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Review

# Microglial heterogeneity in aging and Alzheimer's disease: Is sex relevant?

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ABSTRACT

Neurodegenerative diseases and their associated cognitive decline are known to be more prevalent during aging. Recent evidence has uncovered the role of microglia, the immunocompetent cells of the brain, in dysfunctions linked to neurodegenerative diseases such as is Alzheimer's disease (AD). Similar to other pathologies, AD is shown to be sex-biased, with females being more at risk compared to males. While the mechanisms driving this prevalence are still unclear, emerging data suggest the sex differences present in microglia throughout life might lead to different responses of these cells in both health and disease. Furthermore, microglial cells have recently been recognized as a deeply heterogeneous population, with multiple subsets and/or phenotypes stemming from diverse parameters such as age, sex or state of health. Therefore, this review discusses microglial heterogeneity during aging in both basal conditions and AD with a focus on existing sex differences in this process.



## Introduction

It is now a well-known fact that the likelihood of developing a neurodegenerative disease increases with aging. While there is still much to learn about the processes leading to cognitive decline and brain dysfunction in aged individuals, recent research consistently shows that glial cells are one of the mechanisms driving neurodegeneration. Specifically, studies on microglia, the resident macrophages of the brain, have proposed the role of these cells in related diseases, one of which being Alzheimer's disease (AD).[1–3] Contributing to global rates of dementia, main pathological hallmarks of AD consist of an excessive extracellular formation of amyloid beta (Aß) plaques and intracellular accumulation of neurofibrillary tangles of hyperphosphorylated tau protein.[4] These aggregates further induce neuroinflammation, which contributes to both disease progression and severity.[5]

Remarkably, AD is more prevalent in aged women, a bias that may stem from various factors such as their natural greater longevity, hormonal state, stress response type or genetic profile.[6] The progress and severity of AD also display sex-specific traits. There is evidence that females, once diagnosed with AD, display a faster rate of hippocampal atrophy (hallmark of AD)[7,8] as well as a greater cognitive decline[9] compared to males. Presence of the apolipoprotein E ε4 allele (APOE-ε4), a major AD risk factor, was further identified as associated with an accelerated brain structural atrophy (e.g. in hippocampus, entorhinal cortex or amygdala[8,10]) and a higher probability of transition from a state of health or mild cognitive impairment into AD, especially in female carriers.[9,11,12] This steeper deterioration seems to affect several cognitive domains such as verbal memory or visuospatial ability of affected females, although the reviewed studies partially diverge in final conclusions due to differences in analytical methods and variability

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of AD phenotypes within the population.[13] The velocity of the decline would, however, corroborate with an earlier reached state of dependency of female AD patients on caretakers in daily life activities.[14] On the other hand, as opposed to men who show higher mortality after AD diagnosis, females also tend to live longer, albeit with a disability.[14] Some pathological events such as altered synaptic plasticity, oxidative stress, metabolic processes, inflammation as well as mitochondrial dysfunction contributing to overall brain hypometabolism were suggested to appear in an individual even prior classical histological or clinical hallmarks of AD. Taking into account the limitations of existing transgenic models of AD, female APP/PS1/tau triple-transgenic mice (3xTg-AD) showed mitochondrial dynamics abnormalities in cortex and hippocampus as early as 2 month of age.[15] Further observations in 12–15 months old female mice of the same model; moreover, suggested a presence of more prominent amyloid load, neurofibrillary tangles and neuroinflammation in the hippocampus, accompanied by spatial memory deficits[16] as compared to their male counterparts. This further points out the strong role of sex in the pathophysiology of the disorder and underlying cellular and molecular processes. While the mechanisms behind this sex-specific prevalence are still mostly unknown, recent studies suggest that the life-long sexually dimorphic character of microglia may also contribute to the increased risk of AD in females.[17–19]

Moreover, microglia constitute a heterogeneous cell population whose diversity (molecular, structural and functional) relies on various parameters like their microenvironment, state of individual's health, brain location, sex, external factors or age.[20] Despite the relevance of sex for microglial responses in healthy aging or AD, very few studies actually take both sexes into account, highlighting the need for a deeper insight. Firstly, this review focuses on microglial heterogeneity during normal aging and discusses known sex differences in this process. The second section describes possible similarities and differences of microglia in AD compared to normal aging conditions.

## Microglial heterogeneity in the healthy aging brain

Progressive accumulation of oxidative stress markers, synaptic loss, dysregulation of energetic metabolism and inflammatory environment contribute to a structural and functional impairment of brain with age, elevating the risk of pathology.[21–24] Considering their participation on numerous processes crucial for proper brain development and functioning, such as regulation of immune response or synaptic remodeling, microglia are indisputably potent contributors to aging-related decline.[25,26] However, in what way aging itself affects their intrinsic properties, then reflected in their functions, is yet to be defined.

While microglia are known to form a diverse population throughout life, there is evidence that this heterogeneity fluctuates and possibly increases during early life as well as in aging and pathology.[27–29] Observed differences may originate from microglia effectively dealing with laborious dynamic early-life developmental processes and aging-related events (degenerative or not) in comparison with a more stable and resilient adult state conditions. This variety is reflected in multiple microglial aspects such as their morphology, ultrastructure, metabolism as well as their proteome, transcriptome and potentially their epigenomic profile.[30–32] While only a mild sex-specific effect was reported for microglial diversity in the context of gene expression,[27] this effect was analyzed only in developmental and adult stages, thus not principally excluding occurrence of more pronounced sex differences in later stages of life.

In the healthy adult brain, microglia of both sexes display long and thinly ramified processes that constantly survey the parenchyma, neuronal cell bodies, synapses, blood vessels, as well as other glial cells,[33,34] and are hence called “surveying” microglia. These processes may temporarily enlarge and retract whenever the brain undergoes trauma, injury or an immune challenge, but also revert back to thinly ramified cells once the inflammation subsides.[33,34] By contrast, while surveying microglia are still present in the aged brain, aging microglia take on a reactive or dystrophic morphology with stouter, fewer and less branched processes[35–40] followed by a greater variability in soma size between animals[41] or suggestive of soma enlargement,[37,39] depending on studies. Despite morphology not being an absolute indicator of their functional capability, presence of these phenotypes strongly suggest an interference with microglia-mediated brain homeostasis maintenance and a possible loss of their neuroprotective character with aging. While this aging-related microglial morphology has been mostly assessed in humans and rodents, it is also encountered in other species, including gerbils and dogs.[42] However, effect of species should be strongly considered as microglia of mouse and human were reported to age along distinct trajectories[43] and to showcase more heterogeneity in the case of humans.[44]

In clinical conditions, microglia of a healthy 68 year old male subject (cause of death: neck fracture from motor vehicle accident) displayed a dystrophic morphology as presented above, but further demonstrated abnormalities such as gnarled, apparently fragmented processes and bulbous swellings as compared to microglia of a younger male subject (38 years old, cause of death: acute cardiac dysrhythmia).[45] Another *post-mortem* human study described a rod-shaped microglial subtype[46] whose occurrence increased with aging in the hippocampus and frontal cortex of cognitively intact subjects and in the parietal cortex of demented subjects.[46] Experimentally, this subtype can be induced by disruption of axonal transport due to trauma but its putative origin and functions remain largely unknown.[46] However, it was speculated that rod-shaped microglia might provide neuroprotective effects, trophic support and promote neuronal survival.[46]

At the ultrastructural level, exhibiting signs of cellular stress along with a condensed cytoplasm and nucleoplasm, a newly-defined microglial phenotype named ‘dark microglia’ has been uncovered.[47] Dark microglia seem to be a characteristic of aging, chronically stressed or diseased brain and up to now have been found in several brain regions including the hippocampus and cerebral cortex.[47] This microglial subset seems to also be abundant during normal postnatal development,[48] notably in the hippocampus, where its extensive interactions with synapses suggest an elevated involvement with the remodeling of neuronal circuits and brain parenchyma, among other key functions.[49] These cells have been described in both rodents and humans.[50,51] Sex-specific pattern of their appearance, while not yet deeply explored, especially in context of healthy aging, was proposed in a rodent model of prenatal infection.[52]

Structurally, aged microglia accumulate increasing amounts of lipofuscin granules (i.e. lysosomal lipo-pigments and proteins),[53] lipid droplets[54] as well as cytoplasmic and phagocytic inclusion bodies,[40,55] some of which display features of synaptic elements.[41,56] A progressive accumulation of myelin fragments in the cerebellar white matter of healthy aging mice (both males and females) was suggested to induce an increasing lysosomal overload of microglia with age.[57] It would imply that following reduced capacity for phagocytosis in later stages, failure of internal degradations processes, or both, could have contributed to the occuring cell senescence.[57] However, this myelin-related senescence promoting effect did not take a pronounced place in microglia of grey matter,[57] reflecting the complex impact of local cues and similar but diverse aging pathways within microglial population.

In line with this, a recent study of Burns et al. (2020)[58] showed that aged microglia are characterized by an overrepresentation of proteins involved in various pathways such as autophagy, lipid catabolic processes, mitochondrial dysfunction and most strikingly in endolysosomal biology (phagosome maturation, lysosomal degradation, endosome fusion with lysosomes). This age-related pattern seemed to be qualitatively true for both sexes and also showed a high degree of conservation across species.[58] In humans (healthy and demented patients with 80% of females, mean age of death: 95 years old), aged microglia also showed an enrichment at the proteomic and transcriptomic level for phagocytic pathways.[59] Overall changes in both the proteome and inclusion accumulation over time suggest a possibility of an impaired processing of phagocytosed material and a failure of energetic metabolism (Fig. 1), which may contribute to the microglial hypertrophy described in a subset of microglia with aging.[55,58]

Microglia in a healthy adult brain are homogeneously spread throughout the central nervous system (CNS)/brain parenchyma and their highly motile processes constantly survey the microenvironment.[33,34] Due to disfigured and/or simplified configuration of arborization in aged microglia, reduction in area of surveillance has been observed on several occasions (Table 1.). This insufficient coverage of parenchyma could indirectly promote pathological events, for instance, via reduced phagocytosis of extracellular debris or decreased interaction with pathology-related cues. Although a classical reactive microglial phenotype is usually correlated with an increased cell migration and process motility in response to a stimuli,[60] this is not the case in the aged brain whose microglia frequently display decreased process speed and cell migration both under normal conditions and after tissue injury.[36,37]

Moreover, Damani and colleagues (2011)[36] further showed that, compared to adults, aged retinal microglia spend more time aggregated at the site of injury, which could possibly be true also for microglia in aged CNS. These findings are further paralleled by the fact that microglia in the aging brain seem to be irregularly distributed and were found to accumulate in specific compartments such as the neocortex, visual and auditory cortices.[37,41] This is often accompanied by formation of clusters,[37,61] mostly identified in aged rodents. As microglia clustering was reported in the proximity of the amyloid plaques in mice with AD,[62] their appearance in a normally aging brain may be connected to the aging-related deposition of low amounts of amyloid, which without presence of other deleterious risk factors, do not necessarily have to impact cognition.[63,64]

Microglial accumulation might also hint at an increase in proliferation. Indeed, microglia are capable of self-renewal with very little, if any, contribution from the circulating macrophages.[65–68] While microglial population remains stable during adulthood, in the aging rodent brain (both mice and rats), it was consistently shown that microglial numbers can rise in the retina[36] or various brain regions such as the cortex,[69] the hippocampus,[70] the visual and auditory cortices,[41] the corpus callosum,[71] the substantia nigra or ventral tegmental area.[39]

While this augmentation in microglial density might be solely due to cell proliferation, the blood–brain barrier could also become more permeable over time in both health and disease and allow peripheral macrophage precursors to enter the brain and backup the initial microglial population.[25,72,73] However, it is yet unclear whether such an infiltration in the nervous parenchyma could account for this rise in the number of microglia. In addition, the functional significance of this microglial density increase is not completely understood and could be a compensatory mechanism counteracting a) the inability of aging microglia to revert to basal levels after the inflammation is resolved, b) their declining ability to perform physiological and immune functions, c) increased apoptosis of metabolically overloaded cells, or all of these.[17,58] The transient periods of balanced apoptosis and proliferation could explain why some studies observe no apparent changes in microglial density across ages,[66,74] although this could also be a regional effect.

Comparably to an inflamed adult brain and in agreement with their increased density and morphological features, aged microglia are characterized by heightened inflammatory state under both healthy and diseased conditions. For instance, aging microglia up-

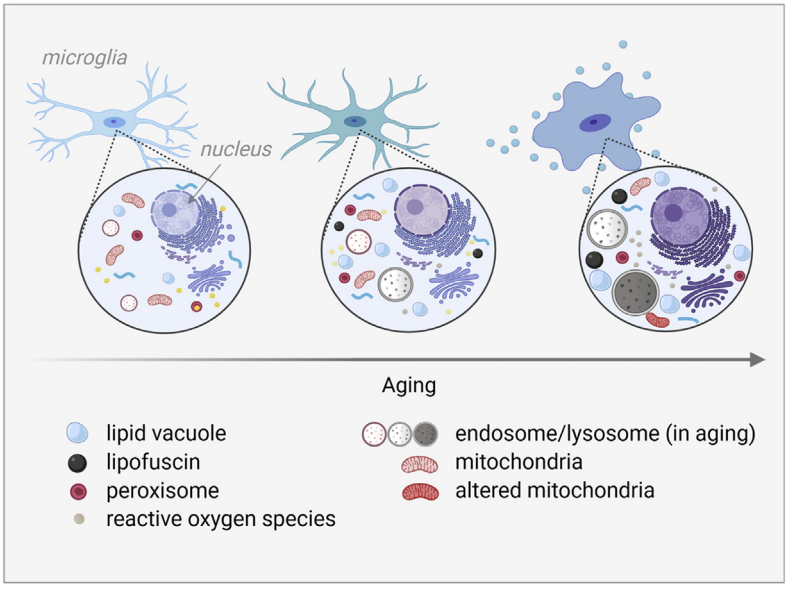


**Fig. 1. Intracellular changes in microglia during aging.** Intracellular composition of microglia is altered with aging, with a possible subset-specific course and features. There is an age-related hypertrophy of lysosomes, endosomes and peroxisomes, accompanied by a progressive accumulation of lipofuscin, lipid droplets and other undegraded debris in the cell. Elevation of oxidative cellular stress and dysfunctional energetic metabolism is, for instance, marked by an increase in Reactive Oxygen Species (**ROS**) and impairment of mitochondrial functions.

**Table 1**
Healthy aging-associated microglial alterations.

| Author(s) | Model | Age | Target area | Methods | Results (In aged animals) |
|---|---|---|---|---|---|
| Streit et al., 2004 | Human ♂ (non-demented) *post-mortem* | 38 and 68 years | Cerebral cortex (grey matter) | - IHC (HLA-DR antigen)<br>- Light microscopy | Dystrophic microglial phenotype<br>- widespread distribution; frequent co-localization with ramified microglia<br>- de-ramified; short, tangled and gnarly processes<br>- cytoplasmic fragmentation (various states), nucleus condensation<br>- cytoplasmic beads and/or spheroids |
| Sierra et al., 2007 | Mouse *c-fms-eGFP* | 2 and 18 months | Whole brain | - IHC<br>- FACS *($eGFP^{+}CD45^{lo}$)*<br>- RT-PCR | - ↑ accumulation of lipofuscin in microglia (and surrounding neuropil)<br>- ↓ complexity of secondary branching, ↑ cellular granularity<br>- ↑ proinflammatory cytokine (*Tnf-α, Il-1ß, Il-6*) and anti-inflammatory cytokine (*Tgf-ß*) expression<br>*in vivo* LPS → robust inflammatory response (*'primed'* phenotype) |
| Frank et al., 2010 | Rat *F344 x BN F1* | 3 and 24 months | Hippocampus | - microglial isolation (Percoll density gradient)<br>- microglia concentration (trypan blue exclusion)<br>- RT-PCR | - no significant difference in microglia number<br>- ↑ expression of microglial '*activation*' markers (*Cd11b, Iba1, MHC-II*)<br>*ex vivo* LPS → exaggerated cytokine response (*Il-1b, Il-6*) |
| Damani et al., 2011 | Mouse *CX3CR1-GFP$^{-/+}$* | 2–3 and 18–24 months | Retina | - IHC<br>- *Ex vivo* time-lapse confocal imaging<br>- RT-PCR<br>- ELISA | - ↑ regional proliferation of microglia, change in distribution pattern<br>- ↓ branching, ↓ surveying area coverage, ↓ process length<br>- ↓ regional motility of microglia<br>*ex vivo* ATP→ microglia process shortening and elimination<br>focal injury→ delayed, but prolonged microglial response |
| Hickman et al., 2013 | Mouse *C57BL/6* | 5 and 24 months | Whole brain (cortex, hippocampus, cerebellum) | - FACS *($CD11b^{+}CD45^{+}$)*<br>- Direct RNA sequencing<br>- RNAScope<br>- Proteomics | - 3503 DEGs between young and aged microglia<br>- shift to neuroprotective priming state in older animals<br>- 13% and 31% of microglial sensome genes were up− and down−regulated, respectively |
| Tremblay et al., 2012 | Mouse *CBA/CaJ C57BL/6J* | <19 and 19–24 months | Primary visual (V1) and auditory (A1) cortex | - IHC and light microscopy<br>- TEM | - C57 and CBA strains undergo progressive auditory and visual function loss, respectively<br>- aged C57 mouse−↑ IBA1$^{+}$microglia, loss of distribution pattern,<br>↓ branching, elongated processes, variable soma size in II/III layers<br>- aged CBA mouse − loss of distribution pattern, trend to ↓ branching, elongated processes<br>- ultrastructural analysis − soma enlargement and rounding, ↑ number of phagocytic inclusions (resemblance to synaptic elements) |
| Hefendehl et al., 2014 | Mouse *IBA1-eGFP$^{+/-}$* | 3, 11–12 and 27–28 months | Neocortex | - *In vivo 2*P imaging<br>- IHC and confocal microscopy | - ↑soma volume and movement (23% cells moved by > 10μm/2 weeks)<br>- ↑ IBA1$^{+}$ microglia (~14%)<br>- ↓ process length (without loss of branching complexity)<br>- ↓ area coverage and slowed process movements<br>- loss of distribution pattern (cell clustering)<br>blood vessel laser damage→ delayed migration and morphological response |
| Bisht et al., 2016 | Mouse *C57BL6/J* | 3 and 14 months | Hippocampus, cerebral cortex, amygdala, hypothalamus | - IHC and light microscopy<br>- TEM | Dark microglia (ultrastructural phenotype)<br>- ↑ density<br>- cytoplasm and nucleoplasm condensation ('dark' appearance in EM)<br>- dilation of endoplasmic reticule and Golgi apparatus, altered mitochondria<br>- cell shrinkage, thin and long processes with acute angles<br>- highly phagocytically active<br>- ↑ interaction with neurons, synaptic components, glia cells, blood vessels<br>- ↓ IBA1$^{+}$ and GFP$^{+}$ (in *CX3CR1-GFP$^{/+}$ model*) signal<br>- basal expression of 4D4$^{+}$ and CD11b$^{+}$; TREM2$^{+}$ (in pathology)<br>- present in non-homeostatic conditions − Alzheimer's disease (*APP-PS1 model*), chronic stress, CX3CR1-KO model |
| Davies et al., 2016 | Human *post-mortem* | 55 ± 4 and 82 ± 10 years | Inferior temporal cortex (grey matter) | - IHC and wide-field imaging (slide scanner) | - no difference in density of IBA1$^{+}$microglia<br>- ↓ process length and overall branching complexity<br>- ↓ surveying area coverage |

| | | | | | |
|---|---|---|---|---|---|
| Grabert et al., 2016 | Mouse ♂ *C57BL/6J Csfr-EGFP* | 4, 12 and 22 months | Striatum, hippocampus, cerebral cortex, cerebellum | - FACS *(CD11b$^{+}$F4/80$^{+}$CD45$^{lo}$)*<br>- Gene expression microarray; qPCR<br>- IHC and confocal microscopy (*in vitro*) | - regional and individual sensitivity of gene modules to aging<br>- ↓ microglia signature genes (e.g. *Tmem 119, P2ry12, P2ry13, Fcrls*)<br>- ↑ gene expression in IFN pathway, transcription regulation, immunoreceptors<br>- ↓ gene expression in migration, motility, endocytosis, immunoreceptors, cell adhesion, cytoskeleton, ligand presentation |
| Askew et al., 2017 | Mouse *C57BL/6 mouse Transgenic mouse models** | 4–6 and 18–24 months | Numerous brain regions | - IHC, light and confocal microscopy<br>- *In vivo* 2P laser-scanning microscopy<br>- Behavioural assay<br>- RT-PCR<br>- FACS*(CD11b$^{+}$CD45$^{+}$)*<br>- RNA seq | - relatively stable number of IBA1$^{+}$ cells (except thalamus – ↑IBA1$^{+}$)<br>- greater microglial density in grey compared to white matter<br>- multinucleated microglial aggregates with ↑ MHC-II$^{+}$ and CD45$^{+}$ expression (possible peripheral origin)<br>- ↑ rate of microglial proliferation/apoptosis (spatio-temporal coupling)<br>- dysfunction in *Csfr1*-signallingpathway |
| | Human *post-mortem* | 20–35 and 58–79 years | Temporal cortex | | - stable number of IBA1$^{+}$ cells<br>- greater microglial density in white compared to grey matter |
| Bachstetter et al., 2017 | Human ♂, ♀ *post-mortem* | 20–96 and 77–100+ years | Hippocampus, frontal cortex | - IHC and slide-scanner imaging | Rod-shaped microglia<br>- narrow cell body, small amount of planar processes<br>- ↑ density IBA1$^{+}$ rod-shaped microglia in hippocampus with age |
| Mangold et al., 2017 | Mouse ♂, ♀ *C57BL/6* | 3, 12 and 24 months | Hippocampus, cortex | - Microarray; qPCR<br>- Immunoblotting<br>- IHC, confocal microscope, inverted research microscope | - sex-specific differences in age-related gene expression<br>- ↑ variety of inter-individual gene expression in aged males<br>- both sexes – highly enriched for inflammatory pathways (greater impact in females)<br>- ↑ expression of microglia-specific genes (complement pathway components, sensome) |
| Raj et al., 2017 | Mouse ♂ *C57BL/6, DBA/2J* | 2–4, 13, 24 and 27 months | Forebrain, cerebellum (white and grey matter) | - Microglia isolation (Percoll density gradient)<br>- qPCR, Microarray<br>- FACS *(CD11b$^{hi}$CD45$^{int}$)*<br>- Phagocytosis assay *in vitro*<br>- Spinning disc confocal microscopy<br>- IHC, slide-scanner imaging<br>- PET imaging | - ↑ expression of genes involved in inflammatory response, phagocytosis, and lipid metabolism<br>- partial correlation between transcriptome and proteome<br>- ↑ autofluorescence (possibly ↑ lipofuscin)<br>- ↑ rates of phagocytosis<br>- ↑ IBA1$^{+}$ signal, microglial clustering and presence of cytoplasmic beads<br>- higher impact of aging on white matter |
| | Human *post-mortem* | <60 and >60 years | | | - ↑ CD68$^{+}$ and HLA-DR$^{+}$ signal in white matter regions<br>- ↑ binding of [11C]-PK11195 (TSPO) in white matter |
| Chan et al., 2018 | Rat *Sprague –Dawley* | 3 and 20 months | Prelimbic area of medial prefrontal cortex | - IHC light and confocal microscopy | - ↑ volume and soma size of OX-42$^{+}$microglia<br>- ↑ volume of non-nuclear microglial glucocorticoid receptor (GR) staining<br>- negative correlation for microglia volume and density of thin spines<br>- negative correlation for non-nuclear microglial GR and total spine density |
| O'Neil et al., 2018 | Mouse ♂ *BALB/c* | 1.5–2 and 16–18 months | Whole brain (hippocampus and cortex for individual experiments) | - FACS *(CD11b$^{+}$CD45$^{lo}$)*<br>- NanoStringnCounter<br>- IHC, epi- and confocal microscopy<br>- RNA seq<br>- qPCR<br>- Behavioural assay | - ↑size of lysosomes and lipofuscin volume in cortex<br>- 511 DEGs (455 up– and 56 down–regulated genes) – major enrichment in inflammatory pathways<br>- brain niche as a contributor to a *primed* microglial phenotype<br>*in vivo* LPS→ prolonged ↓ in social behaviour, exaggerated immune response in the hippocampus |
| Olah et al., 2018 | Human *post-mortem* | 53 ± 5 and 94 ± 1 years | Dorsolateral prefrontal cortex (grey matter) | - FACS *(CD11b$^{+}$CD45$^{+}$7AAD$^{-}$)*<br>- RNA seq<br>- LC-MS<br>- HuMi_Aged gene set | - Transcriptome enriched for DNA repair, telomere maintenance, phagocytosis<br>- ↑ gene expression related to accumulation of amyloid<br>- ↓ gene expression related to *Tgfß*-signalling<br>- considerable correlation of transcriptomic and proteomic data |
| Zoller et al., 2018 | Mouse *C57BL/6JRj* | 6 and 24 months | Frontal cortex | - IHC, light and confocal microscopy<br>- FACS *(CD11b$^{+}$CD45$^{lo}$)*<br>- Microarray | - ↓ IBA1$^{+}$ microglia density<br>- ↓ branching and altered distribution pattern (cell clustering)<br>- ↑ gene expression in active immune response and lipid metabolism pathways<br>- ↑ microglial activation pattern with age (F4/80$^{+}$, CD206$^{+}$, CD36$^{+}$, CD86$^{+}$) |
| Hammond et al., 2019 | Mouse ♂, ♀ *C57BL/6J* | E14.5, P4 – 5, P30, | Whole brain | - FACS *(CD11b$^{hi}$CD45$^{lo}$CX3CR1$^{hi}$)*<br>- Single-cell RNA seq<br>- RNA Scope | - transient or persistent microglia states with a distinct transcriptional program across life<br>- identification of 9 unique states (clusters) |

(*continued on next page*)

**Table 1** (*continued*)

| Author(s) | Model | Age | Target area | Methods | Results (In aged animals) |
|---|---|---|---|---|---|
| | | 3–4 and 18–19 months | | - IHC, spinning disk confocal microscopy<br>- smFISH | - aging caused expansion in existing clusters – most remarkably in cluster 2 (inflammatory signalling) and 3 (IFN pathway)<br>- chemokine Ccl4+ population (2nd cluster) was markedly enlarged and displayed a brain-wide distribution |
| Tejera et al., 2019 | Mouse *CX3CR1-eGFP*$^{+/+}$ *Transgenic mouse model** | 5 and 15 months | Cortex, hippocampus | - *In vivo* 2P laser scanning microscopy<br>- IHC, epifluorescence microscopy<br>- ELISA | - ↓ reduced number, length, and complexity of microglial processes *in vivo* LPS→<br>- 24–48h *post-LPS* – ↑ microglial activity<br>- 10 d *post-LPS* – baseline aged phenotype<br>- ↑ proliferation rates 24 h *post-LPS* |
| Burns et al., 2020 | Mouse *C57BL/6J Transgenic mouse models** *Non-human primates** | < P15 – 24 months | numerous brain regions | - Flow cytometry<br>- FACS*(CD45*$^{dim}$*CD11b*$^{+}$*)*<br>- Imaging cytometry<br>- TEM<br>- Nano liquid chromatography mass spectrometry<br>- qRT-PCR | Autofluorescence positive (AF+) and negative (AF-) microglial subtype<br>- stable bipolar distribution of AF+ and AF- in first 12 months of life<br>- AF source – intracellular organelles (lysosomes)<br>- ↑ AF intensity in AF + population (linearly with age)<br>- ↑ complexity of AF + lysosomes (prominent lipid droplets)<br>- ↑ size of lysosomes in AF + population (9%–23% increase during 3rd to18$^{th}$ month of life)<br>- ↓ total microglia (~31%) at 24 months (dominant in AF + cells with higher intensity)<br>- AF+ (higher apoptotic rates) vs. AF- (higher proliferation rates)<br>- expression of microglia homeostatic markers (*Cx3cr1, P2ry12, Tmem 119*) in both subsets<br>- ↑ CD68$^{+}$, LAMP1$^{+}$ and baseline ROS levels in AF + subset with age<br>- subtype-specific protein expression differences in metabolic, autophagic, lysosomal and mTOR pathways |
| Marschallinger et al., 2020 | Mouse ♂ *C57BL/6J Transgenic mouse model** | 2–4 and 18–20 months | Hippocampus | - TEM<br>- IHC, confocal microscopy<br>- Anti-Stokes Raman scattering (CARS) laser-scanning microscopy<br>- Lipidomics<br>- FACS *(CD11b*$^{+}$*CD45*$^{low}$*)*<br>- RNA seq<br>- CRISP-Cas9 screen | Lipid-droplet-accumulating microglia (LDAM)<br>- ↑ frequency and volume of lipid droplets, ↑ROS production<br>- ↑ proinflammatory cytokine production (at baseline; after LPS challenge)<br>- impaired phagocytosis<br>- genetic modulators of lipid droplet formation - *Slc33a1, Snx17, Vps35, Cln3, Npc2, Grn* |
| | Human *post-mortem* | <35 and >60 years | | | |
| Shaerzadeh et al., 2020 | Mouse ♂ *C57BL/J6* | 1, 6–9 and 18–24 months | Substantia nigra pars compacta, ventral tegmental area | - IHC, confocal microscopy<br>- RNA scope multiplex fluorescent assay<br>- 3D binary masks reconstruction image analysis | - ↑ IBA1$^{+}$ microglia (temporary regional decline at 9 months)<br>- ↓ complexity of microglial branching and process length<br>- ↓ projection area, enlarged soma<br>- ↑ interaction with dopaminergic neurons |

**2P imaging** – two-photon imaging; **4D4** – phagocytic microglia marker; **AF** – autofluorescence; **ATP** – adenosine triphosphate; **APP-PS1** – amyloid precursor protein-presenilin; **CCL4** – chemokine (C–C motif) ligand 4; **CD11b** – integrin αM subunit; **CD36** - cluster of differentiation 36; **CD45** – cluster of differentiation 45; **CD68** – cluster of differentiation 68; **CD86** – cluster of differentiation 86; **CD206** – cluster of differentiation 206; **CLN3** – CLN3 lysosomal/endosomal transmembrane protein; **CSFR1** – colony stimulating factor 1 receptor; **CX3CR1**– fractalkine receptor; **CX3CR1-KO** – CX3CR1 knock-out; **DEGs** – differentially expressed genes; **ELISA** – enzyme-linked immunosorbent assay; **EM** – electron microscopy; **F4/80** – monocyte-macrophage marker; **FACS** – fluorescence-activated cell sorting; **Fcrls** – Fc receptor-like protein 2; **(e) GFP** – (enhanced) green fluorescent protein; **GR** – glucocorticoid receptor; **GRN** – granulin precursor; **HLA-DR** – histocompatibility leukocyte antigen-DR; **IBA1** – ionized calcium-binding adapter molecule 1; **IFN** – interferon; **IHC** – immunohistochemistry; **Il-1ß** – interleukin-1β; **Il-6** – interleukin 6; **LAMP1** – lysosomal-associated membrane protein 1; **LC-MS** – liquid chromatography-mass spectrometry; **LPS** – lipopolysaccharide; **mTOR** – mammalian target of rapamycin; **MHC-II** – major histocompatibility complex II; **NPC2** – NPC intracellular cholesterol transporter 2; **OX-42** - integrin αM antibody; **P2RY12/13** – purinergic receptor P2Y12/P2Y13; **PET** – positron emission tomography; **qPCR** – quantitative PCR; **RNA seq** – RNA sequencing; **ROS** – reactive oxygen species; **RT-PCR** – real-time polymerase chain reaction; **SLC33a1**–acetyl-coenzyme A transporter 1; **smFISH** - single-molecule fluorescence in situ hybridization; **Snx17** – sorting nexin 17; **TEM** – transmission electron microscopy; **TGF-ß** – transforming growth factor β; **TMEM119** – transmembrane protein 119; **TNF-α** – tumor necrosis factor α; **TREM2** - triggering receptor expressed on myeloid cells 2; **TSPO** – translocator protein; **VPS35** – vacuolar protein sorting-associated protein 35.

regulate the expression of pattern recognition receptors (Toll-like receptors, TLRs; nucleotide-binding oligomerization domain-like (NOD)-like receptors, NLRs), antigen presenting receptors (Major Histocompatibility complex II, MHC II), phagocytosis-related receptors (cluster of differentiation (CD) 11b (CD11b/CR3), CD68)[17,25,74,75] and down-regulate mRNA of homeostasis–promoting markers such as CD200R,[76] which keep microglia in a surveying state.[77] As for cytokines, both inflammatory (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6) and, to a lesser extent, anti-inflammatory (IL-10, transforming growth factor (TGF)-β1) products, seem to be synthesized and released in higher quantities by aged microglia.[25,40,42,78,79] Moreover, the peripheral immune system itself shows heightened inflammation in aged individuals[42] and the constant inflammatory state of both the brain and periphery has been shown to have combined deleterious effects on adult neurogenesis and long-term potentiation,[80–82] impair stress resilience[83,84] and lead to cognitive deficits.[81,85–87]

From a genetic and proteomic perspective, genes and proteins that were dysregulated in the aging brain were found to be connected to immune pathways. Highly enriched in microglia were particularly elements of the complement pathway[88] which label cells for microglia-mediated elimination,[89,90] and of the microglial sensome involved in the sensing of endogenous ligands and pathogens.[91,92] In addition, two subpopulations of reactive microglia in the aging brain have been further characterized by a specific genetic signature, namely the chemokine ligand 4 (CCL4) gene, as well as the upregulation of inflammatory signals (IL-1β, CCL3), interferon-response genes (Ifitm3, Rtp4) and transcription factors (Id2, ATF3).[27] With single cell RNA sequencing, Hammond and colleagues (2019) showed these clusters to be already present in both juveniles and adults but in lower quantities, suggesting that aging potentiates the expansion of existing inflammatory subpopulations, as seems to be the case with dark microglia.[48] Furthermore, these clusters only account for a fraction of the entire microglial population, even in the aged brain, which may indicate that changes in microglia are driven rather by local cues, than occurring in a brain-wide shift.

Aging microglia in the healthy brain thus overall become more reactive as well as showcase enhanced diversity in respect to individual aspects (morphology, ultrastructure or transcriptome) with some existing subsets becoming even more prevalent over time.[27,47] Whether the morphological dystrophic phenotype is a subtype of what we consider reactive microglia, or an individual type of a dysfunctional senescent pre-apoptotic microglia,[45] remains to be defined. A summary of the modifications undertaken by microglia in the process of normal aging can be found in Table 1.

With biological drivers of this cellular senescence to be yet properly characterized, the importance of the cellular bioenergetics and metabolism for sustenance of macrophage/microglial diverse activities is being highlighted.[93,94] Specifically, long-term higher basal workload of these cells due to aging-related accumulation of senescent cells may render them more vulnerable to a programming dysregulation in anabolic and catabolic processes. Prostaglandin $E_2$ (EP2), a lipid messenger whose levels increase with aging in human monocyte-derived macrophages (MDMs) and macrophages of aged mice, was identified to decrease glycolysis and functionality of mitochondrial respiration rates and electron transport chain, via EP2 receptor-related mechanisms.[94] Related polarization of these cells toward an inflammatory state was proposed to be mediated via promotion of glycogen synthesis at the expense of glycolysis. On the other hand, genetic knock-down of EP2 reversed the pro-inflammatory polarization and normalized phagocytic abilities of macrophages of aged animals to levels of young animals.[94] Moreover, pharmacological inhibition of EP2 with impact on brain, reduced hippocampal inflammation, levels of phago-lysosomal marker CD68, increased utilization of glucose for glycolysis and tricarboxylic acid chain by IBA1+ cells and optimized morphological features of mitochondria inflammatory factors both in plasma and hippocampus of aged mouse.[94] These contributed to an enhancement of the performance in both memory and cognitive flexibility-based tasks.[94] On the other hand, while both macrophages and microglia were found to be able to utilize also alternative sources of energy such as glutamine, pyruvate or lactate under specific conditions,[94,95] dependency of macrophages on glucose was suggested to increase with aging.[94]

These results depict how the energetic metabolism and mitochondrial respiration play crucial roles in phenotypic changes of both macrophages and microglia, thus critically affecting their functionality along their biological timeline. It should not come as a surprise that sex differences may be also present also in functions of classical macrophages, which in general underlines the overall diverse immune response and susceptibility to diseases of males and females (for review see[96–98]). For instance, a recent study in Albino Oxford rats, showed distinctive patterns of challenge response of thioglycolate-elicited peritoneal macrophages between sexes along the aging timeline.[99] While macrophages of younger females respond to a challenge (lipopolysaccharide (LPS)) by releasing pro-inflammatory cytokines (e.g. IL-1b and IL-6) in a greater manner than in males, this response becomes diminished with aging, possibly due to lower circulating estradiol levels. Capacity of the male rat macrophages, on the other hand, to respond to the challenge with secretion of TNF-α and IL-1b becomes increased with aging, albeit still not reaching the levels of aged-matched females.[99]

In microglia, while both sexes seem to share common traits (e.g. enhanced neuroinflammation, impaired microglial phagocytosis and reactivity or increase in subtype variety),[27,42,46] there is accumulating evidence that microglia and the overall brain do not quite undergo aging process the same way for males and females. For instance, aging females have slightly elevated density of rod-shaped microglia in humans, although this difference is not statistically significant.[46] Moreover, microglia were shown to accumulate more in specific regions of the aged female brain in both mice and rats (dentate gyrus and hippocampal CA1 region[100]; bed nucleus of the stria terminalis[101]). This difference, however, may not be entirely late age-specific, as it was regionally observed already in adulthood.[102]

Compared to males, female mice also seem characterized by an enhanced expression of inflammation-related transcripts in the hippocampus, more specifically elements of the microglia-related complement pathway (*C1q*).[92] This study further confirmed that the sex difference in C1q is maintained even at the protein level. Similarly, the analysis of the gene expression profiles of the hippocampus and other brain regions (entorhinal cortex, superior frontal gyrus, postcentral gyrus) of aged humans also showed a greater immune activation in females.[103]

By comparison, the hippocampus of aged males was observed to display more gene changes (energy production, ribosome-related processes, RNA processing) and a higher inter-subject gene expression variability in humans and mice, respectively.[92,103] However, it is worth noting that a potential challenge of transcriptomic studies are changes in the transcriptome due to the temporal dynamics of microglial states. Thus, this limitation highlights the necessity of capturing the transcriptome at the right place (throughout the brain or in specific regions) and time.[104] Indeed, there could be several context- and region-dependent microglial subtypes at play with different properties, specialized functions as well as unique transcriptomic signature,[48] but it is yet unknown whether some or all of these different subtypes would show differential distribution between males and females.

## Comparison of microglial heterogeneity between AD and normal aging

In human studies, aged patients suffering from AD display microglia with a reactive or dystrophic morphology, similar to what has been described in healthy age-matched controls, albeit in a more striking manner. For instance, microglial cells in the neocortex of AD subjects (52–98 years old) showed a larger reduction of process length and arborized area compared to controls, as well as an increase in discontinuous or punctuated processes.[105] Similarly, the hippocampus of patients with early or progressive stages of AD (both sexes; 71.9 ± 6.8 and 80.0 ± 7.7, respectively) was analyzed and microglial processes also appeared fragmented due to inhomogeneous IBA-1 staining.[106] In two transgenic adult AD mouse models ($APP_{Sw,Ind}$ model: Swedish (K670N/M671L) and Indiana (V717F) mutation of human Aβ precursor protein; *APP/PS1* model: co-expression of Swedish mutation of human amyloid precursor ($APP695_{SWE}$) and a mutant exon 9 deleted variant of human presenilin 1 (PSEN1/dE9)), neocortical microglia also showed twisted and curved processes along with a reduction in branching and process length in $APP_{Sw,Ind}$ mice.[107] A reduction of the area covered by microglial ramification was also observed in both models.[107] In addition, both rod-shaped and dark microglia appear with increased frequency during AD pathology.[46,47]

Studies in mice and humans further showed that, compared to normal aging, microglia could showcase a higher density in the hippocampus of patients suffering from both AD and hippocampal sclerosis of aging[108] as well as a threefold increase in proliferation in the neocortex of an adult mouse model of AD beta-amyloidosis.[109] This enhanced proliferation in the neocortex, however, was not confirmed in another human study.[105] Furthermore, microglial cells in mice models of AD exhibit impaired phagocytosis, both under control and inflammatory conditions,[110–112] with a similar observation made in APP/PS1 mice.[113] Indeed, Hickman and colleagues (2008) showed that microglia in older APP/PS1 mice (14 months old) had a decrease in the expression of scavenger receptor proteins such as *CD36*, scavenger receptor A (*SRA*) and the receptor for advanced-glycosylation endproducts (*RAGE*). This was accompanied by a matching decrease in Aβ-degrading enzymes (e.g. insulysin, neprilysin) and a 2.5-fold increase in proinflammatory cytokines (IL-1β and TNF-α).[113] These data suggest that neuroinflammation in response to amyloid deposition might prevent Aβ clearance in the long run by diminishing microglial uptake.

Over the recent years, numerous microglial subtypes have been uncovered. While some of them are found in both health and disease like dark microglia, others seem characteristic of a diseased brain only. Following RNA sequencing of bulk isolated microglia, a subtype specifically associated with AD –disease-associated microglia (DAM)– was observed near amyloid plaques in the cortex of rodents and humans.[62] Its highly phagocytic character was showcased by a unique signature of genes associated with lipid metabolism and phagocytosis (Apolipoprotein E, *APOE*; lipoprotein lipase, *Lpl*; Cystatin F, *Cst7*) that distinctly isolated them from control microglia.[62]

In comparison with conventional/surveying microglial cells, DAM also upregulate numerous genes known to increase AD risk (*APOE; Lpl*; Triggering receptor expressed on myeloid cells 2, *TREM2*; protein tyrosine kinase-binding protein, *Tyrobp*; Cathepsin D, *Ctsd*) and downregulate homeostatic microglial genes (*P2RY12, Cx3cr1, Tmem 119*). Curiously, this downregulation of homeostatic markers has also been reported for cerebellar microglia in aged mice (24 months old).[114] Overall, this suggests that DAM might be a conserved and initially compensatory protective subtype in both humans and mice that would participate in the clearance of Aβ plaques in AD.[62] Similarly, a microglial population expressing CD11c (~23% of the total IBA-1+ proliferating microglial population based on immunostaining and FACS analysis) was described in the cerebral cortex of a mouse AD model.[115] Just like DAM, this population interacted with amyloid plaques and its transcriptome was further enriched in APOE and genes related to immune signaling (*Il6, Igf1, Spp1*), lysosome activation (*CD63, CD68*) and metabolic processes (*APOE, Lpl*).[115] To some extent, these pathways were also similar to what has been described above during normal aging.

Additionally, Krasemann and colleagues (2017) uncovered microglial subtype associated with neurodegeneration (MGnD) whose transcriptome was enriched with both inflammatory genes (*Csf1, AXL, CCl2, Itgax*), as well as *APOE*, but negatively correlated with microglial homeostatic genes (*Mef2a, Sall1, Tgfbr1*).[116] This subtype was also seen interacting with cortical Aβ plaques in a mouse model of AD and seemed to be induced by phagocytosis of apoptotic neurons via a TREM2-APOE pathway,[116] while DAM would depend on both TREM2-dependent and independent pathways.[62] Following investigation of the microglial proteome of mice, a microglial subtype associated with disease was further found in both aging and AD. Similar to MGnD, it was characterized by a decrease in the expression of homeostatic markers (*Cx3cr1, MerTK, Siglec-H*) and further exhibited increased expression of phagocytosis-associated receptors (*CD14, CD11c*), activation markers (*CD86, CD44*) and the programmed death ligand 1 (*PDL1*).[117]

Even more recently, a subtype called Lipid-Droplet-Accumulating microglia (LDAM) was discovered in the hippocampus of aged mice and humans.[54] LDAM are characterized by impaired phagocytosis, production of high levels of reactive oxygen species (ROS) as well as inflammatory cytokines, and also possess a transcriptome signature associated with cellular dysfunction.[54] LDAM thus seem to be driven by genes involved in phagosome maturation such as a negative regulator of microglial phagocytosis (*Cd22*), lysosomal genes (*Cd63, TUBA1*), genes related to vesicular transport (*Rab5b*, *Rab 7*), but also those linked to nitric oxide and ROS production (*Cat, Jak*) as well as lipid-related genes (*Plin3*, *Acly*).[54] Its distinct transcriptomic signature is comparable to previously mentioned pathological microglial subtypes (e.g. DAM and MGnD), but this subtype also seems to down-regulate genes that are otherwise generally up-regulated in microglia during normal aging (*Cybb*, *Axl*, *Cd74*).[54] Additionally, genes involved in neurodegeneration-based diseases may drive the expression of this microglial phenotype (*Grn*: frontotemporal dementia, *Snx17*: regulation of amyloid precursor protein, *Slc33a1*: injury-induced axonal regeneration, *Vps35*: Parkinson's disease), supporting the emerging link between lipid storage in microglia and neurodegeneration. A summary of the evolution of microglial heterogeneity in health and disease can be found in Fig. 2.

In comparison, microglial subtypes associated with other neurodegenerative diseases show similarities with those present in AD. For instance, in a mouse model of multiple sclerosis, three clusters of microglia, respectively named daMG2, daMG3 and daMG4, were identified using RNA sequencing.[118] Like the DAM and MGnD, these clusters were all characterized by the down-regulation of homeostatic markers (*P2ry12*, *Tmem 119*) but respectively up-regulated different genes such as *Cd74* (MHC class II-related molecule) and *APOE* for daMG2, C-X-C motif chemokine 10 (*Cxcl10*) and chemokine ligand 4 (*Ccl4*) for daMG3, as well as chemokine ligand 5 (*CCL5*) and integral membrane protein 2B (*Itm2b*) for daMG4 subtype.[118] Similar findings were described in humans suffering from multiple sclerosis.[119]

While many different microglial subtypes seem to co-exist during AD, it is worth noting that the distribution of these subtypes between males and females is mostly unknown. A recent study[120] uncovered a new microglial subtype (i.e. Activated

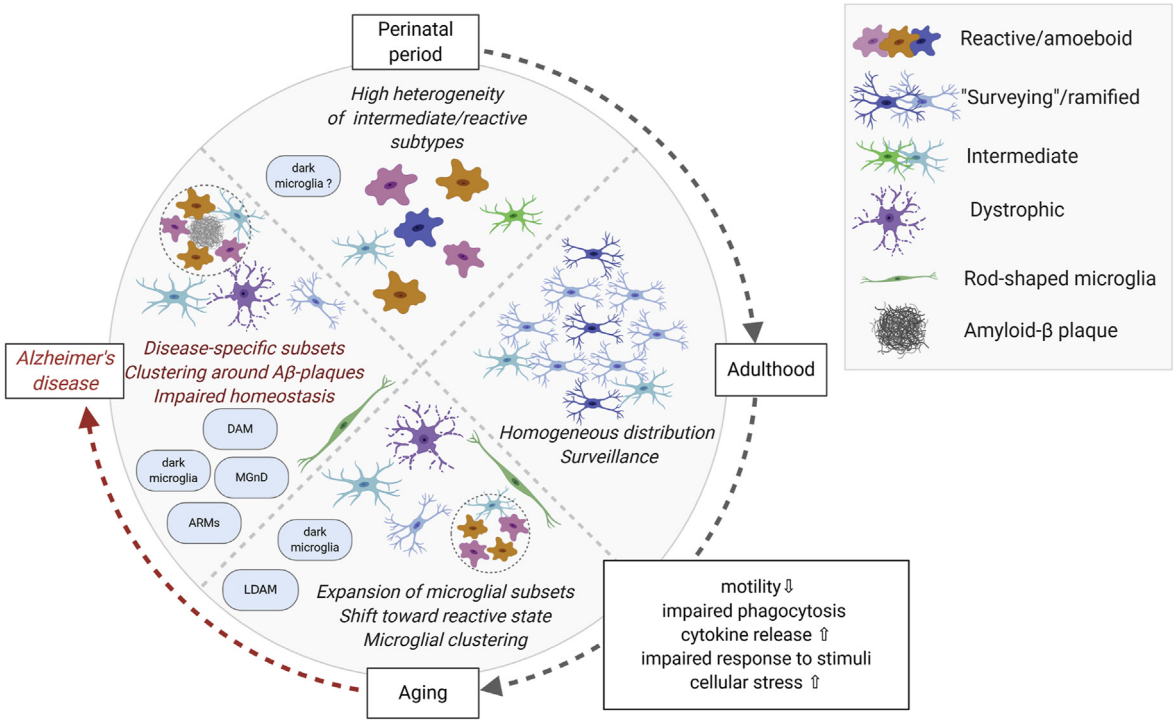


**Fig. 2. Microglial heterogeneity over time.** Microglial population possess a highly heterogeneous character which in addition undergoes significant changes along the aging trajectory. 1) Predominantly present during development are amoeboid microglia, which are thought to be reactive and thus mediate dynamic formation of neuronal circuits. 2) With progressive maturation, microglia slowly adopt more ramified, surveying morphology and maintain homeostasis. 3) During aging, there is a growth in existing subpopulations along with a molecular shift in microglial subsets toward more reactive/pro-inflammatory signatures. Aging promotes appearance of dystrophic microglia which, due to their seemingly fragmented processes and nucleal swelling, may imply an aging-related pre-apoptotic state. Simultaneously, presence of specific ultrastructural/molecular subtypes such as dark microglia or LDAM increases. Microglia proliferate in a brain region-dependent manner, however their even distribution pattern declines as microglial clusters appear. Retraction of their processes combined with their lowered motility thus promotes impaired clearance of debris as well as delayed responses to stimuli. Rising baseline inflammation promotes microglial priming leading to elevated release of inflammatory mediators and exacerbated responses. 4) Microglia in Alzheimer's disease show similarities to microglia in healthy aging. However, novel subsets with disease-specific features also appear as either a compensatory mechanisms or an aberrant consequence of the pathology. Clustering of microglia near amyloid-β plaques frequently occurs. **LDAM** - lipid-droplet-accumulating-microglia; **DAM** - damage-associated-microglia; **MGnD** - neurodegenerative microglia; **ARMs** - Activated Response Microglia; **Aβ** - amyloid beta.

Response Microglia, ARMs) in mice and humans during both normal aging and AD. ARMs are characterized by the expression of genes involved in MHC-II presentation (*Cd74, Ctsb, Cstd*), inflammatory processes (*Cst7, Clec7a, Itgax*) putative tissue repair genes (*Dkk 1, Spp1, Gpnmb*) and AD risk genes like APOE.[120] Strikingly, microglia in female mice progress faster over the ARMs phenotype compared to microglia in males, which hints at the fact that other microglial subtypes could also be sexually differentiated.

Transcriptomic data suggest that microglia of male rodents may be more inclined toward inflammatory response and possess more reactive phenotypes compared to more neuroprotective-focused female microglia in the developing and early adult stage,[121–124] although this depends on the study and brain region assessed.[102,125,126] This is in line with soma enlargement, up-regulated MHC-II expression, regionally increased density of microglia and potentiated response to ATP in regions such as cortex, hippocampus and amygdala of male mouse microglia compared to females.[121] While Guneykaya and colleagues (2018) did not observe any sex difference in phagocytosis, they found that increased levels of P2RY12 signaling were associated with more dynamic motility of microglia in males.[121]

By contrast, microglia in adult females exhibited potentiated signaling of transcription factors such as NANOG or TCF3, which are negative regulators of inflammatory response and promoters of repair mechanisms.[122] In addition, in an adult mouse model of AD (EFAD mice), microglia of females showed decreased Aβ plaque coverage and compaction (a beneficial consequence of microglial interaction with the plaques) as well as reduced TREM2 expression, suggesting a less effective phagocytosis of Aβ burden compared to males.[127] Strikingly, the age-related phenotypic change of female mouse microglia toward a more inflammatory phenotype compared to males in basal conditions[92] and possibly also in response to an immune challenge,[128] could create a possible window of sensitivity to a pathological stimulus or to the development of neurodegenerative diseases. This switch and subsequent sensitivity could be mediated by the loss of neuroprotective estrogens associated with the menopause, as menopaused women are more likely to develop AD.[129] In rodents, estrogens are known to induce sex-specific effects in microglial properties during early life[124,125] and further seem to affect microglia during aging. Indeed, chronic ovariectomy of female rats and mice led to increased microglial reactivity at the baseline level or following immune challenges in the cortex and hippocampus, which was then decreased by estrogen treatment.[130–132] Primary cultures of microglia from adult rats/mice (hippocampus and olfactory bulbs) treated with estrogens also support an anti-inflammatory effect of these hormones on female microglia.[130] It is further worth noting that these neuroprotective effects might rely on several factors such as the dose of estrogens used or the age of the subjects.[130] More specifically for females, the timing of the estrogen treatment is also an important factor to consider. Indeed, studies have shown that the modulation of microglial reactivity by

estrogens seems to only be effective during early aging and depends on how long the brain spent without hormonal exposure.[130,133] While sex differences in aging microglia and their underlying mechanisms are still mostly unclear, this suggests that estrogens might be able to drive sex-specific differences in both microglial phenotypes and functions. However, further insight would be required nonetheless as other metabolic, genomic or epigenomic modulations may be involved as well.[122,134]

These data overall suggest that while microglial cells in females might be more reactive, they might also be less effective or partly dysfunctional, as hinted at in the study of Stephen and colleagues.[127] Conversely, it could be theorized that male microglia, in context of AD, may be more effective in the clearance of plaques due to a better maintenance of their similarly reactive, but still more homeostatic phenotype.[127] Strikingly, this difference may be partly under control of microglial microRNAs (e.g. miR-16–5p, miR-23a-3p, miR-342–3p) as their loss in a knock-out (KO) mouse model (microRNA-processing enzyme dicer KO) specifically affected male microglia and rendered them more reactive both morphologically (amoeboid shape) and functionally (characteristics of DAM).[135]

Moreover, loss of microRNAs decreased the expression of homeostatic microglia markers in males.[135] It is also worth noting that these subsequent more reactive male microglia are further correlated with increased levels of tau, a microtubule-associated protein, and thus a worsening of AD pathogenesis,[135] which is, in the literature, more often seen in females.[136,137] Finally, a recent study suggested that microglia of male mice (60 days old) could be more developmentally delayed compared to females, while exposure to LPS in adulthood specifically allowed males to adopt a more mature transcriptomic profile as well as a less branched morphology.[126] Moreover, human brain samples from patients suffering from AD showed accelerated microglial development, although differences between the sexes could not be assessed.[126] Thus, sex differences in microglial rate maturation over the course of development could also contribute to differential late–life activity and response.

## Conclusion

In conclusion, microglial heterogeneity in both aging and AD is affected by numerous microglial properties ranging from their morphology to their metabolism, as well as their gene expression and protein production. While there exist some similarities between the microglial subsets and/or phenotypes present during normal aging or AD, it is clear that microglia respond differently in each case, with the extent of their diversity adjusting to the current context. However, how these different subtypes would translate to functional changes is still unknown and would thus be worth a more profound investigation in the future. Additionally, microglial heterogeneity depends on the sex of the individuals, with current evidence suggesting male and female microglia might use alternative strategies to respond to their microenvironment in healthy and diseased conditions. These findings are particularly striking as microglial cells are involved in many homeostatic processes as well as in the resolution and progression of neurodegenerative diseases. Yet, sex is rarely incorporated as a variable in this kind of studies, despite most of neurodegenerative diseases like AD being sex-biased. Future research focusing on aging microglia could hence benefit from adding sex as a parameter, since there is little doubt that this would unearth exciting sex differences that would shed new light on the development and potential treatment of neurodegenerative diseases.

## Contributions

C.I. Delage and E. Šimončičová conceived the review. C.I. wrote the manuscript, E. made the table and contributed to writing. MET contributed to writing and revising the manuscript. All authors read and approved the final manuscript.

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## Declaration of competing interest

The authors have no conflict of interest to declare.

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