Tbx21/Dapi (*Fig. S5*) or VGluT2 (*Fig. 2A*). Metamorph software (Molecular Devices) was used to measure the laminar areas (*Fig. 1A*), glomerular diameter, and automatically count granule and mitral cells (*Fig. S5*). NeuN⁺ and TH⁺ PG cells were manually counted on confocal images using ImageJ. Details are provided in *SI Methods*.

Whole-Mount Imaging of M72-GFP Glomeruli. Tissue preparation and glomerular mapping are detailed in *SI Methods*. Intrinsic GFP fluorescence was imaged in the dorsolateral M72-GFP glomerulus with a laser scanning confocal microscope (Leica TCS SL, Leica Microsystems).

Electron Microscopy. Tissue preparation followed established procedures as detailed in *SI Methods*. Synapses were identified with the following criteria: (i) a well-defined synaptic cleft, (ii) a postsynaptic density, and (iii) synaptic vesicles in the presynaptic compartment. Criteria used to classify synapses in the glomerulus and EPL are detailed in *SI Methods*.

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