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# "Microglia derived extracellular vesicles (EVs) in brain aging: a rejuvenation approach"


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# List of abbreviations

AB-MSCs (adult bone marrow–derived MSCs)AD (Alzheimer disease)
AGO2 (protein Argonaute 2)
ALIX (ALG-2-interacting protein X)
ALS (Amyotrophic lateral sclerosis)
AP-1 (activating protein-1)
ARF6 (ADP-ribosylation factor 6)
ARG1 (arginase 1)
ARRDC1 (arrestin domain-containing protein 1)
BAMs (border-associated macrophages)
BBB (blood brain barrier)
BDNF (brain-derived neurotrophic factor)
BMT (bone marrow transplantation)
C1q (complement component 1q)
C4 (complement component 4)
CA2 (carbonic anhydrase 2)
CB1 (type-1 cannabinoid)
CCL2 (chemokine (C-C motif) ligand 2)
CCL3 ((C-C motif) ligand 3)
CCL4 ((C-C motif) ligand 4)
CD206 (Cluster of Differentiation 206)
CD22 (cluster of differentiation-22)
CD55 (complement decay-accelerating factor 55)
Chil3/Ym1 (Chitinase-like-3 protein)
circRNA (circular RNA)
CNS (central nervous system)
CSF (cerebrospinal fluid)
CSF-1 (colony stimulating factor 1)
CX3CL1 (fractalkine)
CX3CR1 (CX3C motif chemokine receptor 1)
CXCL1 (C-X-C Motif Chemokine Ligand 1)
DAM (disease-associated microglia)
DH (dorsal hippocampus)
E2 (estradiol)
EAAT2 (excitatory amino acid transporter 2)
eCB (endocanabinoids)
ER α (estrogen receptor alpha)
ESCRT (Endosomal Sorting Complex Required for Transport)
ESCs (embryonic stem cells)
EVs (Extracellular vesicles)
FMT (fecal microbiota transplantation)
G-CSF (colony-stimulating factor)
GDF11 (growth differentiation factor 11)
GLUT1 (glucose transporter)

GnRH I (Gonadotropin Releasing Hormone)
HB (heterochronic parabiosis)
HCC (hepatocellular carcinoma)
hnRNPs (heterogeneous nuclear ribonucleoproteins)
IBA1 (ionized calcium-binding adapter molecule 1)
IFNγ (interferon gamma)
IGF-1 (insulin-like growth factor 1)
IL1β (interleukin 1β)
IL10 (interleukin 10)
IL4 (interleukin 4)
IL6 (interleukin 6)
ILVs (intraluminal vesicles)
IPF (idiopathic pulmonary fibrosis)
IRG1 (immunoresponsive gene 1)
ISEV (International Society for Extracellular Vesicles)
KLK8 (neuropsin)
LDAM (lipid droplet-accumulating microglia)
LDHA (Lactate dehydrogenase A)
lncRNAs (long-non codingRNAs)
LPS (lipopolysaccharide)
LTF (lactoferrin)
LTP (long-term potentiation)
m/l EVs (medium and larger vesicles)
MCT1 (Monocarboxylate transporter 1)
MHC (major histocompatibility complex)
miRNAs (microRNAs)
MSC (mesenchymal stromal cell)
MT1-MMP (membrane type 1-matrix metalloproteinase 1)
mtDNA (mitochondrial DNA)
MVBs (multivesicular bodies)
MVE (multivesicular endosome)
NG (nanoghost)
NF-kB (Nuclear Factor kappa B)
NMDA (N-Methyl-D-aspartate)
OPCs (oligodendrocyte precursor cells)
OXPHOS (oxidative phosphorylation)
P2RY12 (P2Y12 receptor)
PD (Parkinson disease)
RHOV (GTP-binding protein RhoV)
RISC (miRNA-induced silencing complex)
ROS (reactive oxygen species)
RyR (ryanodine receptors)
SA-β-Gal (senescence-associated beta galactosidase)
sEVs (small extracellular vesicles extracellular vesicles)
SREBF1 (sterol regulatory element-binding proteins)

SOD2 (superoxide dismutase 2)
SYNCRIP (synaptotagmin-binding cytoplasmic RNA-interacting protein)
TIMP2 (metalloproteinases 2)
TLRs (toll-like receptors)
TMEM119 (transmembrane protein 119)
TNFα (tumor necrosis factor α)
TREM2 (triggering receptor expressed on myeloid cells-2)
UC-EVs (neonatal human umbilical cord MSCs)
VAMP3 (vesicle-associated membrane protein 3)
VH (ventral hippocampus)
VPS4 (Vacuolar Protein Sorting 4)
v-SNARE (vesicular SNARE)
VTA1 (Vesicle Trafficking 1)
WHO (World Health Organisation)

# 1. Abstract

Brain aging presents a picture defined "inflammaging", characterized by high oxidative stress, chronic inflammation and elevated production of inflammatory compounds (*López-Otín et al. 2023*). Microglia, the immunocompetent cells of the central nervous system, shift towards a chronic low-grade inflammatory state and become hyper-responsive. Indeed, these cells undergo the most prominent aging-related changes in both the morphological and functional phenotypes; their progressive loss of neuroprotective functions affects the whole brain homeostasis. This condition could result in cognitive functions deterioration, lack of motor coordination and memory loss. Microglial phenotype is impacted by aging in a region-dependent manner with hippocampus being more susceptible to age-related modifications during late aging (*Grabert et al. 2016*). In addition, age-related changes occur differently in aged males and females, since sex hormones are involved in microglial activation and inflammatory response.

Extracellular vesicles are key players of the inter-cellular communication between donor and recipient cells, through which brain cells exchange packages of molecular information con-sisting of lipids, proteins and nucleic acids (*Budnik et al. 2016*). Microglia-derived EVs transportmolecular contents that mirror the inflammatory status of donor cells and modulate the inflam- matory phenotype of recipient microglia and other cell types. The principal aim of the project was to investigate whether exogenous administration of EVs deriving from microglial-like BV2cells could be efficient to slow down neuroinflammation in aged mice. To this aim, BV2-derivedEVs were analyzed for their molecular, cellular and behavioral effects when administered to 16-20 months-old male and female mice. Data obtained demonstrated that the treatment dampened inflammation, restored a juvenile microglia morphology, reduced anxiety-like behavior while increasing spatial learning, with sex-dependent differences. Results of this thesis has been published in 2024 on the journal “Brain, behavior, and immunity” (*Rinaldi & Balietti et al., 2024*).

# 2. Brain Aging

Aging is a universal complex process, which involves the entire organism, determining a progressive decline in physiological functions from subcellular to tissue level. According to World Health Organisation (WHO), "healthy aging" can be defined as "the process of developing and maintaining the functional ability that enables well-being in older age" (*World Health Organization, 2015*). Within the context of the central nervous system (CNS), aging is firstly characterized by alterations in brain anatomy, which typically experiences atrophy and severe volume loss (*Nyberg and Wåhlin, 2020*). Other macroscopic features that can be observed within aged brains include the cortical thinning, that together with degeneration of white and grey matter, contribute to tissue softening. These aged-induced changes, together with cellular and molecular alterations, ultimately results in cognitive impairment, reduced motor performance and other behavioral deficits occurring during aging (*Blinkouskaya et al., 2021*). However, since aging is a phenotypic phenomenon shaped by both genetics and lifestyle, its effect on different organs could diverge across individuals (*Anton et al 2005*). Nonetheless, aging represents a condition of vulnerability and is strictly linked to increased risk of developing various disorders including cancer, cardiovascular, musculoskeletal and neuro- degenerative disease (*Johnson et al., 2015*).

## 2.1 Hallmarks of brain aging

Different cellular and molecular events are affected by aging, thus a classification of the factors driving aging has been recently proposed. Three criteria should be satisfied by each hallmark of aging: (I) the time-dependent manifestation, (II) the capacity of accelerating the aging process by amplifying the hallmark in experimental models (III) the possibility to slow down, stop, or reverse aging by thera-peutic interventions on them (*López-Otín et al., 2023*).

In accordance, different events occur in the brain during aging, among which oxidative stress, chronic activation of the innate immune system, accumulation of aberrant proteins and high production of inflammatory compounds (Fig. 1). These manifestations generate a condition defined "inflammaging" (*Franceschi et al., 2018*). Inflammation is a key feature of brain aging, during which glial cells often exhibit an activated state.

Notably, a growing body of evidence associates age-related brain to **neuroinflammation**, with toll-like receptors (TLRs) playing a crucial role in immune responses to infections. In fact, in experimental models of age-related neurodegenerative diseases, activation of microglial TLR4 receptors worsened neuronal degeneration, while their pharmacological suppression resulted in neuroprotection (*Colonna and Butovsky, 2017*). Microglia activation during

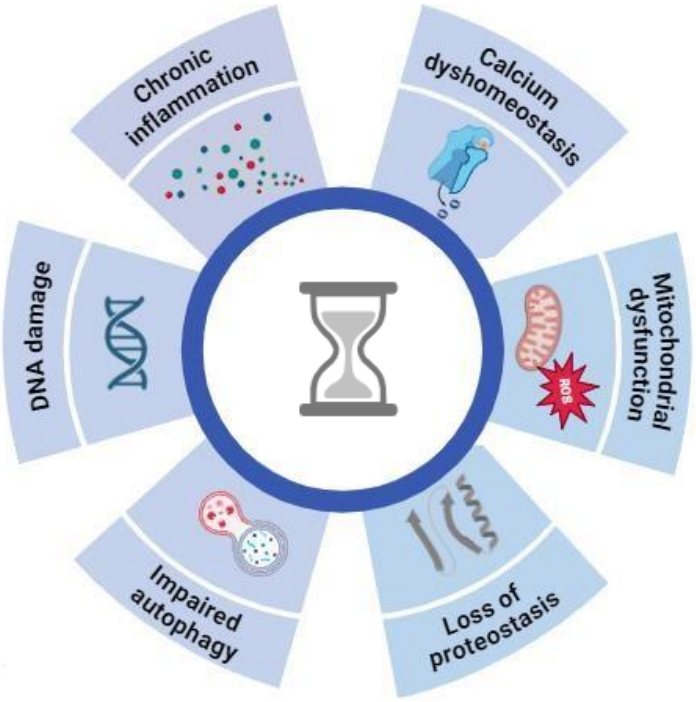



**Figure 1.** Schematic representation based on *López-Otín et al., 2023* of aging hallmarks. They include: chronic inflammation, calciumdyshomeostasis, mitochondria dysfunction, loss of proteostasis, impaired autophagy and DNA damage.

aging leads to high production of pro-inflammatory cytokines including interleukin 1β (IL-1β), interleukin 6 (IL6), and tumor necrosis factor α (TNFα). In humans, it has been found that elevated levels of IL6 in plasma constitute a prognostic biomarkers associated with all- cause mortality among the oldest old population (*Hirata et al., 2020*). Moreover, RNAseq andearly microarray studies of astrocytes in aged mice demonstrated that the cytokines IL1α, TNFα, and the complement component 1q (C1q) produced by microglial cells are capable in turn to induce a neuroinflammatory astrocyte-reactive phenotype when compared to young mice (*Clarke et al., 2018*). Agingastrocytes have been linked to age-related cognitive impairment by facilitating synaptic elimination (*Boisvert et al., 2018*). However, it is noteworthy to mention that in aging brain astrocytes maintain some neuronal and synaptic functions, which include recycling of neurotransmitters, buffering of extracellular potassium (*Allen et al., 2014*) and the uptake, processing, and delivery of nutrients from the blood to neurons (*van Deijk et al., 2017*). In fact, key elements of the lactate shuttle (i.e. lactate dehydrogenase A, LDHA and monocarboxylate transporter 1, MCT1), through which neurons are provided with trophic factors, are not affected by aging as well as the sterol regulatory element-binding proteins (SREBF1), the transcription factor which modulates lipid synthesis. This suggests that genes related to astrocytic core functions, namely neuronal support, are not impacted by age (*Boisvert et al., 2018*). In addition to astrocytes, also oligodendrocytes have been shown to be affected by microglia activation. The number and activity of oligodendrocyte precursor cells

(OPCs) significantly decreased during aging and microglia appeared to inhibit OPCs differentiation into mature myelin-forming cells (*Baror et al., 2019; Allen et al., 2023*).

However, not only glial cells undergo changes due to aging, but neurons as well. It is reported that, starting from the middle-age, adult hippocampal neurogenesis progressively decline along aging (*Snyder et al., 2019*). Moreover, the inflamed microenvironment alters the com plex balance required for synaptic plasticity, by affecting the synthesis of brain plasticity-re lated molecules such as the brain-derived neurotrophic factor (BDNF) and the insulin-like growth factor 1 (IGF-1). Moreover, aging is also associated to alterations in neurotransmitter systems, including dopamine and acetylcholine. In particular, dysfunction of the cholinergic system are often observed in aging and dementia, manifested as deficits in cognitive processes and executive functions such as wakefulness, mood, learning motor function, motivation, short-term memory (*Lee et al., 2022*).

Aging is also characterized by **calcium signaling dysregulation**, which in turn has implication in synaptic plasticity. Consistently, it has been shown in aging models that ryanodine receptors (RyR) on the endoplasmic reticulum, which account for $Ca^{2+}$-induced $Ca^{2+}$ release, become progressively more active in the course of aging (*Stutzmann et al., 2006*). Aged hippocampal pyramidal neurons showed increased L-type $Ca^{2+}$ current that could result in increased slow after hyperpolarization spikes, contributing to inhibition of long-term plasticity (*Thibault et al., 2001; Foster 2007*). In accordance with $Ca^{2+}$ dyshomeostasis in aged animals, it has been demonstrated that aged rats have deficits in both long-term potentiation (LTP) induction and maintenance (*Burke & Barnes, 2006*). An additional classic hallmark of brain aging is the deterioration of protein homeostasis (i.e. proteostasis) and impairment of the phagolysosomal system. Failures in theseprocesses result in the accumulation of misfolded, oxidized, glycated, or ubiquitinylated proteins that form aggregates as intracellular inclusion bodies or extracellular amyloid plaques (*Hipp et al., 2019*). **Lysosomal inefficiency** due to overloading brings to accumulation of lipofuscin, also referred to as *age pigment*, a well-known deposit of highly oxidized cross-linked macromolecules (proteins, lipids, and sugars) traditionally associated with aging (*Brunk& Terman, 2002*). **Lipofuscin granules** have been detected in aged brain samples inside glial cells and neurons (*Panov et al., 2020*). Despite lipofuscin formation in an unavoidable event linked to aging, it has been demonstrated that enhancing proteasome activity in aged mice reduced brain lipofuscin levels, likely preventing new accumulation, and improved cognitivefunctions (*Bobkova et al.,2015*). Moreover, during aging, it has been observed an increased amount of **protein oxidation** markers (carbonyl residues) and a growing body of evidence points out that, as macromolecules become oxidizes, they can no longer be degraded by lysosomes (*Kehm et al., 2021; Brunk et al., 2002*). Therefore, lysosomal enzymes,

unable to digest the cellular material, are usually accumulated and upregulated such as senescence-associated beta galactosidase (**SA-β-Gal**), a common marker of senescent cells (*Cohen & Torres 2019*). Additionally, important changes largely occur with aging at subcellular and molecular level inthe nucleus and mitochondria. Machineries for DNA maintenance and repair allow the cells to handle the genetic lesions such as point mutations, deletions, translocations affecting nuclear and mitochondrial DNA (mtDNA) in order to preserve chromosomal and genome stability. This fine-tuned network has been demonstrated to become less effective with age leadingto accumulation of genomic damage and ectopic DNA in the cytosol (*Miller et al., 2021*). A recent study also showed that immunosenescence caused by **DNA damage** is capable to accelerate aging of organs including the brain, in a mouse model with immune system-specific deficiencies in DNA damage repair (*Yousefzadeh et al., 2021*). MtDNA integrity is affected by age as well and together with reactive oxygen species (ROS) and calcium alterations cause mitochondrial dysfunction. The energy demand required for synaptic transmission, synaptogenesis, and synaptic pruning generally account on mitochondria via oxidative phosphorylation (OXPHOS). During aging the respiratory systems is compromised, with particular destabilization of complexes I and III, which alter the whole cellular bioenergetics (*Amorim et al., 2022*).

Chronic low-grade inflammation during aging has dramatic consequences in remodeling the blood brain barrier (BBB) structure with increasing brain microvessel permeability, basal lamina thickness, and the extended size of astrocyte endfeet (*Bors et al., 2018*). These changeslead to **increasing BBB permeability** and dysfunctions, which hampers the exchanges of nu trients and brain metabolism. For instance, it is reported that altered glucose transport, due todysfunctional glucose transporter 1 (GLUT1), does not provide the brain the adequate energy supply, rendering it more susceptible to neurodegenerative diseases (*Noe et al., 2020*). The aberrant BBB permeability cause the spreading of inflammation from CNS to systemic and viceversa. In fact, an additional feature of aging is **gut dysbiosis**, which consists of change in composition the gut microbial communities, resulting in gut immune dysregulation and in crease of inflammatory status of gut mucosa (*Mou et al., 2022*). In a feedback loop mechanism,inflammaging contributes to gut microbiota dysbiosis via BBB disruption, while pathogenic gut symbiotics increase the production of metabolites and small molecules, which reach the brain and activate the microglia, further sustaining neuroinflammation and neurodegeneration(*Antonini et al., 2019; Fransen et al., 2017*).

Given what has been discussed so far, it is noteworthy to mention that inflammaging is at the crossroad of aging and age-related diseases. However, the molecular changes underpinning

aging brain modifications are still not well understood, representing a challenge for age-related research.

## 2.2. Sexual differences in aged brains

Several studies have documented differential patterns of brain aging between males and females, shaped by hormonal, genetic and environmental factors.

Sex differences have been observed in the age-related volume reduction of specific neuroanatomic structures, even though general agreement on this phenomenon is still debated. Recent studies reported that human aged brains display generalized volume loss, with males experiencing greater loss accompanied by more significant ventricular enlargement than females (*Armstrong et al., 2019*). Moreover, it has been found that the brain regions displaying volume reduction are those mostly prone to aging and Alzheimer's disease (AD), namely the temporal lobe,cingulate, prefrontal cortex, and most medial regions of grey matter (*Sang et al., 2021; Fjell et al., 2014*). Indeed, as reported by Perneczky and collaborators, men affected by AD displayed more severe and widespread pathological changes compared to women at the same stage of disease. Those changes particularly affected the frontal, temporal, and insular cortices, along with the hippocampus in the right hemisphere (*Perneczky et al., 2007*). However, even though different anatomical structures account for specific cognitive domains, the sexual disparities in the age-related relationship between cognitive domains and brain morphological measures still remain to be further investigated. Moreover, it has been demonstrated that sex contributes to immunity and inflammation in a dimorphic manner (*Olivieri et al., 2023*). For instance, a recent study demonstrated that male mice exhibited a higher proportion of infiltrating and pro-inflammatory macrophages compared to females upon bacterial infection. (*Deny et al., 2022*). Additionally in healthy adult humans, the systemic levels of the aging associated cytokines IL1β, IL6, and TNFα were significantly higher in men as compared to women (*Bernardi et al., 2020*).

Concerning inflammaging, Cyr and collaborators also documented that aged male mice exhibit an increase in the activation of inflammasome proteins, which results in the release of inflammatory signaling molecules (*Cyr et al., 2022*). Moreover, priming the immune system with peripheral administration of LPS resulted in an exacerbated immune response, which led to cognitive impairements in aged male rats compared to adult animals; this response was not observed in female rats (*Ince et al., 2023*). It is noteworthy to highlight that sex can act on inflammaging in a cell and region-specific manner. Cytokine and chemokines such as chemokine (C-C motif) ligand 3 and 4 (CCL3 and CCL4) and C-X-C Motif Chemokine Ligand 1 (CXCL1) have been observed to be upregulated hippocampus (*Porcher et al., 2021*) andcortex (*Cyr et al., 2023*) in females rodents more than in males. Possible and indeed partial explana-

tions of these sex-dependent discrepancies involve sex hormones and the X chromosome. Notably, estrogen interact with DNA-binding transcription factors, as nuclear factor kappa B (NF-kB) and activating protein-1 (AP-1), to exert immunomodulatory functions and as a result of this pathway, IL6 production is suppressed (*Klein & Flanagan, 2016*). Moreover, it has been showed the capacity of estrogento delays the onset of cellular senescence, by preventing DNA damage (*Rall-Scharpf et al., 2021*). Interestingly, the microRNA-223-3p (miR-223-3p) linked to X chromosome appear to have a key role in modulating the innate inflammatory response, contributing to macrophage polarization. In fact, inhibition with an antagomiR-223 resulted in increased expression levelof pro-inflammatory cytokine *Il1β* and decrease of the anti-inflammatory *il10* (*Deny et al.,2022*). However, circulating sex hormones decrease during aging, negatively affecting the synaptic function. Indeed, it has been demonstrated that estradiol (E2) is capable to enhance hippocampal-dependent cognitive tasks and prefrontal cortex-dependent learning in aged female animals as well as in post-menopausal women (*Spencer et al., 2007*). Aged brains respond differently to estrogen treatment than the young brain pointing out the neuroprotective role of sex hormones. Indeed, it has been documented that neurodegenerative disorders such as Alzheimer's and Parkinson's disease (PD) tend to manifest after menopause in humans, with earlier menopause being associated with an earlier onset of neurodegeneration (*Li et al., 2002; Corbo et al., 2011*). On the other hand, female aged brain is supposed to be less sensitive to estrogens levels, due to a reduction of estrogen receptor alpha (ER α) expression on CA1 pyramidal cell spines (*Adams et al., 2002*). Accordingly, in cognitive processes not involving sex hormones as verbal memory, elderly females significantly outperformed male capabilities(*Rodriguez-Aranda 2006; Gale et al., 2007*). Conversely, males displayed enhanced efficacy in spatial memory and orientation tasks. In mice, it has been documented an overall decline of cognitive function in spatial memory in 18-20 months old mice of both sexes compared to young (*Benice et al., 2006*). Moreover, research has largely demonstrated a higher vulnerability in females compared to males to experience depression and anxiety (*Gobinath et al., 2017*)All considered, sexual dimorphism affects many aspects of brain aging and, even though it could appear as an additional source of variation, it has fundamental clinical relevance (*Burmistrov et al., 2024*). Indeed, the above discussed differences in brain aging have crucial implications in the sex-dependent incidence of most psychiatric, neurological and neurodegenerative disorders (*Riecher-Rössler, 2017*) and highlight the need of sex-tailored approaches for both prevention and treatment.

## 2.3. Brain rejuvenation strategies

United Nations projections indicate that the proportion of older individuals in developed countries currently stands at 20% of the total population and this rate is anticipated to reach almost 30% over the next three decades. By 2050, the global population of those aged 60 will double, reaching 2.1 billion and the number of people aged 80 or older is expected to triple, rising to an estimated 426 million (*World Population Ageing, 2023*). In light of the above, it becomes increasingly urgent to figure out therapeutic strategies for counteracting the growing prevalence of age-related neurodegenerative diseases, while also pursuing longer lifespan and higher quality of life. Several 'rejuvenating' approaches have been proposed to delay and eventually revert aging, exploiting different strategies such as cell reprogramming, heterochronic parabiosis and bloodborne factors (*Gaspar-Silva et al., 2023*). As conceived by anti-aging approaches, somatic cells can be reprogrammed to pluripotent stem cells, undergo genetic manipulation and re-differentiated into rejuvenated cells. Interestingly, in vivo reprogramming in the CNS, specifically in the hippocampal dentate gyrus, improved the migration of neurogenic progenitors, neuronal plasticity and memory in mice (*Rodriguez-Matellan et al., 2020*). The ability of cellular reprogramming to reverse aging has been documented also in human cell lines, advancing the hypothesis that this approach may be beneficial to target human aging (*Ocampo et al., 2016*). The potential application of stem cells in brain rejuvenation relies also on their production of vesicles carrying pro-youthful factors. Indeed, it has been recently demonstrated that vesicles derived from embryonic stem cells (ESCs) were capable to alleviate the senescent phenotype of hippocampal neural stem cells, such as SA-β-gal activity, in aged mice (*Hu et al., 2021*).

Promising results have been obtained with heterochronic parabiosis (HB), a technically challenging surgery that allows the fusion of the circulating systems of young and aged mouse, with the aim to achieve rejuvenation by exposure to a young systemic environment (*Conboy et al., 2005*). Singe-cell transcriptomic analysis have confirmed the capacity of HB to alleviate the aged associated phenotype and modify the gene expression signature in hematopoietic and immune system, as well as in skin, liver, skeletal muscle, and brain (*Ma et al., 2022*). In aged mice, the parabiotic coupling to young mice induces neuron remyelination and an increased frequency of microglial cells with anti-inflammatory phenotype (*Miron et al., 2013*). Additionally, Villeda and collaborators demonstrated that young blood was capable to enhance dendritic spine number and synaptic plasticity in the aged hippocampus, which ameliorated memory and learning (*Villeda et al., 2014*).

Heterochronic parabiosis provides evidence that young blood, or alternatively bone marrow transplantation (BMT), may reverse organismal aging. Evidences showed that 18-month-old

mice receiving bone marrow transplants from 4-month-old retained more synapses between neurons in the hippocampus consistently with improved learning plus spatial and working memory performances than mice recipients of old bone marrow (*Das et al., 2019*). Interestingly, recent studies highlighted the existence of a cell-type aging heterogeneity within the brain (*Buckley et al. 2023*), suggesting that there could be cell types primarily targeted by rejuvenation interventions that could mediate the beneficial effects (*Mahmoudi et al, 2019*). For instance, studies demonstrated that the replacement of murine aged microglia with young cells had beneficial effects on synaptic plasticity and cognition, restoring the expression of genes involved in neural activity (*Elmore et al., 2018*). However, cellular transplantation entails different challenge in the clinical application, namely the availability of young, suitably matched donors and the invasiveness of the therapy. Systemic rejuvenating strategies take advantage of the gut-brain interaction, acting on intestinal microbiota. Specifically, fecal microbiota transplantation (FMT) of 3-months old mice in older animals (18 and 24-months) efficiently restored BBB and intestinal epithelium integrity as well as reduced microglial activation and systemic inflammation (*Parker et al., 2022*). Conversely, the same aging hallmarks were exacerbate when old to young FMT was performed, as reported in other studies showing a worsening of hippocampal-dependent behavioral performances (*D'Amato et al., 2020*).

These systemic approaches, especially BMT and HB, provided robust evidence that blood factors more than blood cells have a major role in rejuvenating effects. Mice directly injected with human umbilical cord plasma displayed reactivation of genes linked to memory consolidation, significantly increased LTP and improved cognitive functions (*Castellano et al., 2017*). Similarly, infusion of young cerebrospinal fluid (CSF) into aged brain stimulated the regeneration of oligodendrocytes and improved memory in older mice (*Iram et al., 2022*). One hypothesis to explain the beneficial effects of young blood suggests the presence within plasma of pro-rejuvenation factors, among which growth differentiation factor 11 (GDF11), Gonadotropin Releasing Hormone (GnRH I), tissue inhibitor of metalloproteinases 2 (TIMP2) and the pro-longevity factor α-klotho (*Bieri et al., 2023*). Alternatively, it has been proposed that young blood could dilute or inhibit pro-aging factor present in aged blood. Evidences to sustain this hypothesis account on studies demonstrating that lowering the aged plasma level by approximately 50% by dilution with saline-albumin solution, reduced micro-glial activation, and enhanced cognitive function (*Mehdipour et al., 2021*).

Young blood therapy currently represents a promising strategy, which increases the opportunity of translation to human aging and age-associated diseases. In fact, young blood and

plasma exchange-based approaches are now being tested in clinical for Alzheimer's and Parkinson's disease (NCT02256306, NCT02256306). In these trials, plasma from young donors has been intravenously administered to mild and moderate AD or PD patients. Alternatively, other clinical trials (NCT03713957, NCT03765762) assessed the safety and tolerability of highly selected plasma fractions (GRF6021 and GRF6019) intravenously infused to PD and AD patients. (*Boada et al., 2020;Kang et al., 2020*).

# 3. Microglia

In 1856, the German pathologist Rudolf Virchow coined the term *neuroglia* (derived from the Greek word for 'glue'') to describe a compartment of cells in CNS distinct from the neuronal elements. In the early 20th century, Ramón y Cajal also recognized that the brains comprise more cell types besides neurons and astrocytes supported by the observation of poorly stained, seeminglyapolar cells in his preparations, to which he referred to as "the third element" (*Ramón y Ca-jal, 1913*). The first to use the term "microglia" was the Spanish neuroscientist Pío del Río-Hortegain 1919, when for the first time the main three types of glial cells of the CNS were defined, namely astrocytes, oligodendrocytes, and microglia. (*Rio-Hortega, 1919*). In his studies, Río-Hortega proposed that microglia cells were of mesodermal origin and only recently it was confirmed that they actually originate from mesodermal precursors of a distinct lineage, sharing similarities with the functions and features of macrophages (*Sierra et al., 2016*). Microglia are the tissue resident macrophages of the CNS and represent approximately 5-10% of all glial cells in the CNS (*Nayak et al., 2014*). Moreover, quantification studies revealed glia-neuron ratio closer to 1:1 in the total brain, suggesting that microglia can account for the 10% of all brain cells (*von Bartheld et al., 2016*).

## 3.1. Origin, markers and functions

Differently from meningeal, choroid plexus, and perivascular macrophages, also defined border-associated macrophages (BAMs) (*Mrdjen, et al., 2018*), microglia originate from erythromyeloid progenitors in the yolk sac (E7.5–8.5) and invade the neuroepithelium during embryogenesis at E9.5 (*Ginhoux et al., 2010; Prinz et al., 2021*). After brain colonization, microglia proliferation proceed until postnatal stage induced by colony stimulating factor 1 (CSF-1), granulocyte macrophage colony-stimulating factor(G-CSF), neurotrophin-3, interleukin 4 (IL4), and interleukin 5 (IL5) (*Navascues et al., 2000*). Unlike peripheral macrophages,in the adult mouse brain microglia are maintained throughout life by local proliferation independently from bone marrow-derived progenitors, as established by fate mapping studies (*Ajami et al., 2007; Schulz et al. 2012*).

Immature microglia in embryonic development present a round amoeboid morphology with short processes, which promote locomotive migration to occupy brain parenchyma. Contrarily, during postnatal development, microglia start transforming into a ramified morphology with long processes, which indicates a resting state (*Swinnen et al., 2013*).

Notably, microglia modify their characteristics according to environment and external stimuli. Consequently, they are now recognized as the most dynamic cells in the healthy, mature brain (*Tremblay, 2011; Hattori 2023*).

Indeed, microglial cells display a distinct gene expression profile that sets them apart from other CNS cells and resident tissue macrophages. Both their origin and the specific environment of the CNS in which they reside contribute to shape microglia unique identity (*Crotti et al., 2016*). Microglia are highly responsive cells which assume different phenotypes depending on intrinsic factors as species, genetics, sex, and context of health or disease as well as the specific environment they inhabit, including stage of life, CNS region location, and external determinants (such as nutrition, microbiota, pathogens)

The terms "resting" and "activated" microglia were first introduced in the literature in the mid-1970s to describe the morphological differences between cells observed in physiological ("resting") conditions and those in pathological ("activated") conditions. In the early 2000s the classification of M1 and M2 referring to pro-inflammatory and anti-inflammatory phenotype, respectively, took hold (*Michelucci et al., 2009*; *Prinz et al., 2019*) in analogy to the macrophages nomenclature (*Murray et al., 2014*). Specifically, M1 polarized microglia referred to cells characterized by enlargement of the soma, shortening of cellular processes, production of ROS and expression of pro-inflammatory cytokines like IL1β, IL6, TNFα. On the other hand, M2 state described ramified cells involved in wound repair and debris clearance and identified by markers as arginase 1 (Arg1), mannose receptor (Cluster of Differentiation 206, CD206), Chitinase-like-3 protein (Chil3/Ym1) (*Gordon 2003; Cherry et al., 2014*). These different phenotypes can also be elicited *in vitro* by exposing the cells to inflammatory stimuli such as lipopolysaccharide (LPS) or interferon gamma (IFNγ) for pro-inflammatory state and interleukin 10 (IL10) or IL4 for anti-inflammatory state. Overall, microglia activation should not be considered always a negative event but, instead, it represents an adaptive response to damage. However, these dichotomic categorizations appear as an oversimplified interpretation that do not take into account microglia complexity and heterogeneity(*Ransohoff, 2016*). Recent studies that took advantages of single cell RNA-seq attempt to identifymicroglial transcriptional signatures to describe the different microglial states. For studying microglia activation is essential to consider multiple layers of complexity such as epigenetic, transcriptomic, metabolomic, and proteomic, which collectively could integrate coexisting states as represented in Figure 2 (*Paolicelli et al., 2022*). Moreover, it must be considered that microglia assume different features as adaptation to local signals, thence for understanding microglial dynamic it is necessary to address microglial function.

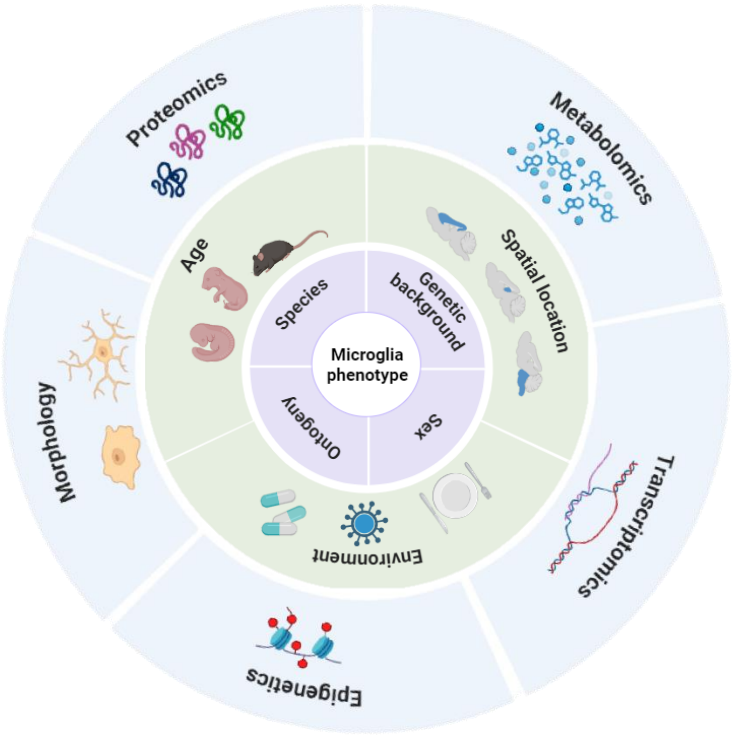



**Figure 2**. Representation of all the features which determinate microglia complexity. Intrinsic determinants (sex, ontogeny, genetic background and species) and specific context (age, spatial location and environment) collectively participate in shaping specific epigenetic, transcriptional, metalobomic, prote- omic and morphological phenotype. Figure based on *Paolicelli et al., 2022.*

Homeostatic microglia, namely microglia in physiological conditions, play a crucial role in immune surveillance of the brain parenchyma by extending and retracting highly motile processes. They are responsible for phagocytosis and elimination of extracellular materials such as microbes, dead cells, and protein aggregates. Notably, microglia have the ability to detect cells undergoing apoptosis and typically migrate to various regions of the CNS before or during the process of programmed cell death. Besides, microglia secrete various soluble factors, including chemoattractants, cytokines, and neurotrophic factors, which contribute to immune responses and tissue repair in the CNS (*Colonna & Butovsky, 2017*). During development and young adulthood microglia shape neurogenesis participating in the process of synapse pruning, through which immature synapses are permanently eliminated while others are established and strengthened (*Sierra et al., 2010; Paolicelli et al., 2011*). In this process, it is involved CX3C motif chemokine receptor 1 (CX3CR1), a chemokine receptor specific to microglia and exclusively expressed by them within the CNS that binds to the chemokine fractalkine (CX3CL1) released by damaged neurons promoting microglial phagocytosis. CX3CR1-CX3CL1 axis ensures effective pruning, which is vital for synaptic plasticityand the overall refinement of neural circuits. In fact, mice lacking CX3CR1 exhibited defects inmotor learning and synaptic plasticity in the postnatal hippocampus (*Rogers et al., 2011*). Also the complement component C1q and C4 are involved in synaptic pruning, thence some CNS

diseases associated to variation of C4, such as schizophrenia, display disturbed microglial phagocytosis (*Sekar et al., 2016*). It is indeed demonstrated that microglia cells influence functional activity of neurons and other glial cells and *vice versa* (*Baxter et al., 2021*). For instance, the release of IL1α, TNFα, and C1q by activated microglia drives astrocytes towards a neurotoxic state, which is commonly observed in the brains of individuals with neurodegenerative and neuroinflammatory conditions (*Liddelow et al., 2017*). Alternatively, astrocytes direct microglia processes to the site of injury by releasing ATP, which activates microglial P2Y12 receptor (P2RY12) (*Davaloset al., 2005*). It has been also demonstrated that microglial processes extend towards neurons when exogenous glutamate or N-Methyl-D-aspartate (NMDA) is applied. This recruitment relieson the activation of neuronal NMDA receptors, which triggers an influx of $Ca^{2+}$ ions and subsequent ATP release. (*Dissing-Olesen et al., 2014*).

Recent studies revealed that the downregulation of P2RY12 in disease-associated microglia (DAM) contribute to increase neuronal excitability and related behavioral changes (*Badimon et al., 2020*). Purinergic dependent microglial activation, namely process extension, chemotaxis, and phagocytosis, relies on ATP, which functions as “find me” or “eat me” signals and involve P2 receptors. In this context, microglial secretion of pro-inflammatory cytokines act on astrocytes via P2Y1, leading to glutamate release, which in turn binds to neuronal receptors such as mGluR receptors to modulate AMPAergic synapse (*Perea, G., & Araque 2007; Shinozaki et al., 2017*). Additionally to chemotactic mechanisms, microglia phagocytosis is also mediated through Non-inflammatory signaling via triggering receptor expressed on myeloid cells-2 (TREM2). Interestingly, TREM2 overexpression can reduce the proinflammatory capacity of microglia (*Nayak et al., 2014*). Currently, there is prevailing agreement that mammalian microglia are recognized by the expression of particular transcription factors, among which transcription factors like Pu.116, cytoplasmic markers such as ionized calcium-binding adapter molecule 1 (IBA1). In particular, the surface markers including the purinergic receptor P2YR12, transmembrane protein 119 (TMEM119), and CX3CR1 might be used to distinguish them from bone marrow–derived macrophage and monocytic subsets (*Hickman et al., 2013; Grabert et al., 2020*).

## 3.2. Microglia sex-related differences

Increasing evidence suggests that male and female microglia differ in their transcriptomic, proteomic, and morphological traits along the various brain regions throughout brain colonization, development, and function, both in healthy conditions and in disease (*Bobotis et al., 2023*). It is noteworthy to point out that sex can modify microglia postnatally, and these variations can last into adulthood. (*Bordt et al., 2020*). Microglia from female young mice that were transplanted into depleted male brains retained a gene expression profile similar to that observed in control

female mice. This suggested that sex-specific transcriptomic fingerprints seem to be intrinsically programmed (*Villa et al., 2018*). Since that microglia express steroid hormonal receptors on their membrane, it is noteworthy that sex hormones have an influence on the functions in these cells as well as in other brain cells including neurons, oligodendrocytes and astrocytes (*Liu et al., 2020*). This enables microglia to be responsive to steroid hormones in different ways and to modulate sex-specific immune responses throughout embryonic development to adulthood. (*Vegeto et al., 2001*). However, no variations were detected in the transcriptomes of whole brain microglia from male and female wild-type mice at E14.5. This suggests that the process of sex differentiation of these cells may start after E18 and extend into the postnatal period (*Hammond et al., 2019*). During microglia cells maturation, male and female brains are differently exposed to estrogens, in fact testicular production of androgens, that will be converted to estradiol by brain aromatase, occurs during embryonic days 16–18 and right after birth, while embryonic and neonatal ovaries are largely inactive, thence females experience a lack of exposure to E2. This dif- ference has been supported from studies reporting perinatal activation of estrogen receptor-α (ER-α) limited to males during early development (*Acosta et al., 2020*). Evidences reported that es- trogens, such as estradiol, significantly mitigate immune functions and reduce microglia inflam-matory potential (*Vegeto et al., 2001; Villa et al., 2016*). Interestingly, microglia are key targets of $E_2$ action, indeed treatments with this sex hormone can drive those cells towards neuroprotective phenotypes (*Nelson et al., 2017*). Notably, *in vitro* treatment with E2 was effective in suppressing LPS-induced M1-like reactive state and enhancing anti-inflammatory cytokine gene ex-pression in microglia cells (*Thakkar et al., 2018*). In addition, in vivo administration of estradiolin newborn female rats (from post-natal day 0 to 2) was able to reverse the numbers of phagocyticmicroglia to male baseline levels in few days (*Nelson et al., 2017*). Besides estrogens, also tes- tosterone has been demonstrated to reduce inflammation in microglia in males (*Shah et al., 2006*),whereas progesterone treatments have been shown to interact with microglia, reducing CNS in- flammation and preventing demyelination in young mice (*Aryanpour et al., 2017; Ghoumari et al., 2020*).

With regard to the differences in the inflammatory response between sexes, transcriptomic analysis carried out by independent research groups revealed that female microglia expressed more genes related to intercellular signaling and had a transcriptome indicative of more neuroprotective state. On the contrary, microglia from males generally showed a pronounced upregulation of genes linked to inflammatory pathways, including those involved in chemotaxis, cytokine production, and the regulation of immune responses (*Guneykaya et al., 2018; Villa et al., 2018*). This suggested that male microglia may be more prone to inflammatory response than females. Presumably, circulating estrogens in females limit the damage due to overreaction to inflammation

in the central nervous system (*Villa et al., 2015*), playing a key role in maintaining neural homeostasis.

Examining the sex-dependent variations in microglia cell number, a recent research showed that young adult male mice had more Iba1+ cells in their cortex, hippocampus, and amygdala than female mice (*Guneykaya et al., 2018*). By contrast, a different investigation demonstrated that microglial density in the somatosensory cortex of two-month-old mice was comparable in males and females (*Thion et al., 2018*). Overall, the lack of direct comparisons of densities between regions results in considerable disagreement regarding sex-related changes in microglia density. Altogether, these variations underline the possible impact of sex in shaping the immune functions of microglia. Furthermore, research indicated that these sex-related differences are likely to become more pronounced with aging (*Kang et al., 2018*), emphasizing significant implications for comprehending sex-specific susceptibility to neuroinflammatory and age-related diseases and potential therapies.

## 3.3 Microglia during aging

As the brain ages, alterations in the inflammatory state of the brain occur and the increase of neuroinflammation is sensed firstly by microglia. Microglia cells undergo the most prominent age-related changes, shifting from a neuroprotective state to a more pro-inflammatory phenotype. This transition is characterized by increased release of cytokines (IL6, IL1β, TNFα), elevated generation of ROS and reduced clearance of cellular debris, indicative of impaired phagocytosis (*Mosher & Wyss-Coray, 2014*). The aged brain produces more myelin debristhan the young brain and microglia actively participate in their degradation. However, while theload of myelin breakdown increases, insoluble lysosomal aggregates build up in the aged brain (*Safaiyan et al., 2016*). Consistently, the transmembrane glycoprotein receptor cluster of differentiation 22 (CD22) which is a negative regulator of phagocytosis, has been observed to be upregulated in microglia of aged mice and its inhibition restores phagocytosis, promote clearance of myelin as well as improves cognitive functions in old mice (*Pluvinage et al., 2019*). In the aged hippocampus, over 50% of all microglia accumulate lipid droplets in their cytoplasm and exhibit a specific transcriptome signature, leading to their classification as lipid droplet accumulating microglia (LDAM) (*Marschallinger et al., 2020*). Given that, during aging microglial cytoplasms accumulate heterogeneous material and assume an amoeboid and dystrophic morphology, characterized by enlarged cell body, diminished process motility and loss of fine branches (*Streit et al., 2004*). Due to these structural alterations, the brain parenchyma covered by individual microglial processes, defined scanning domain, is reduced, thus the immune surveillance functions are compromised (*Damani et al., 2011; Hefendehl et al., 2014*).

During aging, microglia undergo significant changes in their inflammatory profile, showing a primed phenotype (*Norden et al., 2015*), namely an increased propensity to respond more strongly to an inflammatory stimulus (*Niraula et al., 2017*). For example, the brains of elderly mice given peripheral injections of lipopolysaccharide showed a significant increase in the expression of the *Il1β* and *Il6* genes and proteins compared to young mice (*Godbout et al., 2005;Sierra et al., 2007*). Furthermore, it is reported that an immunological challenge such as an is- chemic stroke triggers microglia activation, as evidenced by a higher increase in the density of Iba1-expressing microglia in the brains of elderly mice relative to young animals (*Manwani et al., 2011*). Notably, enhanced responses following immune stimulation have been observed alsoin *in vitro* and *ex vivo*. Specifically, microglia cells isolated from aged mice showed increased basal secretion of TNFα and IL6 (*Njie et al., 2012*) and in old rats displayed a more pronounced upregulation in pro-inflammatory cytokine gene expression when challenged with LPS compared to young animals (*Frank et al., 2010*). The observation that *in vitro* microglia showed increased activation independently from the presenceof other CNS cells, pointed to intrinsic changes in their phenotype as the underlying cause. Nonetheless, it is significant to highlight that the molecular processes behind the *in vitro* aging of microglia may be distinct from those underlying the impairment of microglia in the aging brain *in vivo* (*Stojiljkovic et al., 2019*). Indeed, microglia activated by LPS displayed an upregulated NF-kB signaling, not observed in aged microglia, supporting the idea that the functional state ofreactive microglia due to aging is different from the one induced by an acute inflammatory insult(*Holtman et al., 2015*).

In microglia the acquisition of the aged profile passes through an alteration of gene transcription, as already seen when assuming other phenotypes (*Hammond et al., 2019; Sierra et al., 2007*). Microglia loss of their resting signature has been demonstrated by studies showing downregulation of genes associated with the homeostatic function, like Tmem119 and the purinergic receptor P2ry13 (*Brawek et al., 2021*). Additionally, age-induced mRNAs changes include dysregulated levels of major histocompatibility complex II (MHC II) (*Frank et al., 2006*), cell surface marker CD11c and genes responsible for phagocytic and lysosomal functions as well as antigen presentation (*Wendimu et al., 2022*). Next-generation sequencing studies have demonstrated that the transcriptional reprogramming in the aging brain involves mRNAs associated with mitochondrial function (*Raj et al., 2018*). Consistently, alterations in aged microglia include calcium homeostasis disruption due to deregulation of calcium channels and dysfunction in mitochondria and the endoplasmic reticulum (*Chandran et al., 2019*).

However, evidences suggest that aging does not lead to all microglia in the CNS adopting a primed phenotype in the same way, instead, it is more likely that microglia in sensitive regions, such as the hippocampus, undergo this switch more prominently (*Grabert et al., 2016*). Indeed,

transcriptomic studies highlighted that, across different areas in the aged brain, the white matter-resident microglia showed marked modifications (*Safaiyan et al., 2021*). In particular, murine glial cellsin white matter of corpus callosum showed the highest number of differentially expressed genes with age compared to other anatomical locations (*Allen et al., 2023*). This region-dependent vulnerability has been found even in human aged brains, where the altered expression has been observed for 5029 genes in the superior frontal gyrus compared to 1110 in the entorhinal cortex (*Lu et al., 2004*).

Moreover, it is noteworthy to mention that age-related changes occur in the brain in a sexually dimorphic manner. In accordance with that, a recent transcriptome study showed that most of the age-induced modifications in gene expression are sex-dependent (*Mangold et al., 2017*). In line with previous findings, recent functional *in vivo* researches revealed that microglia in adult male mice are more responsive than those in females are. As a result, the percentage of microglia exhibiting spontaneous $Ca^{2+}$ transients was considerably higher in males underbasal conditions (*Brawek et al., 2021; Olmedillas Del Moral et al., 2020*). Although sex variations have been described in adult microglia, it is still unknown how these differences affect brain aging (*Villa et al., 2018*). Emerging studies emphasize that microglia respond in a sex-biased manner to immunological challenges as infection or damage. And these divergent reactions might explain disparities in susceptibility to neuroinflammatory diseases and may also contribute to thedistinct rates of cognitive decline seen in males and females (*Ince et al., 2023*) as observed for depressive behavior (*Liu et al., 2019*). Consistently, microglia alterations due to aging also affectbehavioral aspects. In fact, it has been demonstrated that age-related cognitive decline is correlated with higher levels of pro-inflammatory cytokines produced by microglia (*Ritzel et al., 2015*)and that the replacement of aged microglia with young cells exerts a positive impact on synapticplasticity and cognition, restoring the expression of genes involved in neural function (*Elmore etal., 2018*). Furthermore, cognitive and memory deficits may result from a lower capability of microglia cells to provide trophic and functional support to neurons and myelin (*Wang et al., 2020*). Interestingly, expression of neuropsin (*Klk8*), an extracellular matrix serine protease involved in promoting neurite outgrowth and modulating Schaffer-collateral LTP (*Tamura et al., 2006*) was notably increased in microglia during aging, indicating that these cells might contribute significantly to LTP formation and synaptic plasticity (*Pan et al., 2020*). Taken together, the compromised microglia homeostatic features and functions can contribute to ageing process and converge on sustaining inflammaging status in the brain.

# 4. Extracellular vesicles (EVs)

The history of extracellular vesicles discovery began with the studies of blood coagulation. Pioneering experiments with platelets conducted by Chargaff in 1945 highlighted the presence of "material in minute particulate form" with clotting function (*Chargaff E, West R., 1946*), later referred to as "platelet dust" (*Wolf, 1967*).

The term "extracellular vesicles" (EVs) was coined by Aaronson in 1971 studying *Ochromonas danica*, a flagellated alga. He observed membrane-bounded structures budding from cells suggesting that those vesicular structures could originate not uniquely from mammals cells, paving the way for further studies on EVs release in other organisms (*Aaronson et al., 1971*).

In the late 80s, Johnstone and collaborators observed that maturing reticulocytes exploited EVs to discard transferrin receptors they no longer needed. Therefore, extracellular vesicles were initially thought to be "cellular garbage", namely a system through which cells shed waste or unwanted material (*Johnstone et al., 1987*). In the following decade, many experiments revealed that these vesicles were not just debris but biological entities with enzymatic and functional potential as well as physical and biochemical characteristics. In this regard, the most groundbreaking discovery was Raposo *et al*'s demonstration that EVs from immune cells were capable of inducing antigen specific T-cell responses (*Raposo et al., 1996*), suggesting EVs had the potential to be harnessed as anti-tumoral vaccines. From that moment on, EVs started to emerge for their functional roles in biological processes and as important mediators of intercellular communication.

Accordingly to the guidelines of International Society for Extracellular Vesicles (ISEV), "extracellular vesicles" or "EVs" is the consensus generic term indicating a heterogeneous group of lipid bilayer membrane-delimited structures released presumably by all types of organisms and cells into the extracellular space (*Théry et al., 2018*).

After their secretion, EVs can reach distant tissue or remain near the site of secretion to promote autocrine or paracrine signaling (*van Niel et al., 2022*). EV-mediated signals can be conveyed by a plethora of different cargoes, among which membrane proteins, cytosolic proteins, lipids and genetic material such as mRNAs and non-coding RNAs, such as long non-codingRNAs (lncRNAs) and microRNAs (miRNAs).

EVs release has been documented for a variety of cells and tissues to regulate a multitude of physiological functions spanning from embryonic development (*de Ávila et al., 2019*) to immune response, inflammation, cell proliferation, tissue homeostasis and repair (*Yáñez-Mó et al., 2015*). Nevertheless, EVs are also implicated in pathological processes including neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS), Parkinson's and Alzheimer disease

(*Thompson et al., 2016*). Since EVs have been isolated from blood, saliva, breast milk, urine, cerebrospinal fluid and other biological fluids, growing interest has been expressed in their use as sources of biomarkers to follow the progression of pathological states or for providing early diagnosis (*Couch et al., 2021*).

Given their ability to carry a broad variety of biological molecules through different biofluids, EVs are thought to represent promising novel therapeutic tools and drug delivery systems. This, together with the above mentioned properties and applications of EVs, led to the explosion of interest in EVs in the early 21st century.

## 4.1. Classification and biogenesis

The definition "extracellular vesicles" covers many types of membranous particles such as exosomes, microvesicles, ectosomes, oncosomes, and apoptotic bodies. Based on their biological origin and role, they can be broadly divided into microvesicles and exosomes, as depicted in Figure 3 (*Raposo et al., 2013*).

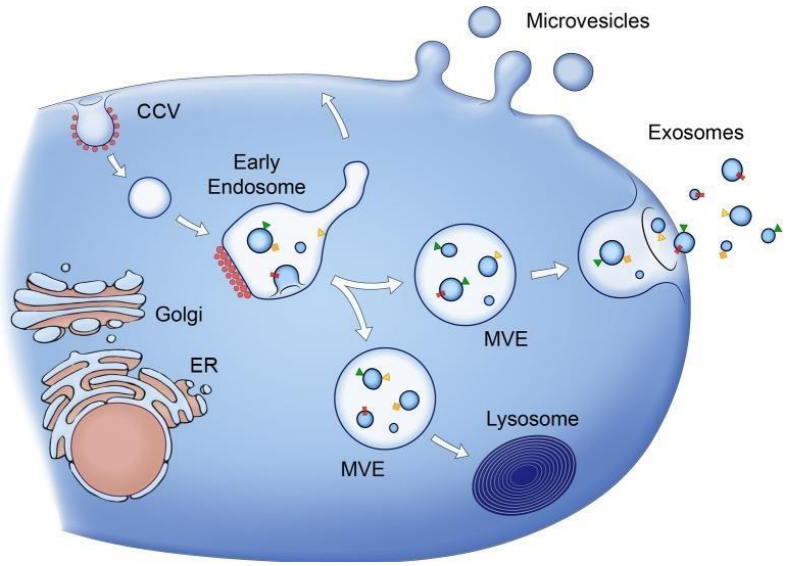



**Figure 3.** Schematic representation of the different populations of extracellular vesicles. Microvesicles originates from direct budding from the plasma membrane, while exosome from fusion of internal multivesicular endosome with the plasma membrane. CCV (clathrin-coated vesicles). Figure from Raposo & Stoorvogel, 2013. "Extracellular vesicles: exosomes, microvesicles, and friends". Copyright © 2024 Rockefeller University Press.

Microvesicles (50–1000 nm) are generated by direct budding from the plasma membrane (*Colombo et al., 2014*), as well as the apoptotic bodies, a subtype of EVs secreted by dying cells (Fig. 3). This biogenesis relies on the generation of plasma membrane microdomain and then budding and fission of it to generate a vesicle. Molecular rearrangements of lipid and cytoskeletal protein composition are required to achieve physical bending of the membrane, favouring membrane blebbing and release of EVs. (*Trajkovic et al., 2008*). An additional factor that concurs to mi-

crovesicles formation is $Ca^{2+}$ concentration. In fact, altered level of $Ca^{2+}$ activates aminophospholipid translocases (flippases and floppases), scramblases and calpain, leading to rearrangements in the asymmetry of membrane phospholipids. (*Suzuki et al., 2010*).

The flipping of specific lipid species, indeed, between the layers of the shedding membrane has been reported as a unique characteristic of microvesicles budding (*van Niel et al., 2018).*

Further to the mentioned mechanisms, molecular factors participate in microvesicles biogenesis as well. Among the molecular machineries involved in this process there is the Endosomal Sorting Complex Required for Transport (ESCRT), which favors the neck region formation, fission of the bud and is disintegrated after the vesicle is extruded, as illustrated in Figure 4A (*Babst M., 2011*). ESCRT complex iscomposed by approximately twenty proteins that assemble into four complexes (ESCRT-0, -I, - II and -III) associated with highly conserved proteins as Vacuolar Protein Sorting 4 (VPS4), Ves-icle Trafficking 1 (VTA1), ALG-2-interacting protein X (ALIX) (*Henne et al., 2011*).

The ESCRT-0 complex recognizes ubiquitylated proteins, whereas the ESCRT-I and -II complexes account for membrane deformation into buds with sequestered cargo, and ESCRT-III components finally drive vesicle scission (*Hurley & Hanson, 2010*).

It is reported that the tumor susceptibility protein 101 (TSG101) of ESCRT-I is relocated from endosomal membranes to the plasma membrane through interaction with the arrestin domain-containing protein 1 (ARRDC1), where it participates in production of EVs called “ARMMs”, ARRDC1-mediated microvesicles (*Nabhan et al., 2012*).

Interestingly, the ESCRT complexes involved in the various stages of extracellular vesicle biogenesis are partially shared between microvesicles and exosomes.

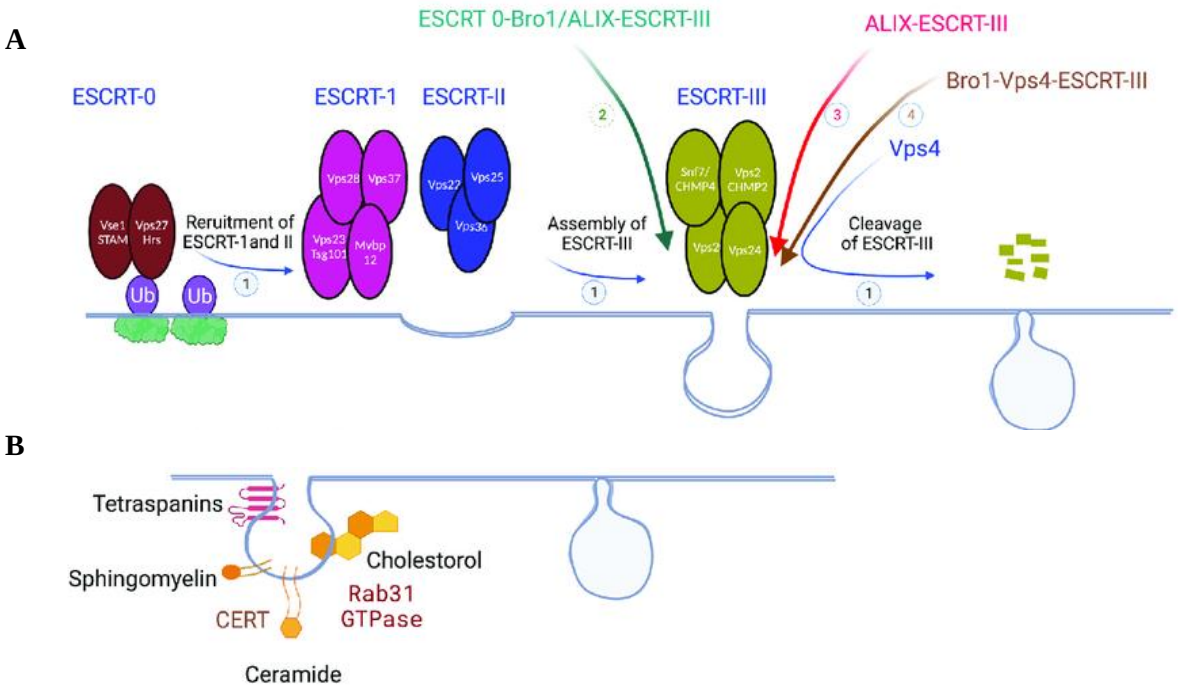


**Figure 4.** Rapresentation of EVs biogenesis pathways. (A) ESCRT-dependent EVs biogenesis 1. Classic pathways from ESCRT 0-III-Vps4. 2. ESCRT 0-Bro1/ALIX-ESCRT III. 3. ALIX-ESCRT III. 4. Bro1-Vps4-ESCRT III. (B) ESCRT-independent EVs biogenesis. Figure from Krylova & Feng (2023). "The Machinery of Exosomes: Biogenesis, Release, and Uptake." Copyright: © 2023 by the authors.

The first to use the term 'exosome' was Rose Johnstone to describe vesicles released following the fusion of multivesicular bodies (MVBs) with the plasma membrane (*Johnstone et al., 1987*). Indeed, exosomes are generated within the cell through the endocytic pathway, where inward budding of the endosomal membrane leads to the formation of tiny vesicular structures (30–200 nm) inside the endosome lumen. The endocytic pathway involves dynamic membrane compartments that internalize extracellular ligands and cellular components. In fact, extracellular proteins are either recycled to theplasma membrane or degraded. Degradation begins with early endosomes maturing into late en-dosomes, which accumulate intraluminal vesicles (ILVs), resulting in structures known as MVBs.Typically, MVBs fuse with lysosomes for content degradation, but they can also merge with theplasma membrane, releasing their contents into the extracellular space (*Raposo & Stoorvogel, 2013*).

Similarly to microvesicles biogenesis, the ESCRT-0 complex coordinates early steps in ILV formation and recruits clathrin to the early endosome membrane through the Hrs subunit, meanwhile the ESCRT-I and -II complexes orchestrate the curving process of the endosomal membrane to form an ILV. ESCRT-III is responsible for the fission events and disassembly of the complex through the AAA ATPase VPS4. The block of the VPS4 activity disrupts the release of both exosomes and microvesicles (*Jackson et al., 2017*). Consistently with these functions, ESCRT components, among which TSG101 and ALIX, are frequently found as proteins within exosomes (*Lischnig et al., 2022*).

Nevertheless, simultaneous depletion of ESCRT subunits from all four ESCRT complexes does not completely prevent MVB formation, suggesting that alternative mechanisms may contribute to the formation of ILVs and exosomes (*Colombo et al., 2013*). For instance, ceramide and other complex lipids can cluster together to create raft-like domains which help initiate the membrane bending required for inward budding and the generation of ILVs (Figure 4B). Indeed, the absence of sphingomyelinase, the enzyme that converts sphingolipids into ceramide, hinders the exosomal releaseof Aβ-peptides in neurons (*Yuyama et al., 2012*). Furthermore, additional factors important in thedynamics of exosome generation are the tetraspanins (CD9, CD81, CD63), a family of transmem-brane proteins enriched in various subtypes of extracellular vesicles. Specifically, CD63 is widelyrecognized as a classical exosome marker due to its predominant localization within intracellularendolysosomal compartments and its incorporation into ILVs destined to become exosomes. In contrast, CD9 and CD81 are primarily found at the plasma membrane but also associate with lateendosomes and are frequently identified alongside CD63 in MVB-derived extracellular

vesicles.(*Mathieu et al., 2021*).

These proteins gather into clusters and dynamic membrane platforms, interacting with other tetraspanins as well as various transmembrane and cytosolic proteins. These tetraspanin-enriched domains have been proposed to function as sorting machineries participating in exosome biogenesis and composition (*Perez-Hernandez et al., 2013*). However, growing evidence indicates that tetraspanin-dependent exosome biogenesis requires other regulators, such as ALIX or syntenin (*Wei et al., 2021*). In sum, the mechanisms concurring in EVs biogenesis are multiple and many are still being revealed.

Such complexity is reflected in the heterogeneity between EV populations, which may display overlapping sizes, and the lack of clear agreement on specific proteins that uniquely define each EV subtype. This variability, together with the absence of methods that can separate specifically the different subclass of EVs, have greatly hindered their classification at the subtype level.

Consequently, controversies about EV nomenclature has sparked debate over time, and many scientists do not agree with the current naming guidelines. (*Witwer & Théry, 2019*).

Current EV isolation methods enable classification based on size, density, or surface antigens, but they do not differentiate EVs by their origin of biogenesis; this limitation prevents the clear identification of EVs as exosomes or microvesicles (*Théry et al. 2018*). Therefore, MISEV 2023 discourages nomenclature based on biogenesis unless such an EV population is distinctly isolated and thoroughly characterized (*Welsh et al., 2024*).

The International Society for Extracellular Vesicles proposes a classification based on size of the vesicles, distinguishing small extracellular vesicles extracellular vesicles, sEVs (<200 nm in diameter) from other medium and larger vesicles, m/l EVs (>200 nm) (*Théry et al. 2018*).

However, the terms ‘sEV’ and ‘exosome’ should not be used as synonymous: small EV populations, in fact, include both small microvesicles and exosomes. Moreover, small EVs (above all that <100 nm in diameter) can eventually originate from the plasma membrane and larger ILVs have been observed in MVBs, which could give rise to larger exosomes. This further complicates the relationship between EV size and biogenesis (*Russell et al., 2019*).

For the reasons above, EVs classification results still challenging and most of the existing ‘exosome’ and ‘microvesicle’ literature refers to a broad population of EVs.

## 4.2. Cargo, release and interaction with target cells

The EVs content varies significantly with respect to the type of origin cell, mode of biogenesis and physiological or pathological state of the donor cell (*Minciacchi et al., 2015*). This has resulted in the creation of public on-line databases as “EVpedia”, where information about EV cargoes is provided (*Kim et al., 2013*).

At the basis of the cell-to-cell communication through EVs lies the idea that cells possess the capability to carefully regulate the loading of specific cargo within extracellular vesicles in order to convey messages made of proteins, lipids, and nucleic acids (Fig. 5). For instance, the positioning of cargo on the plasma membrane rather than the endosome is likely a key factor in determining whether the cargo will be incorporated into a microvesicles or an endosome (*Dixson et al., 2023*). Proteins particularly abundant in EVs, that are often used as markers, are those related to biogenesis such as tetraspanins, ALIX, TSG101, syntenin, and the major histocompatibility complex class I (MHC I) (*Abels et al., 2016*).

The GTPases also play a role in sorting cargoes as well: the small GTPase RAS-related protein RAB22A colocalizes with budding microvesicles and mediates loading of cargo proteins in cancer cells (*Wang et al., 2014*). Similarly, the GTPase ADP-ribosylation factor 6 (ARF6) was identified as a regulator of selective recruitment of proteins, including β1 integrin receptors, MHC class I molecules, membrane type 1-matrix metalloproteinase 1 (MT1-MMP) and the vesicular SNARE (v-SNARE) vesicle-associated membrane protein 3 (VAMP3), into tumoral cells (*Muralidharan-Chari et al., 2009*). Apart from biogenesis related proteins, EVs are loadedwith proteins that undergo post-translational modification (PTMs) like ubiquitination, SUMOylation and neddylation; PTMs play essential part in protein cargo sorting via cooperating with ESCRT complex. (*Ageta et al., 2018*; *Chen et al., 2021*).

Besides proteins, extracellular vesicles also carry nucleic acids, including DNA sequences and RNAs both mRNAs and non-coding RNAs, such as miRNAs, lncRNA and circular RNA (circRNAs).

Incorporation of miRNAs into EVs has been shown to be regulated by heterogeneous nuclear ribonucleoproteins (hnRNPs), which are involved in RNA trafficking and function. Among these, hnRNP A2B1 recognizes the EXOmotif (GGAG tetranucleotide) in miRNAs, controlling their packing into EVs (*Villarroya-Beltri et al., 2013*).

Other machineries responsible for nucleic acidsorting include ESCRT-II subcomplex that could act as an RNA-binding complex, the miRNA- induced silencing complex (RISC) and protein Argonaute 2 (AGO2), the synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP) and the KRAS–MEK signalling pathway (*Rädler et al., 2023*). Concerning mRNAs loading into EVs, evidences suggest that specific se- quences within the 3’ UTR are associated with this process; these sequences may act as a zipcodemotif that targets mRNAs into EVs (*Bolukbasi et al., 2012*). However, the molecular mechanismsof RNAs loading into extracellular vesicles remain to be fully clarified.

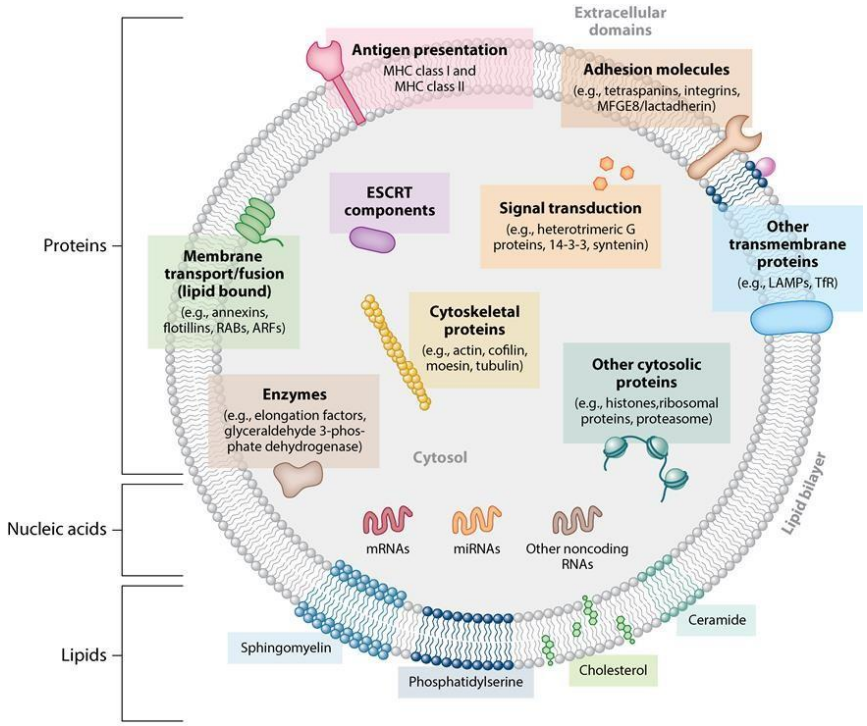



**Figure 5.** Overall composition of extracellular vesicles. Taken from "*Colombo, Raposo and Théry, Ann Rev Cell Dev Biol 2014*". Copyright © 2014 by Annual Reviews.

Regarding EVs secretion, Rab GTPases, the largest family within the Ras superfamily of GTPases, are essential for intracellular vesicle transport interacting with SNARE proteins to induce EVs membrane fusion and secretion (*Pegtel et al., 2019*). Similarly, the small GTPase RhoA with its downstream targets ROCK regulates release of large vesicles by modulating actin cytoskeletal rearrangements through RhoA-cofilin pathway (*Li et al., 2012*).

Once released, the interaction of tetraspanins and integrins with extracellular matrix proteins, likely fibronectin and laminin, has been demonstrated to have an impact on exosome and microvesicles binding to recipient cells (*Purushothaman et al., 2016*). The internalization of extracellular vesicles can occur through different pathways, influenced by the specific cell type and its physiological condition, as well as the interaction between ligands on the EV surface and receptors on the cell membrane. The outcome of extracellular vesicles may also rely on specific structures found on the plasma membrane of the target cell. For instance, lipid raft-like domains,together with integrins and adhesion molecules on the EVs surface, facilitate interaction, attachment, and membrane fusion with target cells. In fact, studies have shown that disrupting lipid rafts by depleting cholesterol decreases the uptake of extracellular vesicles. (*Plebanek et al., 2015*).

Other mechanisms capable to drive extracellular vesicles towards sites of uptake include clathrin-mediated or clathrin-independent endocytosis, phagocytosis and micropinocytosis and filopodia structures (*Emam et al., 2018; Heusermann et al., 2018*).

After being internalized, extracellular vesicles enter the endocytic pathway and typically reach

MVBs, which are generally directed to the lysosome. Alternatively, the vesicles can evade degradation by fusing back with the limiting membrane of the multivesicular endosome (MVE), thereby releasing their contentsinto the cytoplasm of the recipient cell (*van Niel et al., 2018).*

## 4.3. EVs released by microglia and their role in neuroinflammation

As other all cell types, also cell populations of the central nervous system release extracellular vesicles, including astrocytes (*Upadhya et al., 2020*), neurons (*Men et al., 2019*) and microglia (*Verderio et al., 2012*), both *in vitro* and *in vivo*. EVs in the brain favor intercellular interactions between neurons and astroglial cells, playing a pivotal role in preserving CNS homeostasis. For example, exosomes released by mouse primary cortical neuron cultures are uptaken by astrocytes and induce an upregulation of excitatory amino acid transporter 2 (EAAT2), an important mediator of glutamate uptake in the brain (*Morel et al., 2013*). Interestingly, EV release by oligodendrocytes have been demonstrated to support axonal maintenance and mediate neuroprotective function by sustaining neuronal homeostasis (*Frühbeis et al., 2020*; *Krämer-Albers & Werner, 2023*). With regard to brain homeostasis, studies point out that microglia derived microvesicles can also directly target neurons, thus regulating synaptic plasticity and neuronal excitability (*Prada et al., 2013; Antonucci et al., 2012*). Studies have identified microglia-derived EVs as vehicles for the transport of hydrophobic compounds like endocanabinoids (eCB), which stimulate type-1 cannabinoid (CB1) receptor on target GABA neurons, suppressing spontaneous inhibitory transmission (*Gabrielli et al., 2015*). Additionally, *in vitro* studies demonstrated that microglial EVs are capable to enhance glutamatergic transmission contributing to alteration of excitation/inhibition balance (*Antonucci et al., 2012*).

Microglia-derived EVs, consistently with the immune surveillance role acted by the donor cells, are key regulators of many processes and mediators of neuroimmune crosstalk. (*Ruan et al., 2021*). These interactions through EVs are crucial both in CNS physiology and during neuroinflammatory pathogenetic processes. Indeed, pro-inflammatory cytokines like IL1β, IFNγ, TNFα, and caspase 1 can be conveyed by EVs, thereby enhancing an inflammatory microenvironment (*Marostica et al., 2021*).

Therefore, extracellular vesicles can promote either disease progression or tissue repair in CNS insults (*Budnik et al., 2016*). Specifically, microglia-derived EVs can exert both detrimental and protective roles during inflammatory demyelinating diseases as multiple sclerosis. Reactive microglia, in fact, release EVs enriched in proinflammatory cytokines such as IL1β and microRNAs that are associated with synaptic defects and a block of remyelination. On the contrary, EVs produced by pro-regenerative microglia are capable to promote myelin repair (*Lombardi et al., 2019*). Similarly, in neurodegenerative diseases, whose onset is strictly related to insoluble

misfolded protein aggregation, EVs have been proposed to facilitate the propagation of those proteins, spreading neuroinflammation (*Coleman et al., 2015*). In fact, in Alzheimer's disease, EVs mediate neuron-to-neuron propagation of Aß amyloids, contributing to neuronal loss(*Sardar Sinha et al., 2018*) and those released from microglia spread Aβ and tau. By blocking microglial exosome production, the amyloid plaque formation and tau propagation decrease (*Dinkins et al., 2014; Asai et al., 2015).* Notably, EVs derived from microglia were suggested tocontribute to the spread of inflammation, since their levels were found to be elevated in CSF during inflammatory conditions (*Verderio et al., 2012*). An enrichment in Il1β, superoxide dismutase 2 (Sod2) and immunoresponsive gene 1 (Irg1) mRNAs levels was observed in EVs secreted by pro-inflammatory macrophages, while Arg1 and Ym1 were upregulated in m/lEVs originating from anti-inflammatory cells, corroborating the idea that microglia-derived EVs reflect in their cargo the inflammatory state of original cells (*Garzetti et al., 2014*). In brain tumor conditions, EVs from LPS/IFNγ treated microglia injected in the brain of glioma-bearing mice showed neuroprotective effect, remodulating microglia inflammatory state and reducing tumor size (*Grimaldi et al., 2019; Serpeet al., 2021*). On the contrary, mice systemically administred with EVs from IL4 treated microglia showed restored white matter repair and neurological functionality after induced cerebral ischemia (*Li et al., 2022*). Another mechanism through which EVs amplify inflammatory reactions is by carrying factors that boost EVs secretion in recipient cells. Studies reported that ATP represents a further signal that stimulates the production of EVs in microglia, through the activation of the ionotropic ATP receptor P2X7. Indeed, reactive microglia were reported to release EVs bringing the pro-inflammatory cytokine IL1β, the enzyme caspase-1, and the P2X7 receptor (*Bianco et al., 2005*; *Peña-Altamira et al., 2018*).

## 4.4. EVs application: diagnostic and therapeutic tools

Currently, it is well established that EVs play functional roles in several biological processes. This suggested that they could be used as biomarkers for pathological conditions. Notably, since EVs are detectable in all body fluids, liquid biopsy currently represents a minimally invasive technique for disease diagnosis and prognosis. Alteration in EVs content or presence of specific molecular signatures are used as predictive markers in lung diseases (*Afzal et al., 2023*), renal diseases (*Feng et al., 2018*), hepatic diseases (*Lapitz et al., 2018*) and cancers (*Yu et al., 2022*). For example, in IgA nephropathy, the chemokine (C-C motif) ligand 2 (CCL2) mRNA was exclusively upregulated in the urinary exosomes from patients compared to healthy controls (*Feng et al., 2018*). Likewise, three miRNAs, namely miR-142p, miR-33a-5p, and let-7d-5p, were dysregulated in sputum exosomes from idiopathic pulmonary fibrosis (IPF) patients in pulmonary fibrosis diseases (*Njock et al., 2019*).

Concerning tumors, recent studies highlighted the relevance of omics-based approaches in individuating proteins and miRNAs associated with specific human cancers in plasma-derived EVs (*Zhou et al., 2021*). For instance, in serum-derived EVs from patients with hepatocellular carcinoma (HCC) were detected upregulated levels of miRs 18a, 221, 222, and 224 (*Sohn et al., 2015*). Similarly, proteomic studies on plasma-derived EVs identified the carbonic anhydrase 2 (CA2), lactoferrin (LTF) and the complement decay-accelerating factor 55 (CD55) particularly enriched in pancreatic cancer patients, while the rho-related GTP-binding protein RhoV (RHOV) was found to be significantly elevated in plasma-derived EVs from lung cancer patients (*Hoshino et al., 2020)*.

Of particular interest is the use of EVs biomarkers for risk assessment of onset and progression of aging-related disease such as osteoarthritis and osteopenia, cardiovascular disease, type 2 diabetes mellitus and neurodegenerative diseases (*Yin et al., 2021; Quiroz-Baez et al., 2020*). For instance, in a model of Parkinson's, α-synuclein detection in sEVs was higher than in controls *Cao et al., 2019*). In AD patients, proteases (α-, β- and γ- secretases) linked to Aβ biogenesis were found in plasma-derived EVs (*Lee et al., 2022*), while tau containing EVs were found in CSF (*Crotti et al., 2019*). Additionally, in EVs derived from serum or plasma of AD patients many miRNAs, such as miR-223, miR-193b and miR-135, have been detected and used to discriminate AD patients from healthy controls (*Vandendriessche et al., 2022*).

Key features of EVs such as minimal immunogenicity (*Zhu et al., 2017*), due to their biocompatibility, the bi-layered and small lipid structure, which protects the genetic cargo from degradation and their capacity to cross biological barriers, make EVs appealing as therapeutic vectors. Nevertheless, it is fundamental to take into account that extensive reorganization of the surface structure as well as engineering methods used for the loading of exogenous cargo, production and storage can impact EVs immunogenicity (*Sun et al., 2023; Xia et al., 2024*).

The therapeutic use of EVs led to the explosion of interest in EVs in the early 21st century, in fact from 2000 to 2020 more than 500 patents were filed in US for the utilization of EVs (*Couch et al., 2021*) and over 300 EV-based clinical trials have been initiated (*Claridge et al., 2021*). In clinical therapy, the first EVs used were those released from mesenchymal stromal cell (MSC), a multipotent cellcapable of differentiating into various cell types. MSCs-derived EVs offer therapeutic benefits and support tissue regeneration when sourced from tissues like bone marrow, adipose tissue, andumbilical cord (*Aguiar Koga et al., 2023*). Recent evidences showed that neonatal human umbilical cord MSCs (UC-EVs) achieved rejuvenation of senescing adult bone marrow–derived MSCs(AB-MSCs). UC-EVs ameliorated bone loss and slowed aging-related tissue degeneration in aged mice, collectively alleviating aging phenotypes of AB-MSCs (*Lei et al., 2021*). In AD, cytokine-preconditioned MSCs produce neuroprotective EVs, which show antiaging and

anti-inflammatory capacity after intranasal delivery (*Losurdo et al., 2020*). Since the cargo of EVs differs between young and aged cells, the beneficial effect of EVs is strictly influenced by age of the donor (*Sanz-Ros et al., 2022*).

In aged rats, the intravenous administration of EVs derived from cerebral endothelial cells of adult rats increased the differentiation of neural stem cells into neuroblasts, through the increase of miR-1 and miR-146a expression (*Zhang et al., 2022*). Similarly, EVs isolated from young mouse plasma and intraperitoneally injected in aged mice enhance the performances of motor activity (*Yoshida et al., 2019; Sahu et al., 2021*).

The extensive potential of MSCs led to the development of membrane-based nanoparticles termed "nanoghost" (NG). In nanoghosts, MSCs cytoplasmic membranes are isolated to obtain a membranous "ghost" for drug delivery, which retains MSCs surface properties avoiding possible immune activation (*Xiao et al., 2024*). NGs showed to have important immunomodulatory capacity in several diseases included cancers, as well as in regeneration process (*D'Atri et al., 2021*).

# 5. Aim of the study

Inflammaging is a complex event, which has been demonstrated to impact brain homeostasis mainly through microglia hyper-activation. Over time, these cells, (the immune "sentinel" cells of the brain), are affected by major changes in their morphology and inflammatory profile, sustaining a low-grade inflammatory state in the brain. This results in disruption of healthy neuro-glial interaction, which ultimately lead to cognitive deficits. Brain aging occurs differently in males and females, pointing out sex-related disparities in both physiological and pathological responses. Sustained chronic inflammation raises the risk of developing age-associated pathologies such as type 2 diabetes or cardiovascular disease, as well as neurodegenerative diseases.

Many rejuvenating strategies have been proposed, leveraging cell reprogramming, transplantation and young blood factors. The use of extracellular vesicles would suggest a new non-invasive approach to attenuate brain chronic inflammation, as a possible therapy in neuroinflammation related diseases. Indeed, the capacity of EVs to transfer bioactive molecules across the BBB make them suitable for delivering therapeutic agents to the brain.

The interest of this study was aimed at understanding the processes of brain aging in relation to sex, and the search for innovative strategies to slow down inflammaging. In particular, the research focused on the effects of EVs derived from untreated murine microglia-like BV2 cells (untreated-EVs), LPS/INFγ-treated BV2 (LPS/INFγ-EVs) or from IL4-treated BV2 cells (IL4-EVs) or vehicle intranasally administered to late adult (16–20 months of age) male and female mice. Brain inflammation was evaluated in microglial cells in terms of inflammation-related gene expression and morphology; neuronal plasticity was investigated and behavioral tests were performed to evaluate memory and anxiety-like behaviour.

# 6. Materials and Methods

## 6.1. Cell line

BV2 murine microglia-like cells were cultured in DMEM supplemented with heat-inactivated 10% FBS, 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B and grown at 37°C in a 5% $CO_2$, humidified atmosphere.

## 6.2. BV2 cells stimulation

BV2 cells were treated with LPS from *Escherichia coli* O111:B4 (100 ng/mL, Sigma-Aldrich) and recombinant murine IFNγ (20 ng/mL, ImmunoTools GmbH) to induce a pro-inflammatory state or treated with recombinant murine IL-4 (20 ng/mL, PeproTech) to induce an anti-inflammatory state. Untreated microglia- like BV2 cells receive vehicle. All stimulations were carried out for 24 h in EVs-free fraction medium.

## 6.3. Extraction of EVs

BV2 supernatant was collected and centrifuged at 1000 × g for 5 min to remove cell debris. Then, the supernatant was centrifuged at 10000 × g for 30 min at 4°C. The resulting pellets, containing medium/large EVs, extracted from untreated cells (untreated-EVs), from LPS/INFγ-treated BV2 (LPS/INFγ-EVs) or from IL4-treated BV2 (IL4-EVs), were re-suspended in a 0.22 μm filtered PBS (Thermo Fisher Scientific, Waltham, MA, USA). The media used for cell culture were submitted to the above reported steps of centrifugation and supernatants were collected and used as control for *in vivo* administration (vehicle). For tracing and quantification experiments, the same amount of EVs were used and EVs were labelled with PKH26 or PKH67, a lipophilic membrane dye (Sigma Aldrich, Milan, Italy), according to manufacturer protocol. Briefly, PKH26 dye solution ($40^{-6}$ M) was mixed 1:1 with EVs for 5 min at RT. Excess dye was bound with 2 mL EVs-depleted FBS and the EV-containing pellet was washed twice in PBS. PKH26-stained EVs were re-suspended in PBS for *in vivo* ad- ministration.

## 6.4. Nanoparticle tracking analysis (NTA)

The dimension and concentration of EVs were determined using the Nanosight NS300 (Malvern Panalytical, Malvern, UK) technology, both in the scatter and fluorescence mode (488-nm laser). The samples were diluted in PBS to a final volume of 1 mL to obtain a range of 20-100 particles per frame. For each sample, 5 movies of 60 s were recorded. Videos were analysed by the integrated NanoSight Software NTA 3.4 Dev Build 3.4.4. The camera type, camera level and detection threshold were sCMOS, 14 and 4, respectively EVs size distribution is reported as percentage

of EVs below a specific size as D10, D50 and D90.

## 6.5. Western blot

BV2 cells and BV2-derived EVs were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton, 0.1% SDS, 1% deoxycholate, 2 mM EDTA, pH 7.5) containing a protease and phosphatase inhibitor cocktail (Sigma Aldrich, Milan, Italy). Protein samples were quantified and the same amount was separated by 12%SDS-polyacrylamide gel electrophoresis (20 µg/lane) and the nitrocellulose membranes (Amer- sham Protran, Sigma-Aldrich, Milan, Italy) were probed with the following primary antibodies: rabbit anti-mouse CD81 (1:500; Cell Signaling, Danvers, MA, USA, Cat# 10037), CD9 recombinant rabbit monoclonal antibody (1:500; Invitrogen, Waltham, MA, USA, Cat# MA5-31980), mouse monoclonal GAPDH (1:1000; Merck, Darmstadt, Germany, Cat# MAB374). HRP-tagged goat anti-rabbit or anti- mouse IgG were used as secondary antibodies (1:1000; Dako, Cernusco sul Naviglio, Italy). The detection was performed through the chemiluminescence assay with an Amersham ECL Prime Western Blotting Detection reagent (Cytiva, Little Chalfont Buckinghamshire, UK). Densitometric analysis was carried out with the Quantity One software (version 4.6.6, Bio-Rad, Hercules, CA, USA).

## 6.6. Animals

Young (3–6 months) and late adult (16–20 months) male and female C57BL/6J mice were used. Four to six animals per cage were housed under standard environmental conditions (room temperature, 20–22 ◦C; relative humidity, 50–55 %; 12 h artificial light–dark cycle with the light phase starting at 6 a.m.) with food and water *ad libitum* and basic
environmental enrichment. All procedures were conducted in accordance with the European Union legislation (Directive 2010/63/EU) and were approved by the Italian Ministry of Health (number 664/2021).The well-being of the animals was continuously monitored.

## 6.7. *In vivo* EVs intranasal administration

Young and late adult mice were anesthetized with isoflurane while the animals maintained spontaneous breathing. To enhance the permeability of the nasal mucous membrane, mouse nostrils were washed with 10 mg/mL hyaluronidase (4 µL per nostril; Sigma Aldrich, Milan, Italy; *Danielyan et al. 2009*). Thirty minutes later, late adult mice were treated with vehicle, untreated-EVs, LPS/INFγ-EVs or IL4-EVs re-suspended in sterile PBS in 6 µL spurts via a micropipette. Young animals were treated with vehicle. Each mouse received 1.4 x $10^{11}$ EVs (6 µL per nostril; the second 7 days after the first) as in *Grimaldi et al.2019*.

Brain tissue and CSF were isolated 1 h after PKH67-EVs administration for EV quantifications. To isolate EVs (*Crescitelli, et al. 2021*), the brain tissue was cut in small pieces in DMEM with

20 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA), incubated in DMEM with 40 U/ml DNasi I (Thermo Fischer Scientific, Milan, Italy) and 2 mg/ml collagenase D (Merck, Darmstadt, Germany) under mild agitation (30 rpm) for 30 min at 37 °C and then filtered by 70 μm cell strainer (ClearLine MD, Woburn, MA, USA). The medium was centrifuged at 4°C as follows: 300 x g for 10 min; 2.000 x g for 20 min; 16500 x g for 20 min. The EVs-containing pellet was resuspended in PBS and processed for NTA in fluorescence mode. CSF was collected as reported (*Šakić et al 2018*). Briefly, mice were anesthetized and perfused with ice-cold PBS. A sagittal incision was made with a scalpel between the skull base and atlas; subcutaneous tissue and neck muscles were separated to expose the dura mater. A glass capillary was tapped through the dura to aspire CSF from the cisterna magna. The collected sample was centrifuged at 4°C for 15 min at 2000 x g to remove blood contamination. Five to 10 ml of CSF per mouse were obtained. Equal volumes of CSF from 4 animals were pooled, EVs isolated and analysed.

## 6.8. Real time PCR (RT-PCR)

Twenty-four hours or 14 days (for animals whose behaviour was tested) after the second treatment, mice were perfused with ice-cold PBS, brains were isolated and $CD11b^+$ cells were collected by magnetic cell separation (Miltenyi Biotec, Bologna, Italy) as already described (*Grimaldi et al., 2016*). The cells were lysed in Trizol reagent (Invitrogen, Milan, Italy) for RNA extraction, RNAs were quantified and retro-transcribed using IScriptTM Reverse Transcription Supermix (Bio-Rad, Hercules, California, USA). RT-PCR was carried out in an I-Cycler IQ Multicolor RT-PCR Detection System (Bio-Rad, Hercules, California, USA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, California, USA). The PCR protocol consisted of 40 cycles of denaturation at 95°C for 30 s and annealing/extension at 58°C for 30 s. The Ct values from each gene were normalized to the Ct value of *Gapdh*. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method and expressed as fold increase. Primer sequences for mRNA analysis are:

*Il6* NM_001314054.1 forward GATGGATGCTAC-
CAAACTGGAreverse: TCTGAAGGACTCTGGCTTTG;

*Il1β* NM_008361.4 forward: CAACCAACAAGTGATATTCTCCATG
reverse: GATCCACACTCTCCAGCTGCA

*Tnfα* NM_013693.3 forward: GTGGAACTGGCAGAAGAG
reverse: CCATAGAACTGATGAGAGG

*Cd86* NM_019388.3 forward: AGAACTTACGGAAGCACCCA
reverse: GGCAGATATGCAGTCCCATT

*Cd163* NM_001170395.1 forward: GCTAGACGAAGTCATCTGCACTGGG

reverse: TCAGCCTCAGAGACATGAACTCGG

*Cd206* NM_008625.2 forward: CAAGGAAGGTTGGCATTTGT
reverse: CCTTTCAGTCCTTTGCAAGT

*Cx3cr1* NM_009987.4 forward: TGACTGGCACTTCCTGCAGA

reverse: AGGGCGTAGAAGACGGACAG

*Trem2* NM_001272078.1 forward: ATGGGACCTCTCCACCAGTT

reverse: TCACGTACCTCCGGGTCCA

*P2ry12* NM_001357010.1 forward: CACAGAGGGCTTTGGGAACTTA

reverse: TGGTCCTGCTTCTGCTGAATC

*Gapdh* NM_001411843.1 forward: TCGTCCCGTAGACAAAATGG

reverse: TTGAGGTCAATGAAGGGGTC

## 6.9. NanoString nCounter Analysis

Total RNA was isolated from BV2-derived EVs by Total Exosome RNA Protein Isolation Kit (Invitrogen, Waltham, MA, USA) and concentrated using the Microcon10 centrifugal filters (Merck Millipore, Milan, Italy). The analyzed genes are included in the nCounter® Neuroinflammation Panel. Gene expression raw data were normalized considering *Gapdh* as housekeeping gene *via* nSolver Software (NanoString).

## 6.10. Immunofluorescence

Twenty-four hours after the second treatment, mice were perfused with ice-cold PBS, brains were isolated, post-fixed in 4% paraformaldehyde, cryopreserved with 30% sucrose and then snap frozen. Coronal or sagittal brain cryostat sections (20 μm thick) were washed in PBS, blocked (3% goat serum in 0.3% Triton X-100) for 1 h at RT and incubated with rabbit anti-Iba1 (1:500, 4°C, 18 h; Wako Chemicals, VA, USA, Cat# 019-19741), mouse anti-NeuN (1:300, 4°C, 18 h, Abcam, Cambridge, UK, Cat# ab104224) or rabbit anti-GFAP(1:500, 4°C, 18 h; Cell Signalling, Boston, MA, USA, Cat# 12389). The slices were washed in PBS and stained for 1 h with the secondary antibody conjugated to Alexa Fluor 488 (1:500, RT; Thermo Fisher Scientific, Waltham, MA USA). Incubation with Hoechst 33342 (1:1000, 1 h, RT; ThermoFisher Scientific, Waltham, MA, USA) was then performed for nuclei visualization. The slices were mounted with a Dako Fluorescence Mounting Medium (Agilent, Santa Clara, CA, USA). Analysis was performed using a fluorescence microscope (Nikon, Leuven, Belgium) by Meta- Morph 7.6.5.0. For EVs subcellular localization, coronal brain sections were analysed by confo-cal microscopy on a Nikon Eclipse Ti equipped with X-Light V3 spinning disk (CrestOptics, Rome, Italy). Pictures were acquired with a 100x/1.45 Plan E oil objective. The used Z step sizewas 0.2 μm. Orthogonal view and 3D reconstruction have been processed with the open-source software Image J.

## 6.11. Analysis of microglia activation

Microglia activation was investigated evaluating cell morphology in the hippocampal and cortical regions. For morphological analysis of $Iba1^+$ cells, 20 µm z-stacks were acquired at 0.5 µm intervals using an Eclipse Ti-S microscope (Nikon) at × 60 objective. Maximal intensity projections of each image were generated, binarized, and skeletonized using the Skeletonize 2D/3D plugin in ImageJ, and the Analyze Skeleton plugin (https://imagej.net/AnalyzeSkeleton) was applied. The average branch number (process end points per cell) and length per cell were recorded for each image with a voxel size exclusion limit of 150. The number of single and multiple junction points was additionally calculated to give an indication of branching complexity. The areas of the cell scanning domain and soma were measured for each cell. For each animal we analysed 4-5 slices and 3-7 cells for slice in the region of interest. In each experimental group, from 4 to 6 animals were analysed.

## 6.12. Behavioral tests

The day after the first dose of treatments, mice were handled and habituated to the test room 5 min/day for 6 consecutive days. The test room was dimly and diffusely illuminated from the bottom up by light sources located in the room's corners. The 7th day, animals received the second dose of treatments and the day after they started the behavioural evaluation. Tests were performed from 8:00 to 12:00 am where not differently indicated, and EthoVision XT10 software (Noldus) was used to record performances. Multiple separate cohorts of animals were tested for each treatment and age group to ensure the reproducibility of the results.

### 6.12.1 Open Field test

This test was applied to evaluate locomotor activity to exclude those animals with physical deficits (*Alves et al., 2018; Komsuoglu et al., 2014*). Each mouse was released in the middle of a square arena (60 cm wide, 60 cm long and 30 cm high) digitally divided into 16 areas (15 x 15 cm) and allowed to explore for 5 min. Total distance moved, number of rearings, and number of line crossings were recorded. To reduce odour cues, the arena was cleaned with 70% ethyl alcohol between each animal.

### 6.12.2 Elevated Plus Maze test

The apparatus consisted of two open arms (5 cm wide, 30 cm long and 2 cm high) and two enclosed arms (5 cm wide, 30 cm long and 15 cm high) extending from a common central platform (5 x 5 cm) raised 60 cm above the ground. Each mouse was placed on the platform facing an open arm and was allowed to explore for 5 min with the experimenter out of view. The percent

time spent in the open arms, the number of entries into the open arms and the number of head dipping were recorded.

### 6.12.3 Morris Water Maze test

The apparatus consisted of a circular pool (110 cm wide and 25 cm high) digitally divided into 4 quadrants (North, West, South, East), filled with opaque water (22°C ± 2°C) and containing a transparent platform (10 cm wide) submerged 1.5 cm below the water level. During the training phase (3 days), mice were released facing the wall from 4 different start sites once in the morning and once in the afternoon (from 2:00 to 6:00 pm), for a total of 8 releases/day. The start sites, whose sequence was changed daily, were chosen semi-randomly to prevent the development of search strategies with specific right or left turns and to balance the shorter and longer paths to the goal (*Vorhees and Williams, 2006*). The platform's position was fixed. Mice were allowed to swim for 60 s or until they reached the platform. Animals that failed to find the platform within 60 s were guided to it. In either case, they were left on the platform for 15 s. Performance was assessed in terms of path efficiency (PE) (*i.e.*, Euclidean distance/distance swum by each mouse from the release site to the platform) because it allows weighting the feat in each trial and seems to better reflect spatial accuracy (*Fragoulis et al., 2017; Faraji et al., 2018; Balietti et al., 2021*). On the 4$^{th}$ day (probe phase), 24 h after the last release of the training period, the platform was removed, mice were released from a new position and allowed to swim for 60 s (*Maei et al., 2009; Pereira and Burwell, 2015*). Performance was measured as proximity, defined as the average distance in centimeters of mice from center of the former platform location across the 60-s test. As a control procedure to exclude that cognitive performance could be biased by visual deficits, the day after the probe phase, mice were set a cued task where a flag was placed on the platform. The task involved 4 releases, each with a different release point and goal, so thatmice were unable to use distal cues to solve the problem. Latency and mean velocity were meas-ured.

## 6.13. Hippocampal slices preparation

To perform electrophysiological experiments, 3 days after the second treatment, acute hippocampal slices were collected. The animals were anesthetized by inhalation of halothane (Merck KGaA, Darmstadt, Germany) and then decapitated. The brain was rapidly removed from the skull and submerged for 10 min in ice-cold artificial cerebrospinal fluid (ACSF) solution containing (in mM): NaCl 125, KCl 4, $CaCl_2$ 2.5, $MgSO_4$ 1.5, $NaH_2PO_4$ 1, $NaHCO_3$ 26 and glucose 10. The solution was continuously oxygenated with 95% $O_2$ and 5% $CO_2$ to maintain a proper pH value of 7.35.

Upon removal, the brain was hemisected along the longitudinal fissure to separate the two hemispheres. Brain dissection was carried out according to the slicing plane chosen and the structure to be investigated. Specifically, for experiments examining the ventral hippocampus, transversal slices were cut from the temporal pole of the brain. Coronal slices were cut from the frontalpole for experiments on the dorsal hippocampus. Dorsal and ventral slices were identifiedas the distance, in μm, from the frontal and temporal poles, respectively (approximately from 400to 1750 μm). The brain tissues were blocked on the stage of a vibratome (Thermo Scientific, USA), then 350 μm thick slices were cut in ice-cold ACSF.

## 6.14. Extracellular field potential recordings

After 2 h of recovery at 30°C in an incubation chamber containing oxygenated ACSF, individual slices were transferred to the interface slice-recording chamber (BSC1, Scientific System Design Inc) where they were maintained at 30-32°C and constantly superfused with oxygenated ACSF at the rate of 2 mL/min. Solutions were applied to the slices using a peristaltic pump (Bio-Rad). Slices were visualized with a Wild M3B (Heerbrugg, Switzerland). Experiments were performed from 1 to 7 h after slicing. At the beginning of each recording, a concentric bipolar stimulating electrode (SNE-100X 50 mm long Elektronik–Harvard Apparatus GmbH) was placed in the hippocampus CA1 stratum radiatum (SR) for stimulation of the Schaffer collateral pathway projections to the CA1. A glass micropipette (0.5-1 MΩ) filled with ACSF was placed in the CA1 hippocampal region, at 200-600 μm from the stimulating electrode, to record orthodromically-evoked field extracellular post-synaptic potentials (fEPSP). fEPSPs were recorded and filtered (low pass at 1 kHz) with an Axopatch 200A amplifier (Axon Instruments, CA) and digitized at 10 kHz with an A/D converter (Digidata 1322A, Axon Instruments). Stimuli consisted of 100 μs constant current pulses of variable intensity, applied at 0.05 Hz. In each experiment, stimulus intensity was adjusted to evoke ~50% of the maximal fEPSP amplitude without appreciable population spike contamination. Evoked responses were monitored online and stable baseline responses (variation in the amplitude values under 10%) were recorded for at least 10 min. Only slices showing stable fEPSP amplitudes were included in the experiments. Long-term potentiation was induced by high-frequency stimulation (HFS, 2 trains of stimuli at 100 Hz of 1 sduration each, 3 s intertrain interval). To analyse the time course of fEPSP amplitude, the rec- orded fEPSP was routinely averaged over 1 min (n = 3 traces). fEPSP amplitude changes following the LTP induction protocol were calculated with respect to the baseline (35 min *versus* 1 min before LTP induction). Data were stored on a computer using pClamp 10 software (Axon Instruments) and analysed offline with Clampfit 10 program (Axon Instruments).

## 6.15 Statistical analysis

Data are expressed as means ± SEM. Student *t*-test, one-way or two-way analysis of variance (ANOVA) or Kruskal-Wallis test were performed. Dunnett's multiple comparison or Tukey's multiple comparisons tests were were applied for *post hoc* comparisons. Variables normality was evaluated by Shapiro-Wilk test. A value of $p < 0.05$ was considered significant.Statistical analyses were performed using the Prism GraphPad Software (v9.3.1, San Diego, CA,USA). All statistic tests are specified in the figure legends.

# 7. Results

## 7.1 BV2 cells polarization

Microglial cells, like other macrophages, react differently to environmental stimuli. We firstly evaluated the effect of pro- (LPS/INFγ) or anti-inflammatory (IL4) treatment on murine microglia-like BV2 cell line compared to untreated cells (CTL).

As previously documented in our laboratory (*Grimaldi et al., 2019*), we confirmed that different treatments of BV2 cells triggered unique gene expression patterns, indicative of either pro-inflammatory or anti-inflammatory phenotypes; in fact, typical pro-inflammatory markers *Il6, Il1β, Tnfα and Cd86* were increased by LPS/INFγ treatment and the typical anti-inflammatory markers *Cd206* and *Cd163* were overexpressed upon IL4 stimulation (Fig. 6).

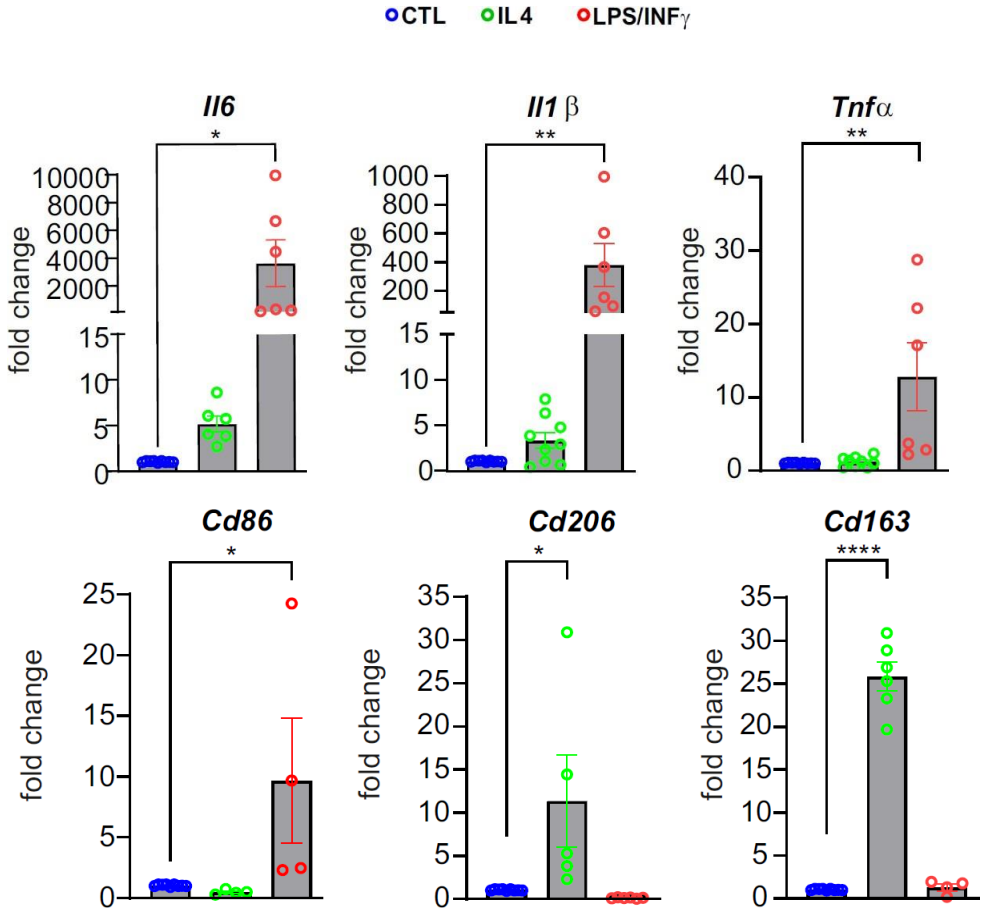



**Figure 6**. RT-PCR analysis of Il6, Il1β, Tnfα, Cd86, Cd206 and Cd163 gene expression in BV2 microglial cells unstimulated (CTL) or stimulated with IL4 or LPS/INFγ. Data are the mean ± SEM of fold increase normalized to Gapdh and expressed compared to CTL; n ≥ 4; Shapiro-Wilk test passed; *p < 0.05, **p < 0.001, ****p < 0.0001; oneway ANOVA, Dunnett's multiple comparisons test.

## 7.2 BV2-derived EVs characterization

In order to explore the mechanisms underlying age-related microglial changes, we investigated the effects of BV2-derived EVs on microglial cells in late adult mice. EVs were isolated from BV2 cells and analysed by NTA. As reported in Figure 7 A the size of EVs was 204.5 ± 5.2 nm with a concentration of 1.66 x $10^8$ ± 1.68 x $10^7$/mL and with the following size distributions: D10: 128.0 nm ± 1.2 nm, D50: 172.1 ± 2.6 nm, D90: 311.7 ± 15.1 nm. Western blot on BV2-derived EVs confirmed the enrichment of specific EVs markers (CD9 and CD81) compared to the cytosolic protein GAPDH, indicating a successful EVs purification (Fig. 7 B). The stimulation of BV2 cells did not modified the size distribution number of released EVs (*Grimaldi et al., 2019*) and led to distinct patterns of RNA expression related to inflammatory markers in EVs. Nanostring analysis demonstrated that 28 of the 243 inflammatory genes analysed were modulated in EVs obtained from IL4 or LPS/INFγ-treated BV2 cells (Fig. 7 C). Collectively these data corroborated previous observations of our group (*Grimaldi et al., 2019; Serpe et al., 2021*) indicating that EVs content parallels the different inflammatory phenotype of the donor cells.

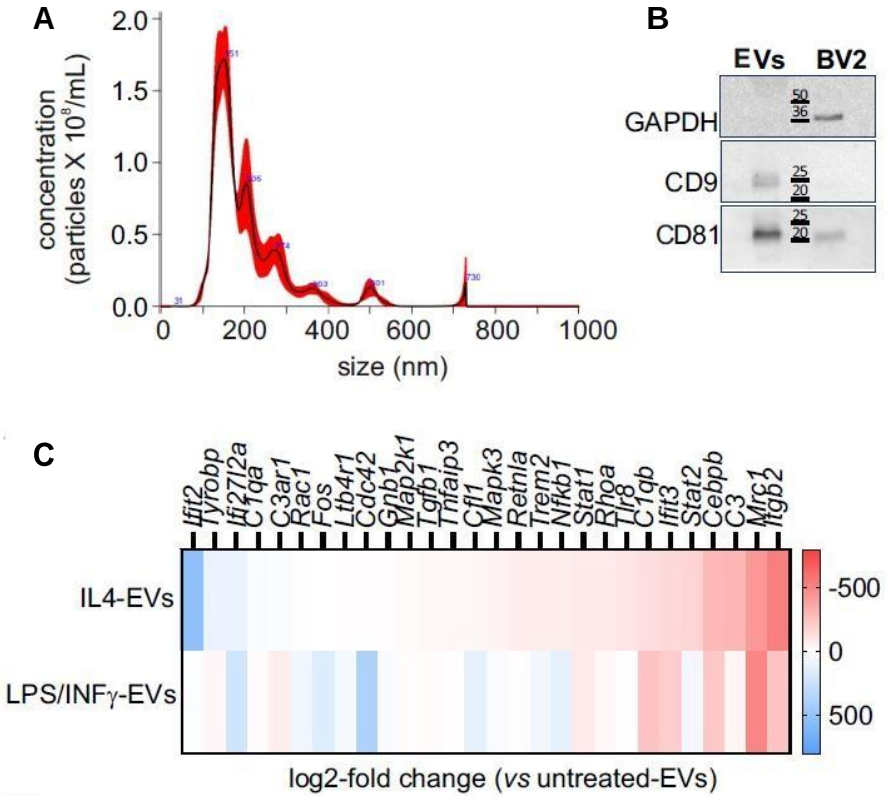
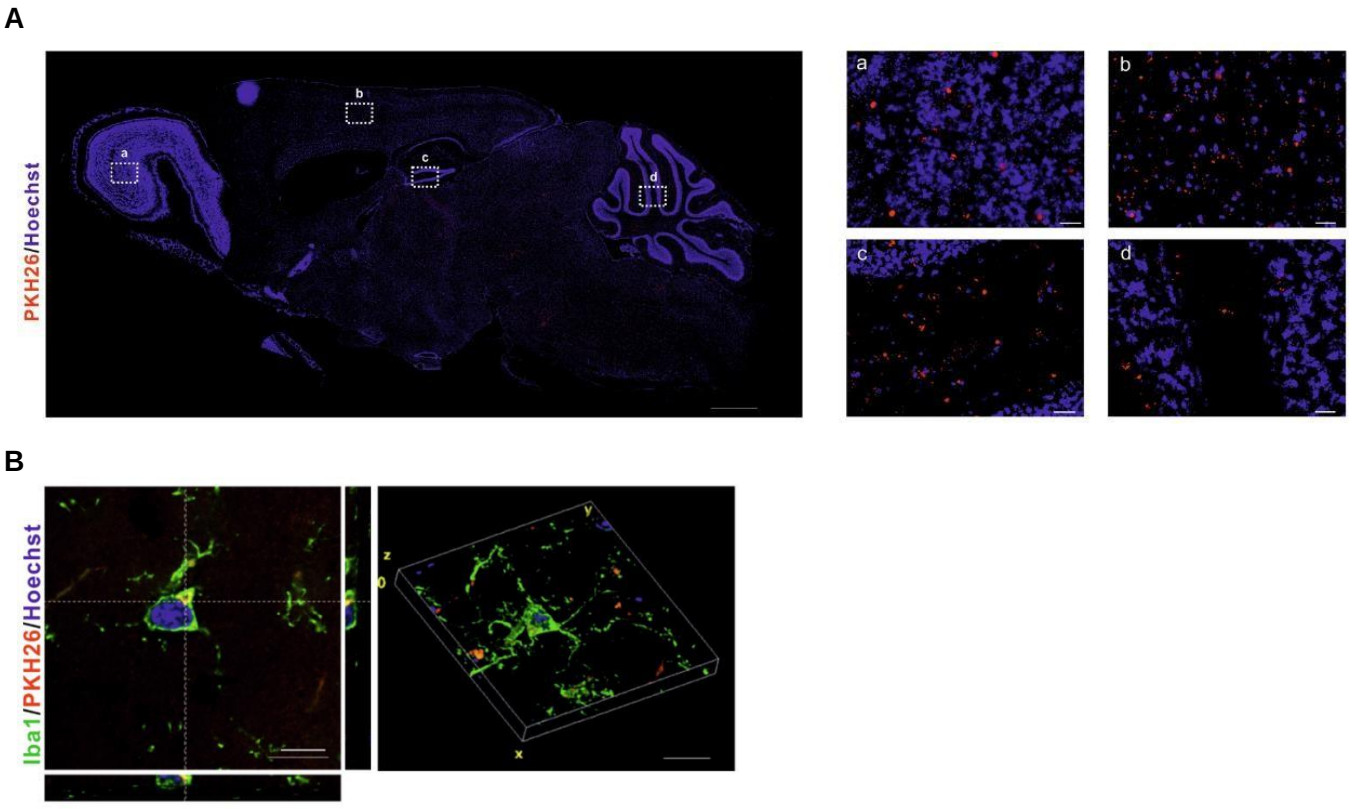



**Figure 7.** (A) Representative concentration/size graph from the NTA of BV2-derived EVs. The mean concentration (particles/mL) and the mean size of EVs are reported in the main text. (B) Western blot on BV2-derived EVs and BV2 cell lysates for the vesicles markers CD9 and CD81; GAPDH was used as a negative control (in the middle, molecular weights are indicated). (C) The heatmap shows the significant fold change expression of 28 inflammatory-related genes expressed in EVs derived from IL4-stimulated BV2 (IL4-EVs) or LPS/INFγ-stimulated BV2 (LPS/INFγ-EVs) compared to EVs derived from untreated BV2 (untreated-EVs). n = 4; differences were significant with at least 2.8-fold changes ($p < 0.05$); Wilcoxon-Mann-Whitney test (U=2). The signals were normalized to *Gapdh* and transformed to the log2 scale. The colour scale represents the relative expression levels (blue for low expression and red for high expression levels).

## 7.3 Distribution and uptake of intranasal administered BV2-derived EVs in CNS

We then moved to *in vivo* experiments. We labelled BV2-derived EVs with PKH dye to track EVs distribution and uptake from brain cells after intranasal infusion to late adult mice. The amount of EVs reaching the brain parenchyma was evaluated 1 h after infusion, by NTA in fluorescence mode and represents 25.14 ± 5.60 % of the input, while 0.07 ± 0.01 % of EVs were localized in the CSF. PKH staining was evident in different brain regions (olfactory bulbs, cortex, hippocampus and cerebellum), as shown in Figure 8 A.

Being the hippocampus the brain region most strongly impacted by aging (*Murphy et al., 2006; Jimenez Peinado et al., 2023; Blackmore et al., 2023*), we focused on this area to investigate the cellular specificity of EVs uptake. We demonstrated that in the hippocampus, EVs are taken by microglial cells (Fig. 8 B), neurons and astrocytes (Fig. 8 C). Data in Figure 8 D show that most of the intranasally delivered EVs was taken by Iba1+ cells (Iba1+/PKH+ cells: 57.05 % ± 3.38 %; NeuN+/PKH+ cells: 28.04 % ± 1.98 %; GFAP+/PKH+ cells: 14.99 % ± 0.42 %). Taken together, these results suggest that intranasal administration was successful to deliver EVs into the brain and that microglia cells are mostly involved in their uptake, consistently with their phagocytic role.



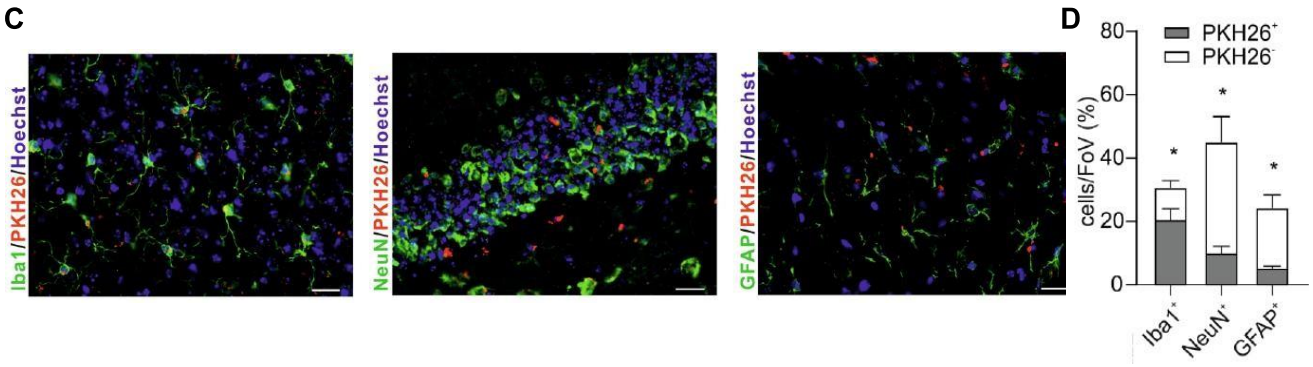



**Figure 8**. (A) Immunofluorescence analysis of sagittal brain section of male mice intranasally infused with PKH26-stainedBV2-derived-EVs (red signal) and nuclei (Hoechst, in blue), scale bar = 1 mm. Left panels, magnification of olfac-tory bulbs (a), cortex (b), hippocampus (c) and cerebellum (d) areas; scale bars = 20 µm. (B) Immunofluores-cence analysis of coronal hippocampal brain sections of male mice intranasally infused with PKH26-stained BV2-derived-EVs(red signal) and stained for Iba1 (in green; Hoechst in blue). Left panel, orthogonal view of a single confocal plane acquisition showing the presence of EVs into a hippocampal microglial cell; scale bar = 10 µm. Right panel, 3D rendering of the same microglial cell showing the intracellular localization of the EVs; scale bar = 10 µm. (C), representative immunofluorescence analysis for Iba1, NeuN and GFAP (in green; Hoechst in blue) of coronal hippocampal brain sections of male mice intranasally infused with PKH26-stained BV2-derived-EVs; scale bar = 20 µm. (D),quantification of Iba1+ or NeuN+ or GFAP+ cells co-stained or not with PKH26 (PKH26+ or PKH26-), data are themean ± SEM, n = 4 animals; Shapiro-Wilk test passed; *$p < 0.05$, Student's *t*-test between PKH26+ and PKH26- cells in each group.

## 7.4 BV2-derived EVs modulate microglia inflammatory profile in male and female late adult mice

To evaluate if BV2-derived EVs could elicit some effects on the microglia/macrophages cell phe-notype, we treated male and female late adult mice twice (at days 0 and 7) with vehicle or EVs derived from control, IL4- or LPS/INFγ-stimulated BV2 cells. After 24h from the last ad- min-istration, the CD11b+ cells were isolated from the brain and tested for the expression of a panel of representative genes (*Il6, Il1β, Tnfα, Cd86, Cd206 and Cd163*), already used in other studies as inflammation-related markers (*Han et al., 2022; Sierra et al., 2007; Kohman et al., 2013*), comparing with young mice as control. In vehicle-treated microglia, we observed that these genes were differently expressed as a function of age and sex, and that there was a significant interaction between the two variables for the expression of all the genes but *Tnfα* (*Il6*: $F(1,12) = 187.70$, $p < 0.0001$; *Il1β*:$F(1,12) = 10.44$, $p = 0.0072$; *Tnfα*: $F(1,17)$, $p = 0.2325$; *Cd86*: $F(1,13) = 82.87$, $p < 0.0001$; *Cd206*:$F(1,13) = 6.34$, $p = 0.0257$; *Cd163*: $F(1,9) = 5.95$, $p = 0.0374$); two-way ANOVA. Firstly, we observed comparable expression levels of *Il6, Il1β, Cd86, Cd206 and Cd163* between young male and female mice, while their expression was significantly higher in late adult males compared to females; interestingly, *Tnfα* was differently expressed in young male and female mice and equally expressed in late adult mice (Fig. 9 A). These results point out a different level of microglia/macrophage inflammation between male and female late adult mice, therefore a dif-ferent microglial phenotypeat the time of EVs treatment in the two sexes.

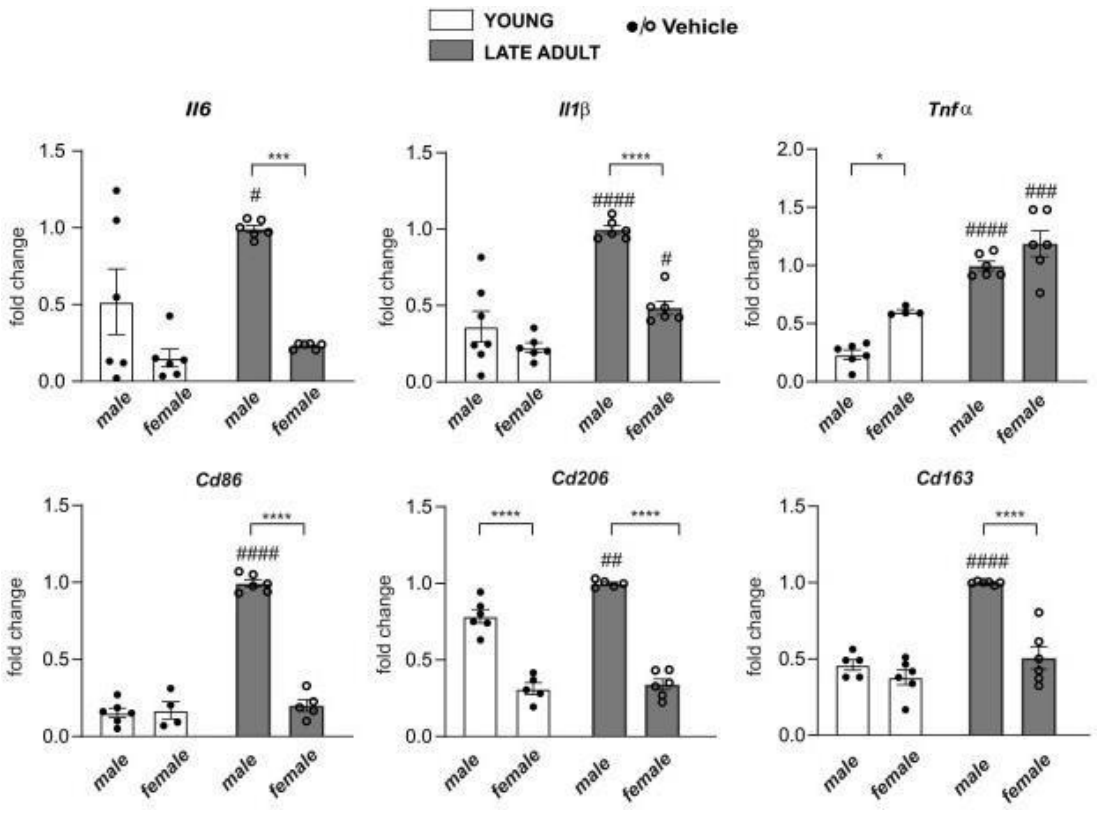



**Figure 9.** RT-PCR expression analysis of inflammation-related genes *(Il6, Il1β, Tnfα, Cd86, Cd206, Cd163*) in $CD11b^{+}$ cells isolated from young and late adult male and female mice treated with vehicle. Data are the mean ± SEM of fold increase (*vs* late adult male, gene expression was normalized *vs Gapdh*); n ≥ 5 animals/experimental group. Shapiro-Wilk test passed, **p* < .005, ****p* ≤ .001, **** *p* ≤ .0001 male *vs* female mice and #*p* < .05, ##*p* < .005, ###*p* ≤ .001, ####*p* ≤ .0001 young *vs* late adult; one-way ANOVA; Tukey's multiple comparisons test.

In late adult male mice, the expression level of *Il6, Il1β, Tnfα, Cd86, Cd206* and *Cd163* was significantly reduced by all EVs treatments (Fig. 10 A). In female late adult mice, the expression of *Il6, Il1β, Cd86, Cd206* and *Cd163* was reduced by all EVs treatments, while *Tnfα* expression was reduced by IL4-EVs treatment and unchanged by LPS/ INFγ-EVs and untreated-EVs (Fig. 10 B). These data suggest that the effect of EVs is almost overlapping in the two sexes. In addition, since the observed effect of EVs in reducing the expression of inflammation-related genes in CD11b+ cells is similar for IL4-EVs, LPS/INFγ-EVs and untreated-EVs, it is not dependent on a diverse vesicular cargo.

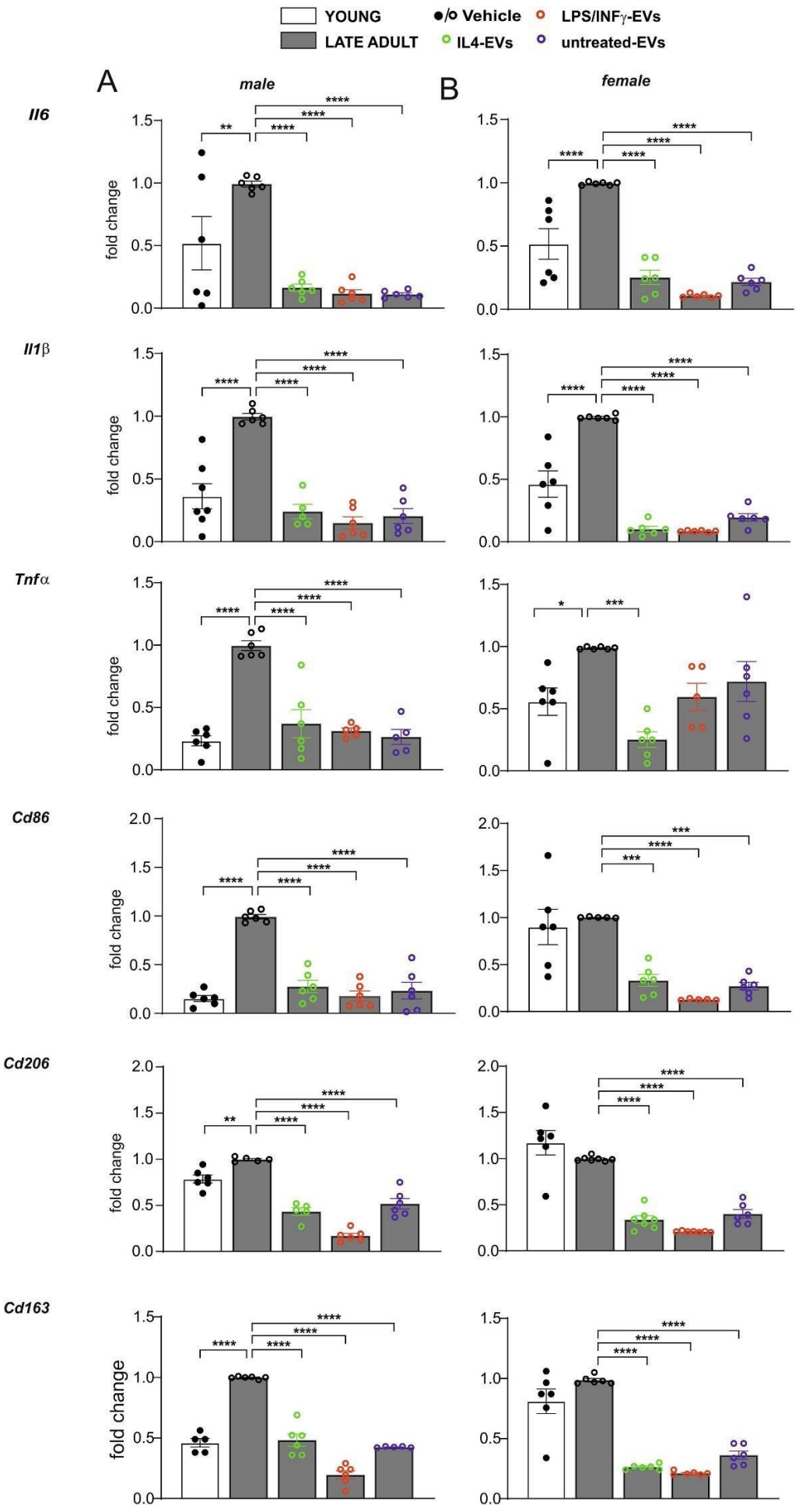

YOUNG
LATE ADULT
●/○ Vehicle
○ IL4-EVs
○ LPS/INFγ-EVs
○ untreated-EVs
A
male
B
female
Il6
Il1β
Tnfα
Cd86
Cd206
Cd163
fold change
0.0
0.5
1.0
1.5
2.0
*
**
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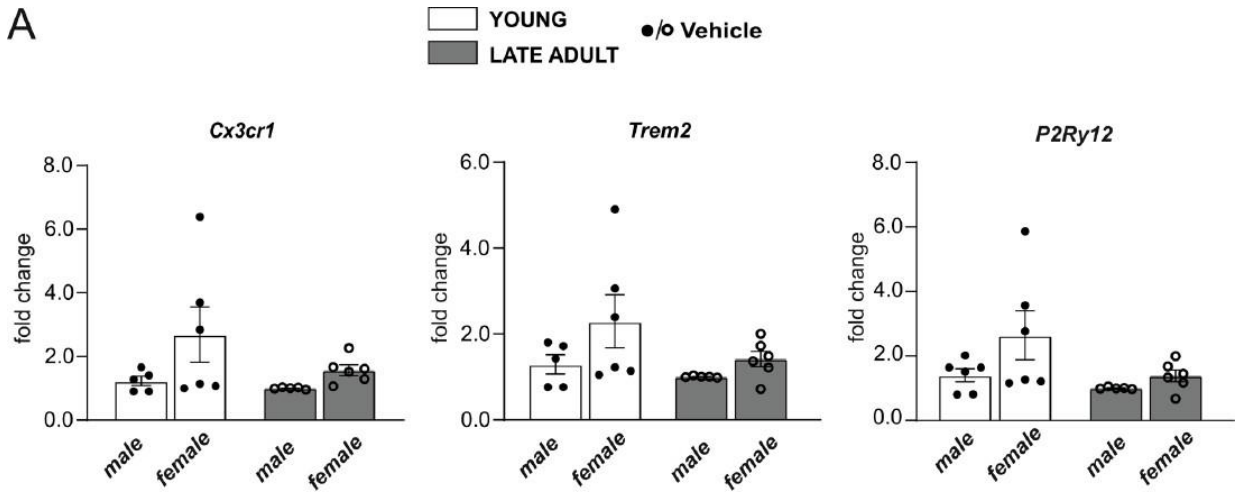
**Figure 10**. (A) RT-PCR expression analysis of inflammatory-related genes (Il6, Il1β, Tnfα, Cd86, Cd163, Cd206) inCD11b+ cells isolated from late adult male mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Data are the mean ± SEM of fold increase (gene expression was normalized to Gapdh); n ≥ 5 animals/experimental group; Shapiro-Wilk test passed; *p < 0.05, **p ≤ 0.005, ***p ≤ 0.0003, **** p < 0.0001 vs late-adult vehicle treated mice; one-way ANOVA; Dunnett's multiple comparisons test. (B) RT-PCR expression analysis of inflammatory-related genes (Il6, Il1β, Tnfα, Cd86, Cd163, Cd206) in CD11b+ cells isolated from late adult female mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Data are the mean ± SEM of fold increase (gene expression was normalized vs Gapdh); n ≥ 5 animals/experimental group; Shapiro-Wilk test passed; *p < 0.05, **p ≤ 0.005, ***p ≤ 0.0003, **** p < 0.0001 vs late-adult vehicle treated mice; one-way ANOVA; Dunnett's multiple comparisons test.

In order to rule out a possible unspecific effect of EVs on the transcriptional process of microglial target cells, in the same CD11b+ cells, we analysed the expression of genes associated with homeostatic functions in microglia, namely *Cx3cr1, Trem2 and P2ry12*. RT-PCR analysis revealed that their expression did not change with age in both sexes (Fig. 11 A). Moreover, the expression of these genes was not dependent on sex or age and there was no interaction of the two variables for their expression (*Cx3cr1*: F(1, 15) = 0.28, p = 0.6039; *Trem2*: F(1,15) = 0.05, p = 0.8241; *P2ry12*: F(1,16) = 0.14, p = 0.7094). Moreover, *Cx3cr1*, *Trem2* and *P2ry12* expression was unchanged upon administration of IL4-, and untreated-EVs in both male and female treated late adult mice (Fig. 11 B-C). Variations were only observed in *Cx3cr1* and *Trem2* expression in male mice upon LPS/INFγ-EVs treatment. These results suggest that EVs treatments do not disrupt indiscriminately the homeostatic profile of late adult microglia. Nonetheless, we cannot exclude the possibility that gene expression changes may also affect BAMs, which are present alongside microglia in the CD11b+ cell population (*Sun et al., 2024*).



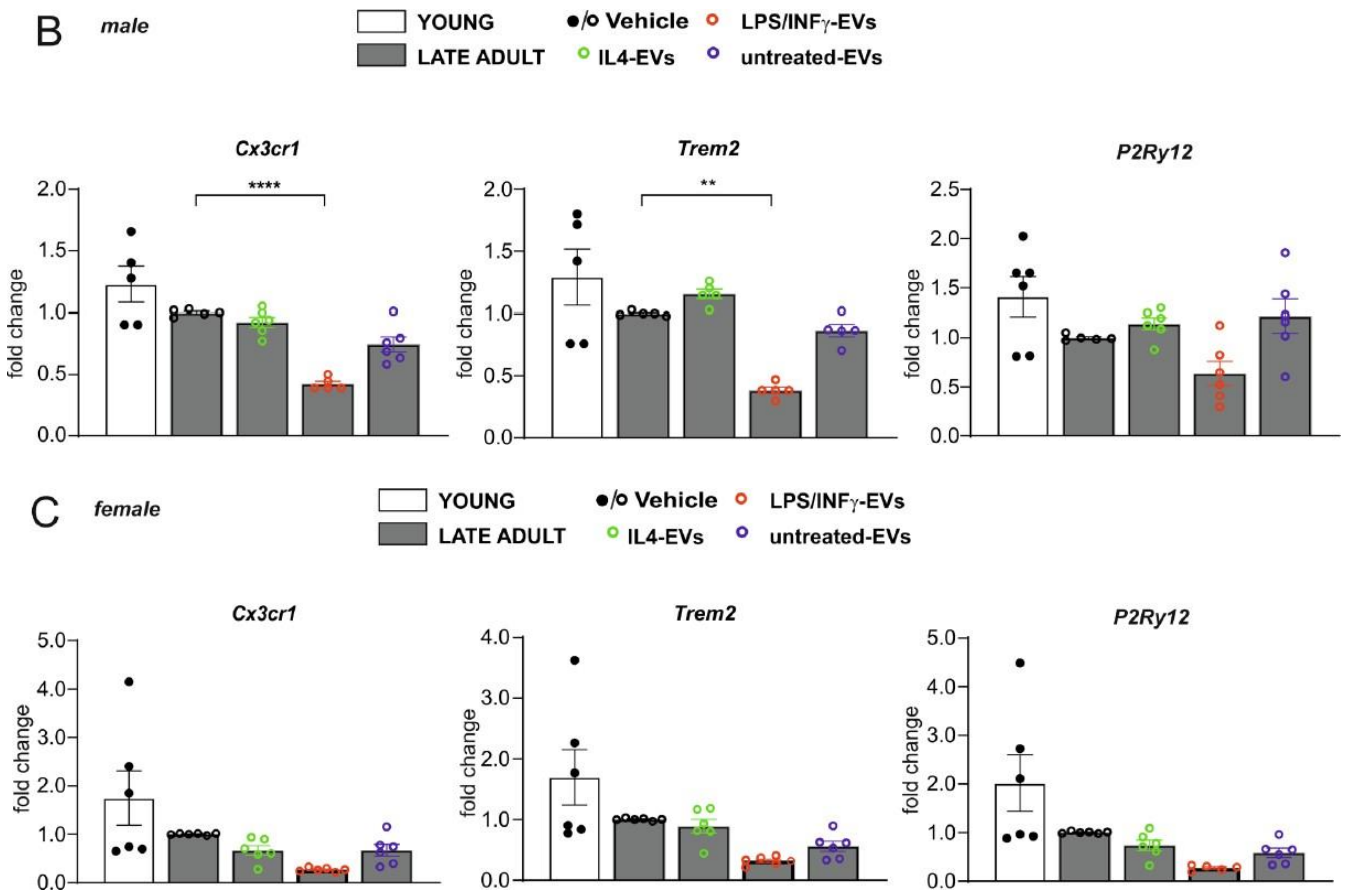



**Figure 11.** (A) RT-PCR expression analysis of homeostasis-related genes (*Cx3cr1, Trem2, P2ry12*) in CD11b+ cells isolated from young and late adult male and female mice treated with vehicle. Data are the mean ± SEM of fold increase (gene expression was normalized to Gapdh); n ≥ 5 animals/experimental group; Shapiro-Wilk test passed; one-way ANOVA; Dunnett's multiple comparisons test. (B) RT-PCR expression analysis of homeostasis-related genes (*Cx3cr1, Trem2, P2ry12*) in CD11b+ cells isolated from late adult male mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Data are the mean ± SEM of fold increase (gene expression was normalized to Gapdh); at least n ≥ 5; Shapiro-Wilk test passed; **p ≤ 0.005, ****p < 0.0001; one-way ANOVA; Dunnett's multiple comparisons test. (C) RT-PCR expression analysis of homeostasis-related genes (*Cx3cr1, Trem2, P2ry12*) in CD11b+ cells isolated from late adult female mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Data are the mean ± SEM of fold increase (gene expression was normalized to Gapdh); at least n ≥ 5 animals/experimental group; Shapiro-Wilk test passed; one-way ANOVA; Dunnett's multiple comparisons test.

## 7.5 BV2-derived EVs modify microglia morphology in male and female late adult mice

To investigate whether BV2-derived EVs could have an impact on microglial phenotype of aged mice, we analyze the morphology of microglial cells in the hippocampus of EVs-treated late adult male and female mice. Young animals of both sexes were used as controls. Firstly, we assessed the age-related differences of microglia phenotype and confirmed that late adult male Iba1+ cells have reduced number of branches, average and maximum branch length, and area of the scanning domain in comparison with young microglia (Fig. 12). In late adult females, microglia had reduced number and junctions of branches, average and maximum branch length, and of scanning domain in comparison with young microglia (Fig. 12). Conversely, no differences were observed in the microglial soma area of male and female late adult mice. Collectively, there are no sex-related differences in the effect of aging on microglia morphology.

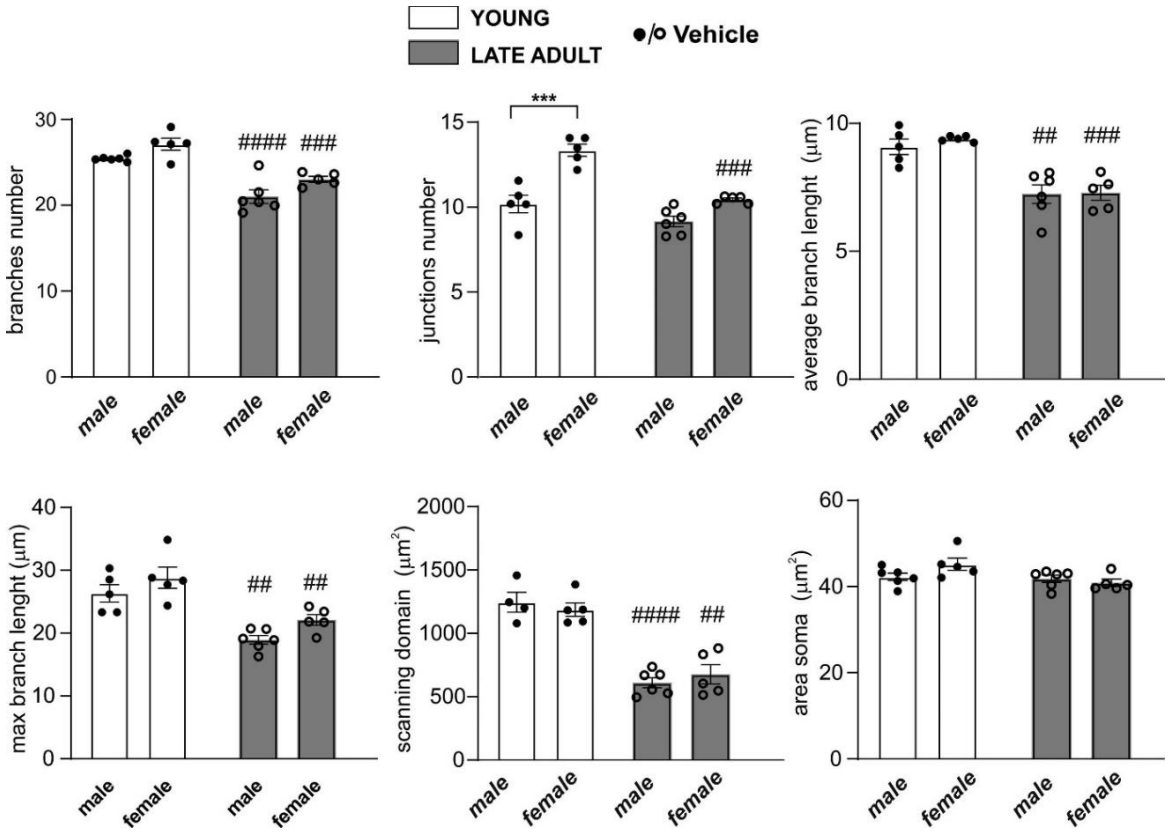



**Figure 12.** Quantification of number of branches, number of junctions, maximum branch length, average branch length, scanning domain area, and soma area in Iba1$^{+}$ cells of young and late adult male and female mice treated with vehicle. Data are the mean ± SEM; n ≥ 3 cells/animal and n ≥ 5 animals/experimental group; Shapiro-Wilk test passed; ***$p \leq .001$ male *vs* female mice and ##$p < .005$, ###$p \leq .001$, ####$p \leq .0001$ young *vs* late adult; one-way ANOVA; Tukey's multiple comparisons test.

As shown in Figure 13, after IL4-EVs administration, late adult male mice exhibited an increased number, junctions and maximum length of branches as well as greater area of the scanning domain of microglia in comparison with vehicle-treated mice (Fig. 13 A-B). Similarly, in late adult

female mice, IL4-EVs significantly increased the number of branches and junctions, the average branch length, and the scanning domain area of microglia, but no effects were observed on the maximum branch length and the soma area (Fig.13 C-D**)**. Of note, in late adult males, untreated-EVs increased the junction number and the scanning domain; in females, untreated-EVs increased the average branch length and LPS/INFγ-EVs reduced the junction number (Fig. 13 B-D). In summary, these findings show that IL4-EVs treatment reverted the age-related alterations in microglia morphology, with few differences between the two sexes.

Differently from results on gene expression, the morphological changes in microglia are dependent on EVs content.

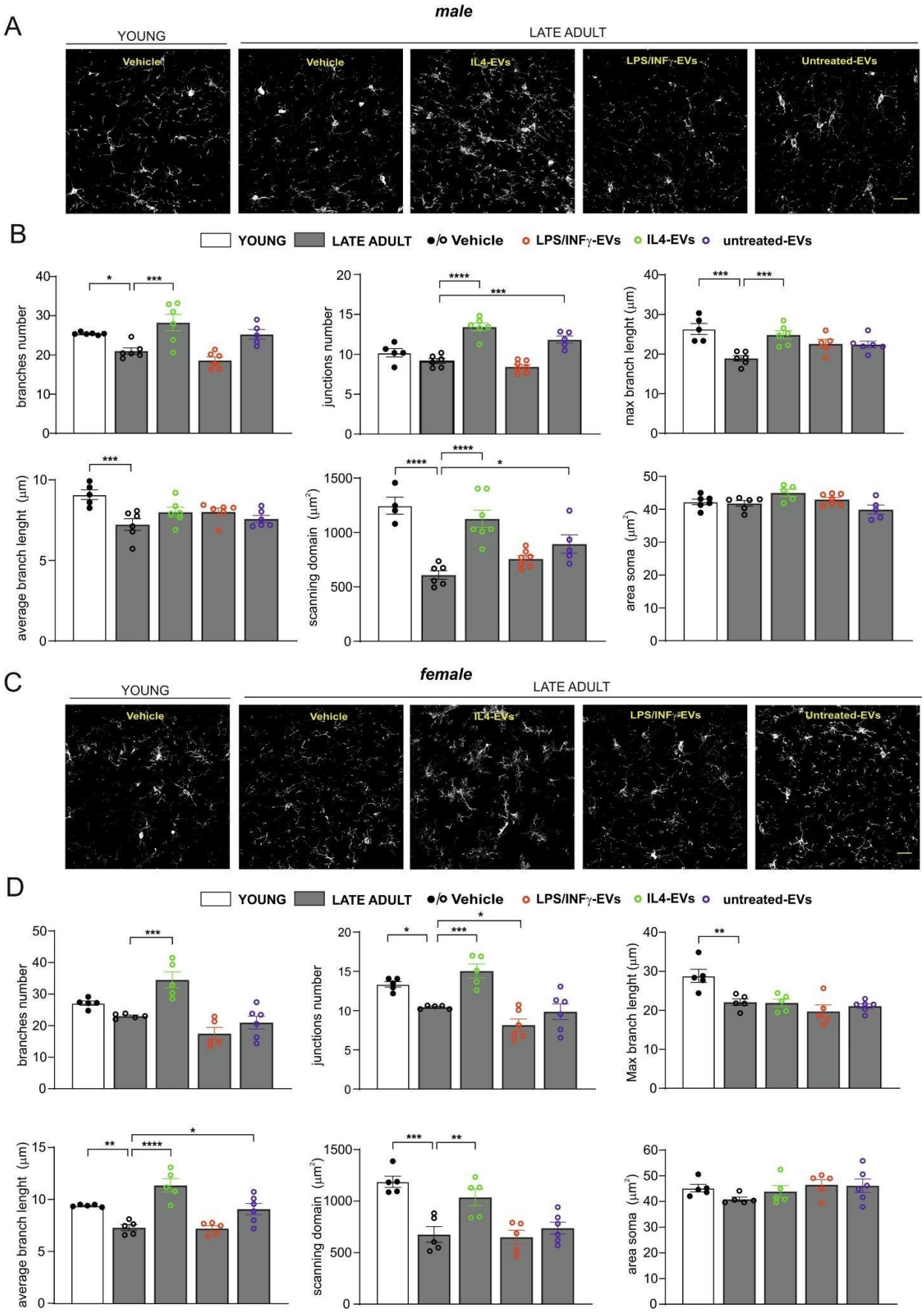

A
male
YOUNG
LATE ADULT
Vehicle
Vehicle
IL4-EVs
LPS/INFγ-EVs
Untreated-EVs
B
YOUNG
LATE ADULT
Vehicle
LPS/INFγ-EVs
IL4-EVs
untreated-EVs
branches number
junctions number
max branch lenght (µm)
average branch lenght (µm)
scanning domain (µm²)
area soma (µm²)
C
female
YOUNG
LATE ADULT
Vehicle
Vehicle
IL4-EVs
LPS/INFγ-EVs
Untreated-EVs
D
YOUNG
LATE ADULT
Vehicle
LPS/INFγ-EVs
IL4-EVs
untreated-EVs
branches number
junctions number
Max branch lenght (µm)
average branch lenght (µm)
scanning domain (µm²)
area soma (µm²)

**Figure 13.** (A) Representative immunofluorescence images of Iba1+ microglia (in white) in the hippocampus of male mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Scale bar = 20 μm. (B) Quantification of number of branches, number of junctions, maximum branch length, average branch length, scanning domain area and soma area in Iba1+ cells of mice treated as in (A). Data are the mean ± SEM; n ≥ 4 animals/experimental group; Shapiro-Wilk test passed; *p < 0.05, ***p ≤ 0.001, ****p ≤ 0.0001 vs late-adult vehicle treated mice; one-way ANOVA; Dunnett's multiple comparisons test. (C) Representative immunofluorescence images of Iba1+ microglia (in white) in the hippocampus of female mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Scale bar = 20 μm. (D) Quantification of number of branches, number of junctions, maximum branch length, average branch length, scanning domain area and soma area in Iba1+ cells of mice treated as in (C). Data are the mean ± SEM; n ≥ 4 animals/experimental group; Shapiro-Wilk test passed; *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 vs late-adult vehicle treated mice; one-way ANOVA; Dunnett's multiple comparisons test.

## 7.6 BV2-derived EVs influence the behavioral and cognitive performances of late adult mice in sex-specific manner

We then explored the outcome of EVs administration on the age-dependent behavioral and cognitive alterations. No differences were observed in male and female mice in the parameters evaluated in the open field test (total distance moved, number of rearings and number of line crossings), neither due to aging, nor to treatments (IL4-EVs, LPS/INFγ-EVs and untreated-EVs), as summarized in Table 1. This suggests that all the tested mice maintained good physical performances upon aging and EVs treatment.

| | YOUNG | LATE ADULT | IL4-EVs | LPS/INFg-EVs | Untreated-EVs | *p value* |
|---|---|---|---|---|---|---|
| | ***Males*** | | | | | |
| | n = 10 | n = 9 | n = 8 | n = 8 | n = 7 | |
| Total distance moved (cm) | 1372 ± 170 | 1443 ± 122 | 1429 ± 163 | 1395 ± 167 | 1287 ± 92 | [a] = 0.9902<br>[b] = >.9999<br>[c] = 0.9982<br>[d] = 0.8931 |
| Number of rearings | 27.10 ± 1.44 | 22.56 ± 3.76 | 20.50 ± 1.77 | 16.88 ± 3.64 | 16.86 ± 3.22 | [a] = 0.5891<br>[b] = 0.9634<br>[c] = 0.4449<br>[d] = 0.4743 |
| Number of line crossings | 179.1 ± 25.6 | 219.1 ± 23.45 | 231.4 ± 22.28 | 244.6 ± 7.62 | 186 ± 15.40 | [a] = 0.5461<br>[b] = 0.9892<br>[c] = 0.8686<br>[d] = 0.7567 |
| | ***Females*** | | | | | |
| | n = 10 | n = 10 | n = 10 | n = 9 | n = 8 | |
| Total distance moved (cm) | 1661 ± 215 | 1673 ± 125 | 1496 ± 116 | 1355 ± 89 | 1493 ± 149 | [a] = >.9999<br>[b] = 0.8018<br>[c] = 0.3721<br>[d] = 0.8248 |
| Number of rearings | 21.7 ± 0.96 | 19 ± 2.47 | 22.2 ± 2.37 | 18.67 ± 2.35 | 21 ± 2.75 | [a] = 0.7950<br>[b] = 0.6869<br>[c] = >.9999<br>[d] = 0.9298 |
| Number of line crossings | 208 ± 28 | 227 ± 23 | 237 ± 24 | 193 ± 18 | 228 ± 13 | [a] = 0.9342<br>[b] = 0.9932<br>[c] = 0.6802<br>[d] = >.9999 |

**Table 1. Open field test performances showed no differences in male and female mice**. The total distance moved, number of rearings and number of line crossings performed by young and late adult male and female mice treated with vehicle or EVs. [a], LA vs. Y; [b], LA vs. IL4-EVs; [c], LA vs. LPS/INFg-EVs; [d], LA vs. untreated-EVs, Kolmogorov-Smirnov test passed, ordinary one-way ANOVA. LA: late adult; Y: young

The inflammatory profile of aged microglia correlates with increased anxiety-like behaviour in aged mice (*Shoji et al., 2016; Norden et al., 2015*). To test if BV2-derived EVs could modulate anxiety-like behaviour, we investigated the effects of IL4-EVs, LPS/INFγ-EVs and untreated-EVs on mice in the elevated plus maze test (EPM). Behavioral tests were started 24h after the last treatment (day 8) and carried out until day 20. Data in Figure 14 A showed that the effect of EVs treatment on the anxiety-like behaviour was different between males and females in young mice. However, this difference was not maintained with aging (Fig. 14 A). In late adult mice, IL4-EVs treatment increased the number of head dipping in males and the number of entries into the open arms and of head dipping in females. LPS/INFγ-EVs and untreated-EVs had no effect in modulating anxiety-like behaviour (Fig. 14 A). These results indicate that the effect of IL4- EVs treatment is sex-dependent, only partially reducing the anxiety-like behavior in late adult males.

As discussed in Chapter 1, spatial learning and memory are also compromised upon aging. We performed the Morris water maze (MWM) test on the same animals to analyze these cognitive domains. As depicted in Figure 14 B, the path efficiency increased from the 1st to the 3rd day in all groups, even if late adult male mice showed a lower path efficiency on the 3rd day in all groups, demonstrating that young and late adult mice of both sexes learned how to solve the tasks (males: young mice, $p < 0.0001$; late adult mice treated with vehicle, $p < 0.05$; late adult mice treated with IL4-EVs, $p < 0.001$; late adult mice treated with LPS/INFγ-EVs, $p < 0.001$; late adult mice treated with untreated-EVs, $p < 0.01$; females: young mice, $p < 0.0001$; late adult mice treated with vehicle, $p < 0.0001$; late adult mice treated with IL4-EVs, $p < 0.001$; late adult mice treated with LPS/INFγ-EVs, $p < 0.001$; late adult mice treated with untreated-EVs, $p < 0.01$). Nonetheless, late adult male mice showed a lower path efficiency on the 3rd day compared to the other groups while young males outperformed young females. The proximity was significantly higher in late adult in comparison with young males, while was unchanged among young and late adult females (Fig. 14 B). In late adult male mice, all EVs treatments recovered the path efficiency but had no effects on proximity (Fig. 14 B) indicating a mild effect on spatial learning. In late adult female mice, all EVs treatments were ineffective on path efficiency and proximity (Fig. 14 B). These findings show that, while aging negatively impact spatial learning and memory in male mice with a partial recovery of their performances because of EVs treatments, late adult females do not experience age-related deficits and EVs do not affect the *status quo.*

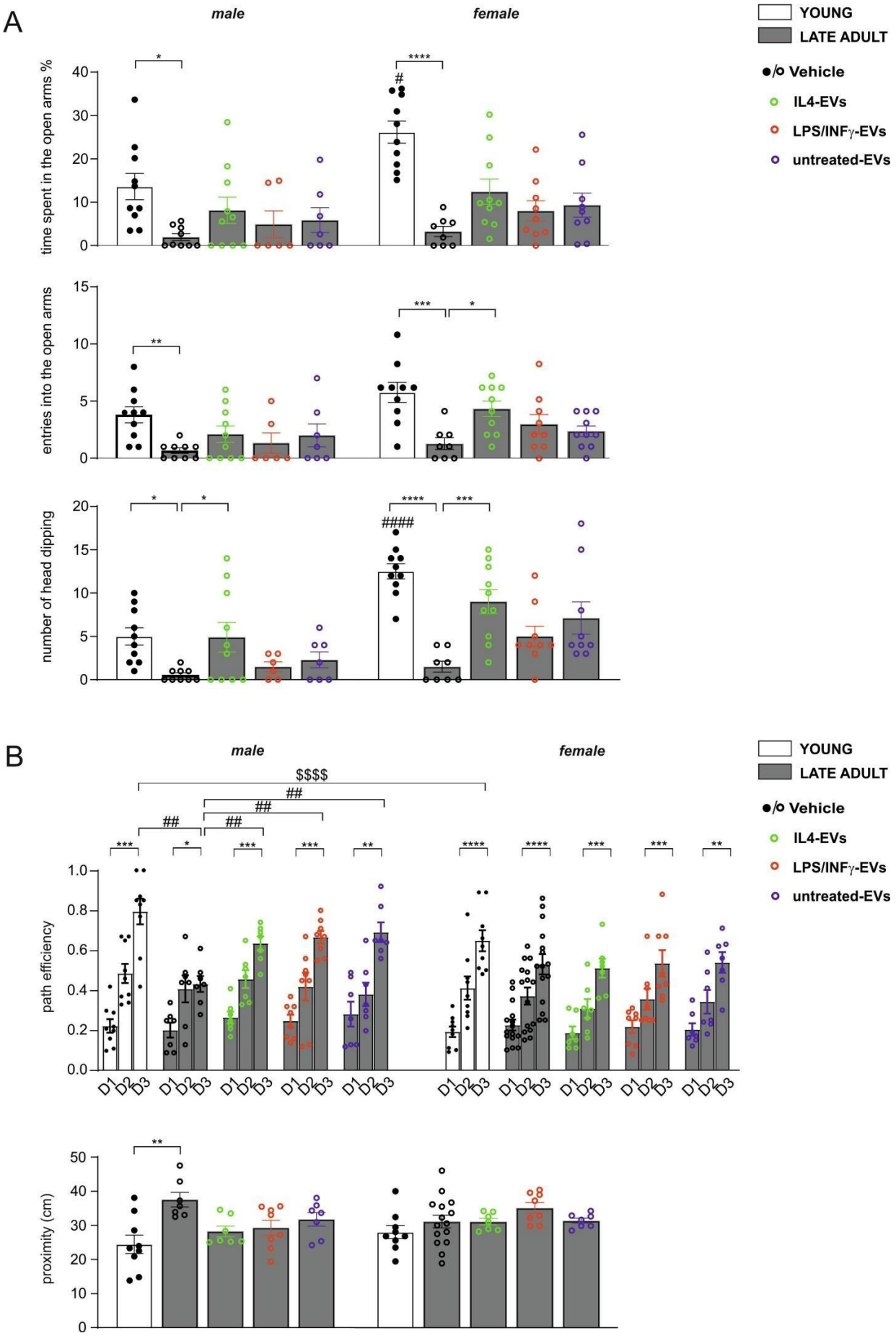

A
male
female
YOUNG
LATE ADULT
●/○ Vehicle
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
time spent in the open arms %
entries into the open arms
number of head dipping
B
male
female
YOUNG
LATE ADULT
●/○ Vehicle
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
path efficiency
proximity (cm)
D1
D2
D3

**Figure 14. (**A) Time spent in the open arms (%), number of entries into the open arms and number of head dipping in the elevated plus maze test performed by late adult male and female mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Data are the mean ± SEM, n ≥ 6 animals/experimental group, D'Agostino & Pearson test passed, *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001 vs late adult vehicle treated mice and #p < 0.05, ####p ≤ 0.0001 males vs females, one-way ANOVA, Kruskal-Wallis multiple comparisons test. (B) Path efficiency and proximity in the Morris water maze test performed by young and late adult male and female mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as control. Data are the mean ± SEM, n ≥ 7 animals/experimental group, Shapiro-Wilk test passed. For path efficiency, two-way ANOVA, Tukey's multiple comparisons test *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 D3 vs D1 in each experimental group and ##p ≤ 0.01 vs D3 of late adult vehicle treated mice between groups, $$p ≤ 0.0001 vs D3 of females vs males. For proximity one-way ANOVA, Dunnett's multiple comparisons test **p ≤ 0.01 vs late adult vehicle treated mice.

## 7.7 Long term effects of BV2-derived EVs on microglia inflammatory profile

To evaluate if the effect of EVs treatment on inflammatory genes expression in aged microglia/macrophages was long-lasting, the expression levels of *Il6, Tnfα, Il1b, Cd86, Cd206, Cd163, Cx3cr1, Trem2* and *P2ry12* were analysed in vehicle- and EVs-treated late adult mice 14 days after the last administration of EVs. We observed that the reduced expression of inflammatory-related genes was maintained only upon IL4-EVs treatment in male mice (Fig. 15 A). Only in IL4-EVs treated female mice, *Cd206* and *Cd163* were maintained over time, demonstrating an additional sex-dependent difference (Fig. 15 B). Of note, no modification of homeostatic genes was observed upon EVs treatment even after 14 days in both sexes (Fig. 15). In summary, this result suggest that the effect of IL4-EVs persist, with sex specific differences, after two weeks from the last administration.

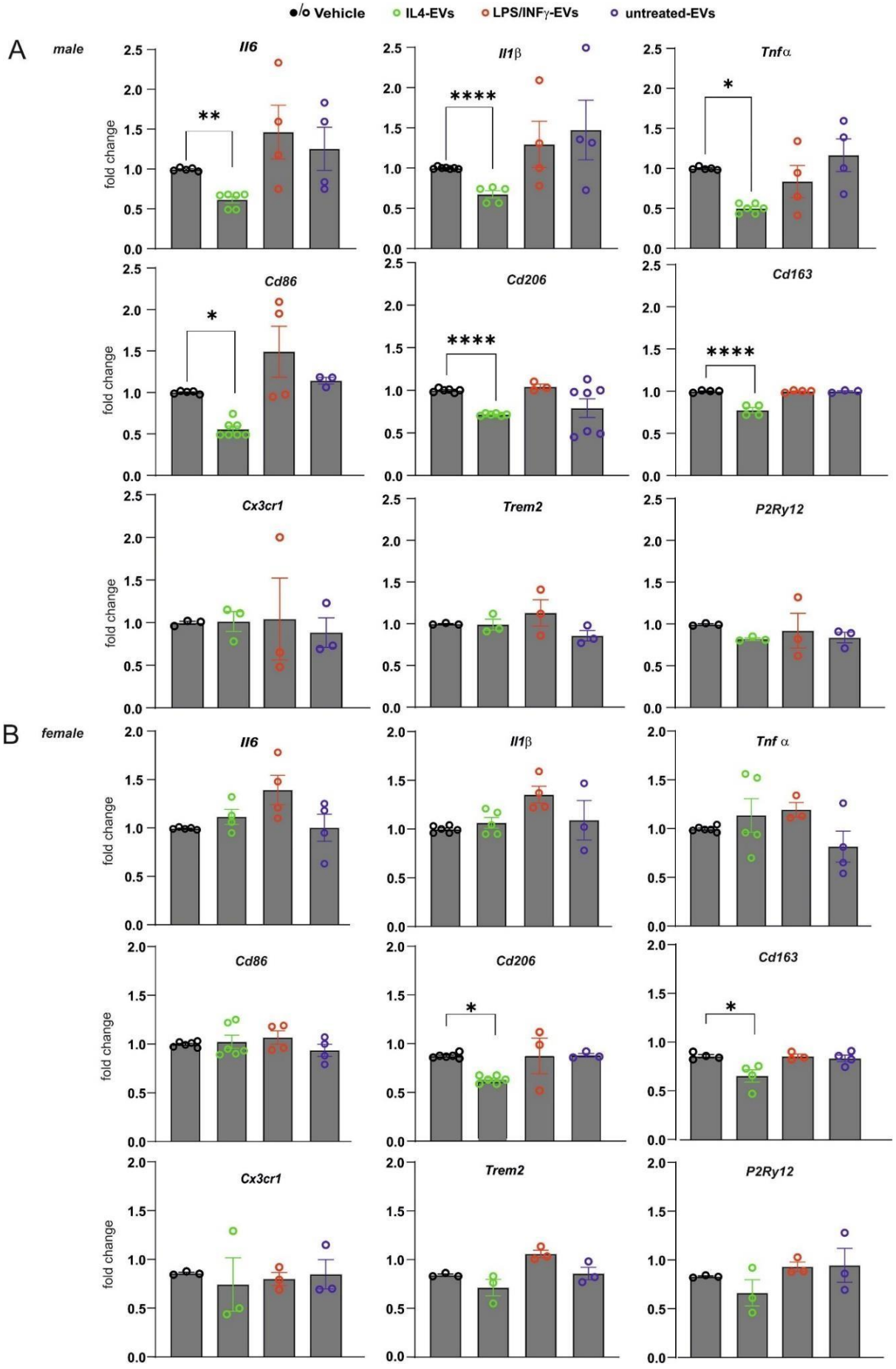

Vehicle
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
A
male
Il6
Il1β
Tnfα
Cd86
Cd206
Cd163
Cx3cr1
Trem2
P2Ry12
fold change
B
female
Il6
Il1β
Tnf α
Cd86
Cd206
Cd163
Cx3cr1
Trem2
P2Ry12
fold change

**Figure 15.** RT-PCR of inflammatory-related genes and microglia homeostatic genes (*Il6, Il1β, Tnfα, Cd86, Cd206, Cd163, Cx3xr1, Trem2, P2ry12*) in CD11b+ cells isolated from late adult male mice treated with vehicle, IL4-EVs or LPS/INFγ-EVs. Data are the mean ± SEM of fold increase (gene expression was normalized to Gapdh); $n \geq 3$ animals/experimental group; Shapiro-Wilk test passed; *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$; Student's t-test. (B) RT-PCR of inflammatory-related genes and microglia homeostatic genes (*Il6, Il1β, Tnfα, Cd86, Cd206, Cd163, Cx3xr1, Trem2, P2ry12*) in CD11b+ cells isolated from late adult female mice treated with vehicle, IL4-EVs or LPS/INFγ-EVs. Data are the mean ± SEM of fold increase (gene expression was normalized to Gapdh); $n \geq 3$ animals/ experimental group; Shapiro-Wilk test passed; *$p < 0.05$, **$p \leq 0.01$; Student's t-test.

## 7.8 BV2-derived EVs modulate hippocampal LTP with sex and region dependent differences

Cognitive and behavioral performances could correlate with neuronal hippocampal plasticity. Specifically, anxiety-like behaviour and spatial learning/ memory are mainly dependent on the ventral (VH) and dorsal (DH) hippocampus, respectively (*Moser et al., 1995; Bannerman et al., 2014*). We investigated whether EVs treatment affects synaptic plasticity by performing LTP experiments on acute ventral and dorsal hippocampal slices and measuring changes in fEPSP amplitude with time after a high-frequency stimulation protocol. First, we observed a sex- and region-dependent fEPSP impairment with age: compared to young controls, late adult female mice had a decreased fEPSP amplitude in the VH (Fig. 16, C-D, left), while late adult male mice had a decreased fEPSP amplitude in the DH (Fig. 16, A-B, right). Furthermore, in both sexes, we observed that in the VH all treatments increased the LTP amplitude compared to the late adult mice, but only in females EVs restored young LTP level (Fig. 16 A-B-C-D left). These data suggest that the effect of EVs on LTP in the VH is not dependent on the vesicular content, being similar for IL4-EVs, LPS/INFγ-EVs and untreated-EVs, and support the evidence on anxiety-like behaviour. In males, young and late adult mice differ in DH plasticity and EVs treatment had no effects (Fig. 16 A-B right). Differently, in female DH, LTP is similar in young and late adult mice and again EVs treatments had no effect (Fig. 16 C-D right) resembling what observed in spatial learning and memory performances (see Fig. 14 B).

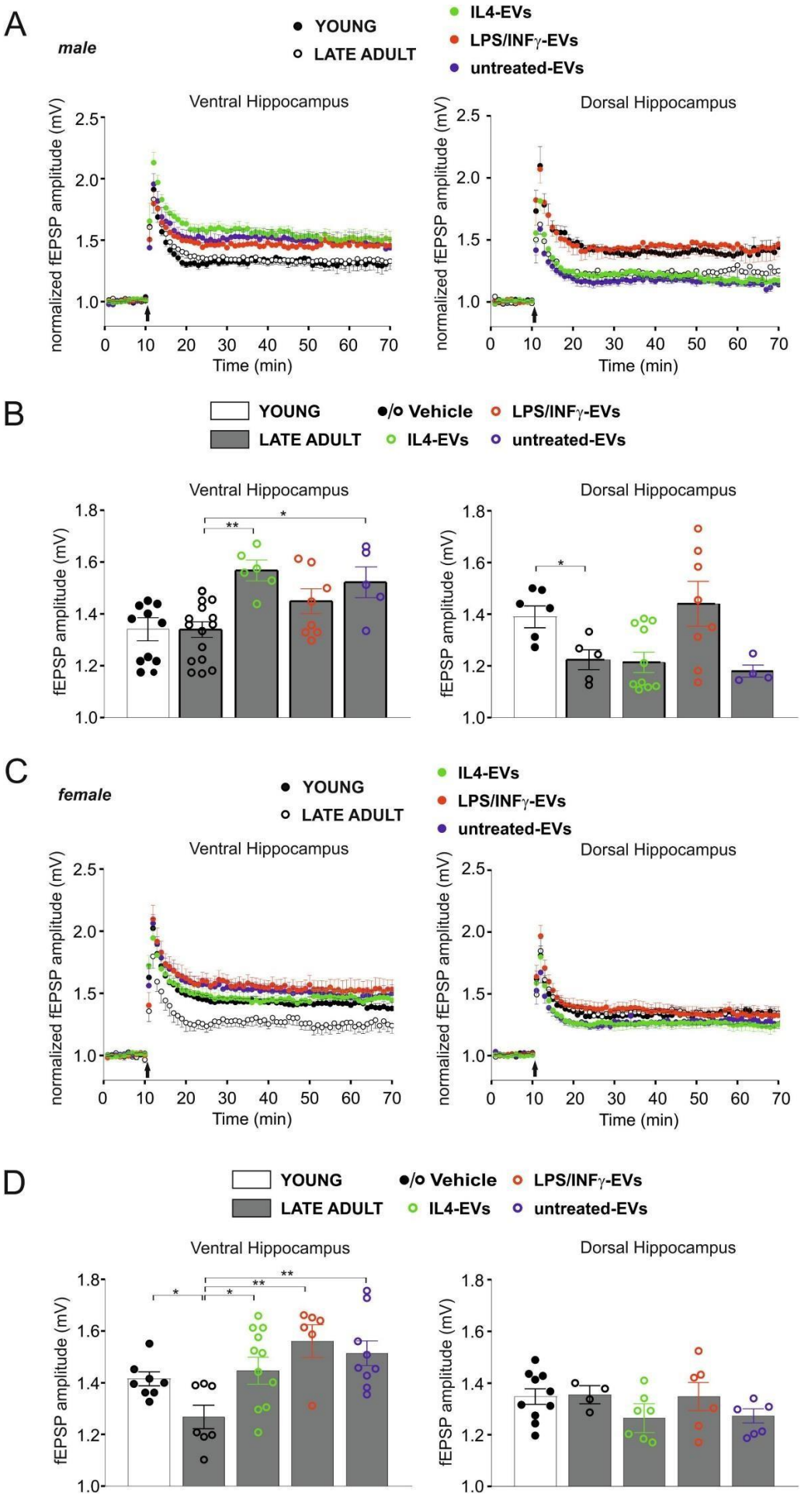

A
male
YOUNG
LATE ADULT
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
Ventral Hippocampus
Dorsal Hippocampus
normalized fEPSP amplitude (mV)
Time (min)
B
YOUNG
LATE ADULT
●/○ Vehicle
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
Ventral Hippocampus
Dorsal Hippocampus
fEPSP amplitude (mV)
C
female
YOUNG
LATE ADULT
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
Ventral Hippocampus
Dorsal Hippocampus
normalized fEPSP amplitude (mV)
Time (min)
D
YOUNG
LATE ADULT
●/○ Vehicle
IL4-EVs
LPS/INFγ-EVs
untreated-EVs
Ventral Hippocampus
Dorsal Hippocampus
fEPSP amplitude (mV)

**Figure 16.** (A) Time course of normalized averaged amplitudes of fEPSPs recorded from CA1 region of VH (left) and DH (right) following Schaffer collateral HFS (2 trains of stimuli at 100 Hz of 1 s duration each, 3 s inter-train interval, arrow) in late adult male mice treated with vehicle (VH: n/N=15/6; DH: n/N=5/3), IL4-EVs (VH: n/N=6/3; DH n/N=: 10/4), LPS/INFγ-EVs (VH: n/N=8/3; DH: n/N=8/3) or untreated-EVs (VH: n/N=5/2; DH: n/N=4/3). Young animals (VH: n/N=11/5; DH: n/N=6/2) were used as controls. n/N refers to the number of slices (n) on the total number of mice (N) analysed. Symbols represent the mean ± SEM of the normalized fEPSPs amplitude evoked every 20 s. (B) Comparison of the mean LTP amplitudes, recorded at 35 min after HFS, in the VH (left) and DH (right) oflate adult male mice treated with vehicle, IL4-EVs, LPS/ INFγ-EVs or untreated-EVs. Young animals were used ascontrols. n/N refers to the number of slices on the total number of mice analysed. Data are the mean ± SEM, dots represent individual slices. Shapiro-Wilk test passed; *p < 0.05, **p < 0.005, vs late-adult vehicle treated mice; one-way ANOVA; Holm-Sidak for post-hoc comparison. (C) Time course of normalized averaged amplitudes of fEP-SPsrecorded from CA1 region of VH (left) and DH (right) following Schaffer collateral HFS (as in A) in late adult female mice treated with vehicle (VH: n/N=7/2; DH: n/N=4/2), IL4-EVs (VH: n/N=11/3; DH: n/N=7/3), LPS/INFγ-EVs (VH: n/N=6/3; DH: n/N=6/3) or untreated-EVs (VH: n/N=9/3; DH: n/N=6/3). Young animals (VH: n/N=8/4; DH: 10/5) were used as controls. n/N refers to the number of slices on the total number of mice analysed. Symbols represent the mean ± SEM of the normalized fEPSPs amplitude evoked every 20 s. (D) Comparison of the mean LTP amplitudes, recorded at 35 min after HFS, in the VH (left) and DH (right) of late adult female mice treated with vehicle, IL4-EVs, LPS/INFγ-EVs or untreated-EVs. Young animals were used as controls. n/N refers to the number of slices on the total number of mice analysed. Data are the mean ± SEM, dots represent individual slices. Shapiro-Wilk test passed; *p < 0.05, **p < 0.005, vs late adult vehicle treated mice; one-way ANOVA; Holm-Sidak for post-hoc comparison.

# 8. Discussion

Maintenance of normal brain functions account on microglial cells, thus age-related changes of these cells represent cause and/or effect of dysfunctional neural processes as demonstrated in many neurodegenerative diseases (*Rim et al., 2024; Malvaso et al., 2023*). Deciphering the aging processes has long been considered a possible tool to contrast aging progression. In the brain of male and female mice, aging is characterized by increased levels of inflammatory cytokines, also with the contribution of microglial cells (*Porcher et al., 2021*). In the human brain, degeneration of microglia progressively occurs over time, leading to a gradual weakening of brain cells interactions and communication (*Streit et al., 2004*). EVs represent a novel mechanism of cell-to-cell communication that directly delivers molecular information from donor to target cells under physiological and pathological conditions (*Budnik et al., 2016*).

In the present work, we investigated the hypothesis that microglial intercellular communication through the release of EVs plays a role in the aging-related inflammatory process. To verify this hypothesis, we took advantage of a non-invasive intranasal administration protocol to deliver EVs isolated from microglial-like BV2 cells treated to induce inflammatory or anti-inflammatory phenotypes. Indeed, previous studies reported that intranasal administration was more efficient in delivering EVs cargoes to the rodent brain in comparison with intravenous administration (*Bet-zer et al., 2017*). Although EVs concentration reaching the brain might be reduced due to lungs and gastrointestinal tract dilution, intranasal administration represents a route easily reproducible in humans. EVs distribution upon intranasal administration revealed successful delivery to the brain, however it must be considered that PKH labelling may impact the size of EVs compared to unlabeled EVs, interfering with the biodistribution of nanoparticles *in vivo* (*Dehghani et al., 2020*). Nevertheless, our NTA analysis demonstrated that there were not significant differences in the size of PKH-labeled and unlabeled EVs (data not shown).

Yang and collaborators documented that EVs derived from BV2 cell lines stimulated with LPS contain cytokines like TNFα and IL6, along with ribosomal components, focal adhesion molecules, extracellular matrix elements, and membrane proteins. (*Yang et al., 2018*). On the other hand, BV2-derived EVs expressing the cytokine IL4, were capable to propagate an anti-inflammatory response, in a mouse model of multiple sclerosis, by upregulating microglia and macrophage expression of *ym1* and *arg-1*, which dampened neuroinflammation and provided protective effects to tissues (*Casella et al.,2018*). In accordance, we reported that the composition of EVs released by microglia-like BV2 cells treated with an inflammatory stimulus (i.e. LPS/ IFNγ and IL4) mirrors the distinct inflammatory profiles of the donor cells. Consistently, previous work documented that macrophages and dendritic cells-derived vesicles resemble the inflammatory state of original cells (*Viaud et al., 2009; Garzetti et al., 2014*). The EV preparations that we used

for intranasal treatments were not homogeneous but enriched in medium/large EVs; according to recent reports, the complete separation between medium/large and small EV populations is not achieved either by centrifugation or by other isolation methods (*Welsh et al. 2024*). However, we confirmed that this treatment efficiently targets EVs into the hippocampal microglia, as already shown for other brain regions (*Attaluri et al., 2023*), supporting the efficacy of this experimental approach also in aged mice.

## *EVs restored juvenile microglia in both sexes*

In this study, we observed that female microglia progressively alter the expression level of inflammation-related and homeostasis-related genes with age, while male microglia exhibit marked variations among young and late-adult ages. These results are in line with transcriptomic and gene ontology studies, which reported a sex-biased overexpression of genes in male microglia linked to "defense response to bacteria" and other inflammatory processes over time (*Guneykaya et al., 2018; Li et al., 2023*). On the contrary, female microglia genes were more associated to tissue repair, morphogenesis and development (*Villa et al., 2018*). This reparative phenotype as well as the differential responsiveness of the female immune system upon bacterial and viral infections might be likely explained by an anti-inflammatory activity of female sex hormones (*Vegeto et al., 2001; Soucy et al., 2005; Habib et al., 2013*). Moreover, aging affects the kinetics of transcription and the structure of transcripts at a molecular level in both sexes (*Debès et al., 2023*); for instance transcript expression decreases with gene length during aging in humans and other animals (*Soheili-Nezhad et al., 2024*). This, together with epigenetics features, such as low accessibility of chromatin, loss of histones and heterochromatin, could underline the increase in inflammatory genes observed in aged microglia (*Zhang et al., 2021*). In our experiments, we documented sex-related differences in the responses to BV2-derived EVs. Interestingly, the observed effect on microglia inflammatory gene expression and morphology were independent on the inflammatory status of the donor cells. Indeed, among the 248 inflammatory mRNAs examined only a small fraction differed in EVs from pro- and anti-inflammatory cells when compared to EVs from unstimulated cells. We speculate that the effects on the microglia inflammatory profile may not always depend on the specificity of the cargo translocated by EVs. In fact, we cannot rule out the transfer, through EVs, of additional elements, such as membrane or signalingproteins, from large EVs. Therefore, additional -omics analysis on all the EVs components wouldbe necessary to identify the common element involved. Nevertheless, cargo-specificity effects have been observed in the morphology of microglial cells, which was differently modulated by EVs released by IL4- or LPS/INFγ-treated microglia.

### *EVs treatment ameliorated brain functionality*

Aging comes with progressive deterioration of cognitive functions, which affects sensory, motor, and intellectual capabilities. Among the main age and inflammation-associated alterations, there are the anxiety-like behaviors (*Shoji et al., 2016*) as well as the spatial learning and memory (*Singhal et al., 2020*). In line with that, our findings confirmed that both male and female late adult mice develop anxiety-like behavior, as assessed using the elevated plus maze test. Notably, the intranasal administration of IL4-EVs significantly reduced this behavioral impairment in females, almost entirely reversing it; on the contrary, the effect observed in males was only partial. This sex-related diverse outcome could likely mirrors the more severe inflammatory profile observed in late adult male mice. Moreover, we observed that aging did not affect spatial learning and memory in late adult females, thus they do not benefit from EVs treatment. Conversely, late adult male mice displayed an impairment in those cognitive domains and a partial recover upon EVs treatments. Interestingly, in microglia of male mice, which experienced only a partial reduction in anxiety-like behavior, we observed a persistent reduction of the inflammatory gene expression even after 14 days after EVs administration, specifically induced by IL4-EVs. At difference, the effects on inflammatory gene expression profile are almost completely extinguished in females, whose anxiety-like behaviour almost totally disappeared. This observed sex-specific difference in IL4-EVs impact between the sexes may reflect distinct variations in the response timing in males versus females, suggesting the need of additional behavioral studies in older micefor further clarification.

Several studies reported the functional discrimination of hippocampal regions, which account for anxiety-like behaviors and spatial learning and memory: namely lesions of the VH are responsible for modifications of innate anxious behaviors, while DH damages impact mainly spatial memory (*Kjelstrup et al., 2002; Potvin et al., 2006*). However, these complex cognitive domains are indeed related to multiple cerebral regions; nevertheless, hippocampal LTP in the dorsal region is considered a good neurophysiological correlate of spatial learning and memory processes. In fact, aging has been linked to reduced hippocampal LTP in rodent brains, which impair their capacity for consolidating long-term memory. (*Lister & Barnes, 2009*). Consistently, previous studies in rats correlated the age-dependent rapid decay of hippocampal LTP with poorer performance on spatial memory tasks (*Barnes & McNaughton, 1980*). Our electrophysiological measurments, confirmed the effect due to aging in DH LTP in males, but no differences were observed upon EVs treatments, differing from what observed in spatial and memory test, where EVs treatment partially modified the age-dependent impairment. On the other hand, DH LTP in females was not altered by age or by EVs treatment, resembling data obtained in the spatial learning and memory behavioral tests.

In line with a role of VH in regulating anxiety-like behaviors, our finding showed that EVs treatment abolished the anxiety-like behavioral defects and rescued the LTP impaired due to age only in females. Collectively, IL4-EVs produce the largest effects in almost all experiments performed, particularly at the behavioral level, where no effects were observed from untreated-EVs and LPS/INFγ-EVs. We speculate that the observed effects of BV2-derived EVs could rely on a common cargo content that modulates molecular, morphological and plasticity outcomes on aging. Interestingly, untreated-EVs have no effect on DH LTP in either sex, but do impact VH LTP, confirming evidence that the hippocampus is regulated by microglia-derived factors in a region dependent manner (*De Felice et al., 2022*).

## *Conclusion*

To conclude, this study suggest that EVs can be exploited to modify inflammaging in late adult mouse model. In accordance with other studies, our results support the hypothesis to use microglial derived EVs as tools to modulate the inflammatory responses in the brain (*Carandini et al., 2015; Aguzzi & Rajendran, 2009*), with potential therapeutic applications (*Grimaldi et al., 2019; Pan et al., 2023*). Strategies taking advantage of EVs delivery would benefit not only patients with aging-related disorders but with neurodegeneration and inflammatory CNS conditions as well. Therefore, the use of a cell line as a source of EVs would enable the availability of large-scale product for future applications. Moreover, we measured the LPS carried over in the EVs preparation and found out that contamination of LPS/INFγ-EVs used for treatments was neglectable (0.015 ± 0.003 pg/mL, data not shown). In addition, nanostring analysis showed that IL4 e IFNγ are not differently expressed in EVs derived from stimulated cells compared to EVs released from untreated cells.

Exploring sex-related differences in neuroinflammation and microglia activation would mean a progress toward personalized medicine and an important point to address the under-representation of women in clinical trials, which still represents a public health burden (*Labots et al., 2018*).

Even though further experiments are needed to better understand the mechanisms underlying EVs-mediated cell-to-cell communication and EVs specific cargoes, the approach proposed in this research paves the way to new possible strategies to contrast the aging process.

# 9. Collaborations

NTA analysis of EVs has been performed in collaboration with Dr. Igea D'Agnano's Lab (Institute of Biomedical Technologies, CNR, 20054, Segrate (MI), Italy) and Prof. Roberto Piacentini (Department of Neuroscience, Università Cattolica del Sacro Cuore, Rome, Italy*)*

Behavioral tests have been performed in collaboration with Prof. Fiorenzo Conti's Lab (Università Politecnica delle Marche, Department of Experimental and Clinical Medicine, Ancona 60126, Italy).

Electrophysiological analysis has been performed in collaboration with Prof. Laura Maggi (Department of Physiology and Pharmacology, Sapienza University, Rome, Italy*).*

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