Integration of unpaired transcriptome and epigenome data of mouse whole brain and computational analysis for finding clues of aging

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Integration of unpaired transcriptome and epigenome data of mouse whole brain and computational analysis for finding clues of aging

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Soli Deo Gloria.

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ABSTRACT

Integration of unpaired transcriptome and epigenome data of mouse whole brain and computational analysis for finding clues of aging

Aging is associated with changes in cellular composition and signaling pathways in the brain, leading to an increased risk of neurodegenerative diseases. While the risk factors of specific aged cell types or the changes in the specific brain regions related to disease are well-studied, little is known about the intrinsic features of whole mouse brain aging.

We performed single-nucleus RNA sequencing (snRNA-seq), single-cell RNA sequencing (scRNA-seq), and single-nucleus ATAC sequencing (snATAC-seq) and integrated an unpaired multiomics dataset to explore the transcriptional and epigenomic alterations in the aged mouse brain. Focusing on alterations within cell types, we uncovered age-related populations, such as age-related microglia (Micro-3), age-related capillary endothelial cells (Endo-C2), and Meis2 high-expression GABAergic neurons. Our findings also revealed significant shifts in intracellular interaction and signaling pathways, including GRN (Granulin) and RELN (Reelin), which show increased neuronal involvement in the aged brain. Notably, Meis2 high GABAergic neurons and ENDO-C2 endothelial cells exhibit prominent alterations in signal reception and transmission, correlating with upregulated inflammatory markers and compromised vascular integrity.

All of these changes highlight the critical roles of inflammatory microglia and signaling between neurons and endothelial cells in aging-related neurodegeneration, providing potential markers of cognitive decline and brain diseases.

Key words: aging, brain, multiomics, single cell transcriptomics, single cell epigenomics, integration, microglia, endothelial cells, GABAergic neurons, neurodegenerative disease

1. Introduction

1.1. The Study of Aging

While human lifespan has increased dramatically in recent years, improvements in healthspan, the period of life in which a person is disease-free, have been more modest.[1] Aging is a complex and multifaceted process characterized by a progressive decline in organic function, an increased vulnerability to disease and death. Aging is driven by specific hallmarks that fulfill the following three criteria: (1) they exhibit changes associated with aging, (2) their experimental enhancement accelerates aging, and (3) therapeutic interventions targeting them can decelerate, halt or even reverse aging. Based on these criteria, twelve hallmarks of aging have been proposed: genomic instability, telomere shortening, epigenetic alterations, loss of proteostasis, disabled macroautophagy, dysregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. [2] These hallmarks are interconnected and also related to the hallmarks of health, which encompass features such as spatial compartmentalization, homeostasis maintenance, and proper stress responses. [Figure 1]

1.1.1. Genomic Instability

Genomic instability refers to the increased tendency of the genome to acquire mutations and alterations over time. This process is fundamental to aging and occurs due to various factors : 1) DNA damage from external sources like UV radiation or chemical exposure, 2) interal sources of damage like ROS, and 3) errors in DNA replication and repair mechanisms. As we age, the accumulation of these genomic alterations can lead to cellular dysfunction, senescence, or cancerous transformations. Research has shown that interventions targeting DNA repair mechanisms can potentially show down the aspect of aging.[3,4][Figure 2]

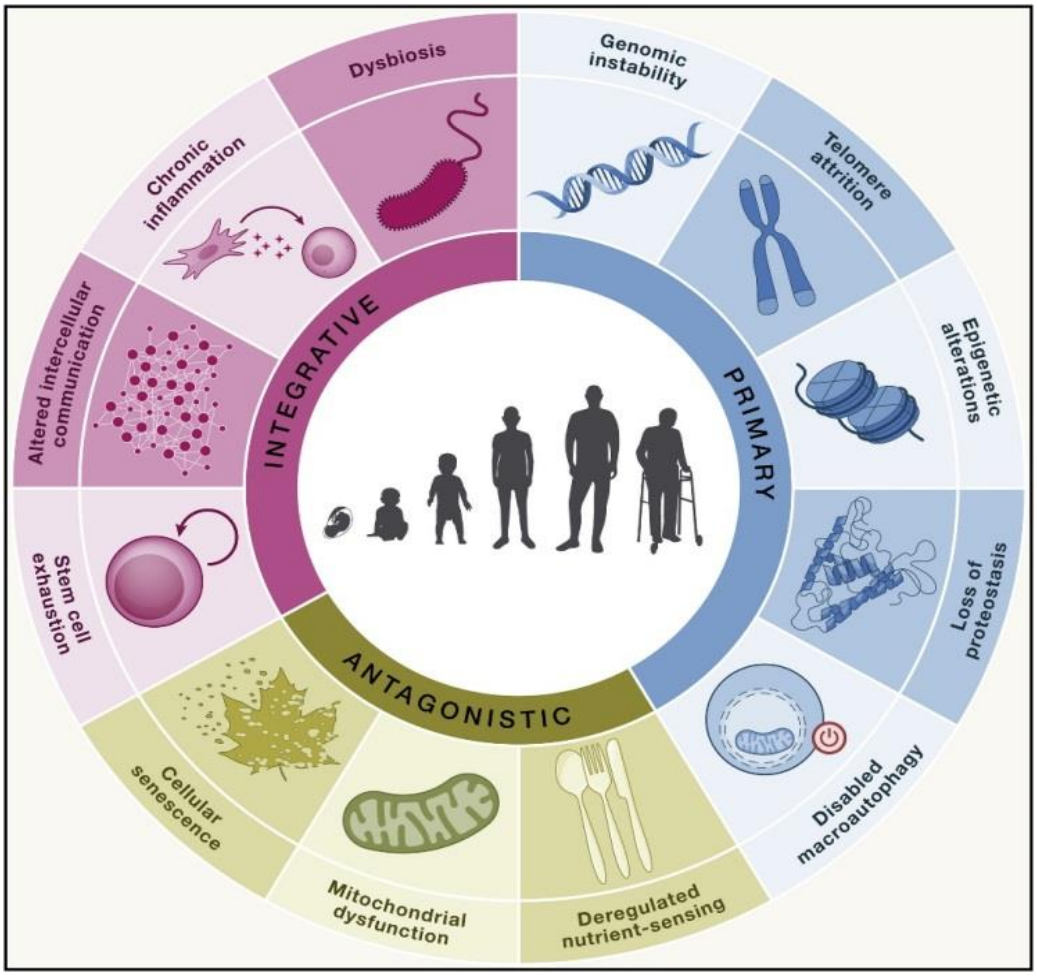

Figure 1. The Hallmarks of aging.

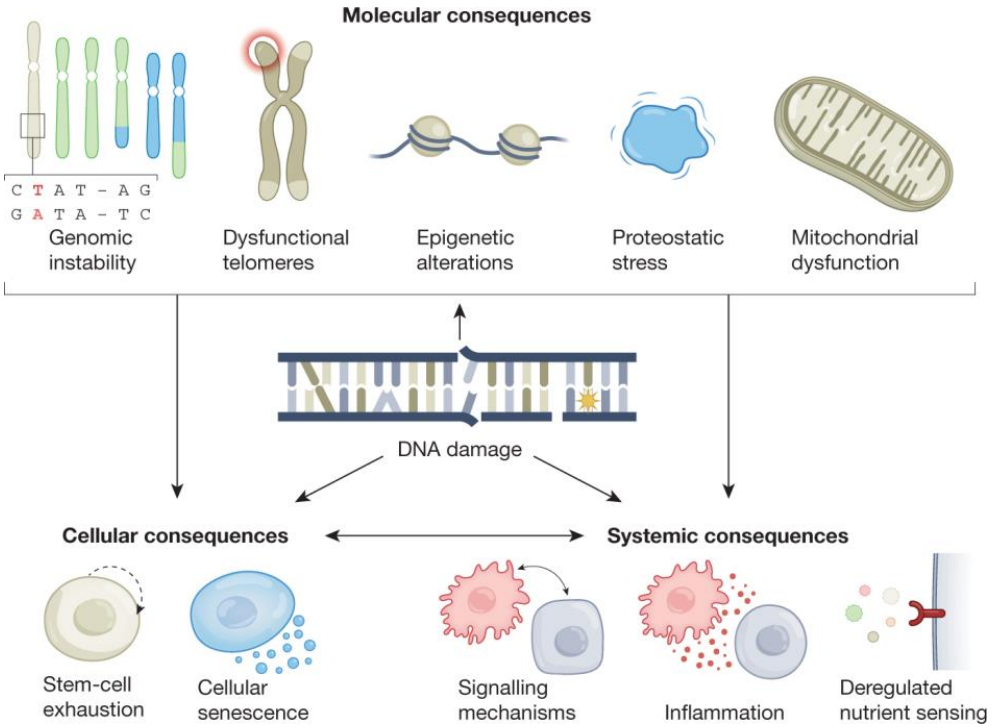

Figure 2. The central role of DNA damage in aging.

1.1.2. Telomere shorteneing

Telomeres are repetitive nucleotide sequences at the ends of chromosomes that protect them from degradation.Key points include : 1) telomeres shorten with each cell division due to the end-replication problem, 2) when telomeres reach a criticalll length, cells enter senescence or undergo apoptosis, and 3) telomere length is considered a biomarker of cellular aging. Studies have demonstrated that telomere shortening is associated with various age-related diseases and that telomerase activation can potentially reverse some aspects of cellular aging. [5,6] [Figure 3]

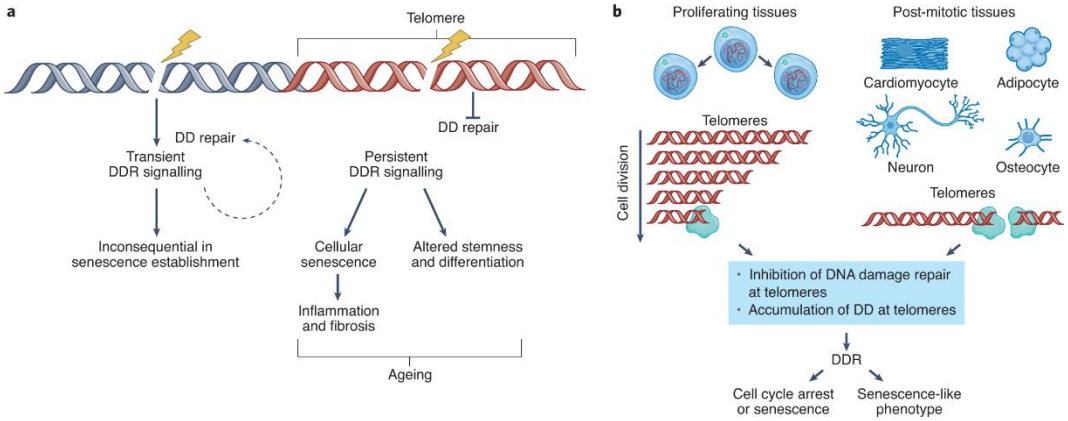

Figure 3. Telomere dysfunction and aging.

1.1.3. Epigenetic Alterations

Epigenetic changes involve modifications to gene expression and protein translation that don't affect the DNA sequence itself. These include :1) DNA methylation patters, 2) histone modifications, and 3) chromatin structure modifications. With age, there is a general trend towards global DNA hypomethylation and site-specific hypermethylation. These changes can lead to altered gene expression profiles associated with aging. Researches have shown that epigenetic reprogramming can potentially reverse age-associated epigenetic marks.[7] [Figure 4]

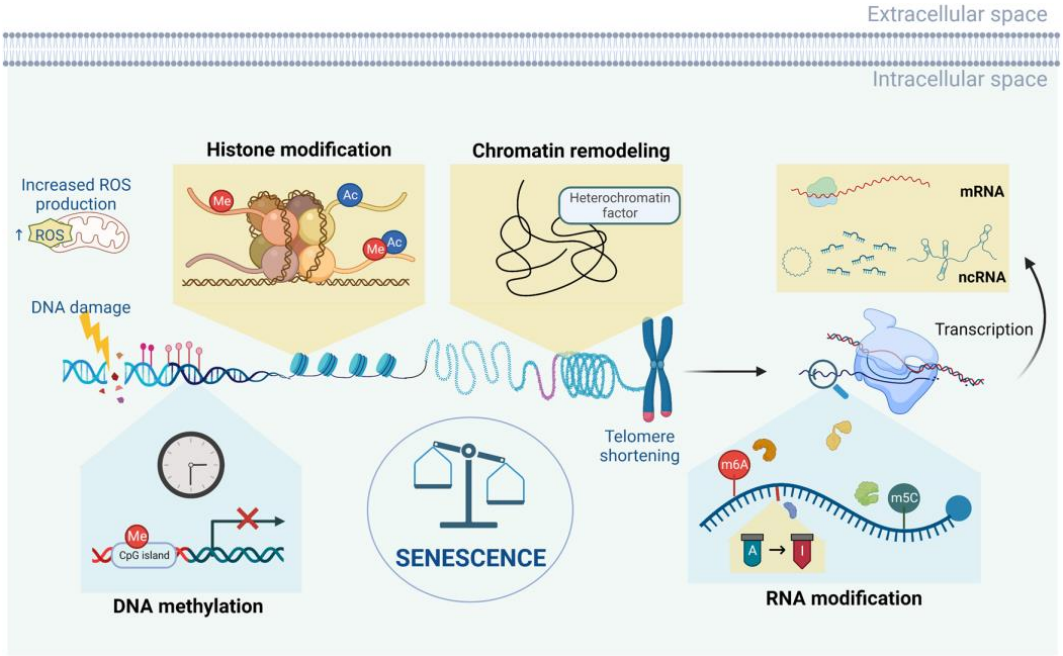

Figure 4. Epigenetic regulation of aging.

1.1.4. Loss of Proteostasis

Proteostasis, or protein homeostasis, refers to the cell's ability to maintain the proteome through regulation of protein synthesis, folding, trafficking and degradation. Age-related decline in proteostasis includes : 1) Decreased efficiency of chaperone proteins, 2) reduced activityttt of protein degradation systems(ubiquitin-proteasome and autophagy-lysosome), and 3) accumulation of misfolded or aggregated proteins. These can lead to various age-related pathologies, such as neurodegenerative disease. Some studies showed that enhancing proteostasis mechanisms can extend lifespan in model organisms. [8,9]

1.1.5. Disabled Macroautophagy

Macroautophagy, often simply called autophagy, is a cellular 'recycling' process that degrades and recycles cellular components. With age :1) autophagy efficiencyyy declines, 2) accumulation of damaged organelles and protein aggregates increases, and 3) cellular stress resistance decreases. Impaired autophagy is implicated in various age-related diseases. Some has been published to show that stimulating autophagy can extend lifespan and healthspannnn in various model organisms. [10]

1.1.6. Dysregulated Nutrient-Sensing

Nutrient-sensing pathways play crucialll roles in metabolism and energy homeostasis. Key pathways are 1) insulin and IGF-1 signaling, 2) mTOR pathway, 3) AMPK and 4) Sirtuins. With age, these pathways become less sensitive and dysregulated, leading to metabolic imbalances. Some papers have published studies showing that modulation of thesepathways particularly through dietary restriction, can extend lifespan in various species.[11]

1.1.7. Mitochondrial Dysfunction

Mitochondria, known as the powerhouses of cells, become less efficient with age: 1) decreased ATP production leads to reduced energy supply for cellular functions, 2) increased reactive oxygen species(ROS) can contribute to cell damage through higher oxidative stress condition, 3) accumulation of mitochondrial DNA mutations and 4) altered mitochondrial dynamics because of the imbalance between fushion and fission processes can cause mitochondrial disability. These changes contribute to various age-related disease, and some rejuvenation experiments proved that changing old mitochondria to young mitochondria slows aging processes. [Figure 5] [12]

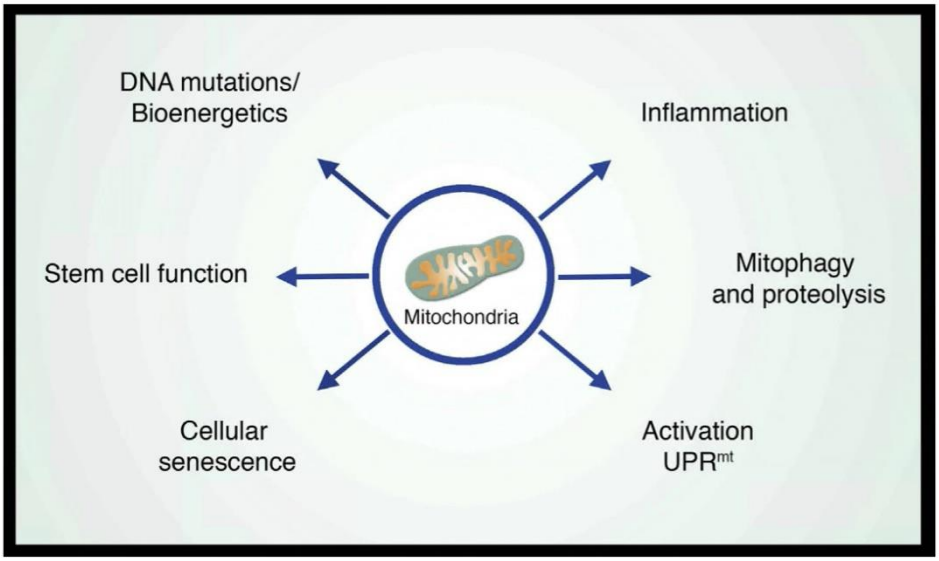

Figure 5. Mitochondria as Regulators of Organismal Aging.

1.1.8. Cellular Senescence

Senescent cells accumulate with age, no longer dividing but remaining metabolically active: 1) cell cycle arrest with inhibitor(p16 and p21), 2) morphological changes (enlargement and altered nuclear shape), 3) senescence-associated secretory phenotype (SASP) such as cytokines, growth factors and proteases, 4) telomere shortening, and 5) DNA damage. The accumulation of senescent cells accelerates aging process and impairs tissue function, sometimes with chronic inflammation. These days, senolytics which remove senescent cells are big interests for aging studies. [13]

1.1.9. Stem Cell Exhaustion

The regenerative capacity of tissues declines as stem cell function and number decrease with age : 1) reduced stem cell pools, 2) impaired self-renewal capacity, 3) altered differentiation potential, 4) changes in the the supportive microenvironment for stem cells and 5) epigenetic alteration of stem cell. These changes compromise tissue homeostasis and repair capacity, fastening the aging rate. Rejuvenating stem cell experimentally reversed the aging feature with some studies. [14]

1.1.10. Altered Intercellular Communication

Changes in signaling between cells contribute to tissue dysfunction. In immune system, increased infammatory signalling like NF-kB promotes immune cells nearby secreating cytokines and induces chronic inflammation. In endocrine system, aged endocrine cells fail to control to secreting hormones or become dysfuntional, else responder cells become dysfunctional. This can happen in brain, causing neuroendocrine deregulation and leading neurodegeneration. Extracellular matrix(ECM) alteration can affect cell-cell interactions physically. Modulating intercellular communication pathways can influence aging process, so some pathway studies through multiple cell types are important to understand age-related disease. [15]

1.1.11. Chronic Infammation

Low-grade, chronic infammation, often termed “inflammaging”, increases with aging. The elevated level of inflammatory cytokines, like IL-6 and TNF-a, is the hallmark of aging. This can enhance oxidative stress by increasing ROS production and decreasing ROS clearance. Immune cells changed their proportion and function with aging and can influence several organic microenvironment. Furthermore increased autoimmune responses by production higher autoantibodies and cause age-related autoimmune disease. [16]

1.1.12. Dysbiosis

Changes in gut microbiome composition and function occur with age. The diversity of microbiome reduced, changing the ratio of beneficial bacteria and harmful barteria. Following this, altered metabolite is producted and influence aging process. Also gut microbiome associate immune system and modulate body health. The potential of microbiome modification in influencing aging and longevity is shown through several reports.[17]

The process of aging is influenced by complex molecular mechanisms involving genetic, epigenetic, and environmental factors that inhibit cell proliferation, alter metabolism and gene expression and promote age-related disease.[18]Many strategies for decelerating age-related diseases have been actively studied, including calorie restriction through reduced calorie intake and increased exercise[19]and pharmaceurical treatments targeting senescent cells and associated molecules. [20]Animal studies, especially mouse model, can provide valuable insights into aging-related research by making it easier to examine these complex molecular mechanisms in a controlled environment, thereby helping to identify potential age-related changes.[21] [22]

1.2. Aging-Related Disease in Brain

In the brain, aging is associated with changes in cellular composition, synaptic plasticity and brain structure, which can contribute to declines in cognitive abilities like learning, memory and behavioral habits.[23] Cellular senescence, hyperexcitability, inflammatory response, and alterations in proteolysis are some of the hallmarks of aging that impact both neuronal and non-neuronal cells, leading to change the expression or protein level and celllular communication.[24]

Among these, age-related neurodegenerative diseases such as Alzheimer's disease(AD), Parkinson's disease(PD) and frontotemporal dementia(FTD) are widely studied, as they lead to progressive cognitive and motor dysfunction. [25] [26] [27,28] [Figure 6] The pathogenesis of these diseases often involves the selective vulnerability of certain neuronal populations, accumulation of pathological protein aggregates (e.g., amyloid-beta, tau, TDP-43), and widespread neuroinflammation. To elucidate the mechanisms driving these changes, several studies of neurodegenerative diseases have explored the cellular and molecular landscapes of the brain from genetics to mechanisms. In AD, the hippocampus and cortical neurons are predominantly affected, while in PD, the dopaminergic neurons in the substantia nigra are primarily impacted. One study showed that accumulation of somatic single-nucleotide variants (sSNVs) increase with age.[29] For example, SNCA; which is an important locus in PD, and APOE; which is a genetic risk of both AD and PD are prevalent patients with neurodegenerative disease and it suggests that genomic alterations in brains might be linked to neurodegeneration. Some studies showed GRN loss of function with age can cause both AD and frontotemporal dementia (FTD), and they are characterized with over-accumulation of amyloid-beta, tau or TDP-45 in brain and lead cognitive decline and dementia. [30,31] [Figure 7]

Recent research suggests that aging-related changes in neuronal and glial cell states, as well as alterations in brain vasculature, contribute to the disease's progression. Therefore, dissecting the specific changes in cellular composition and gene expression profiles

associated with brain aging is key to understanding the early molecular events that predispose individuals to neurodegenerative conditions.

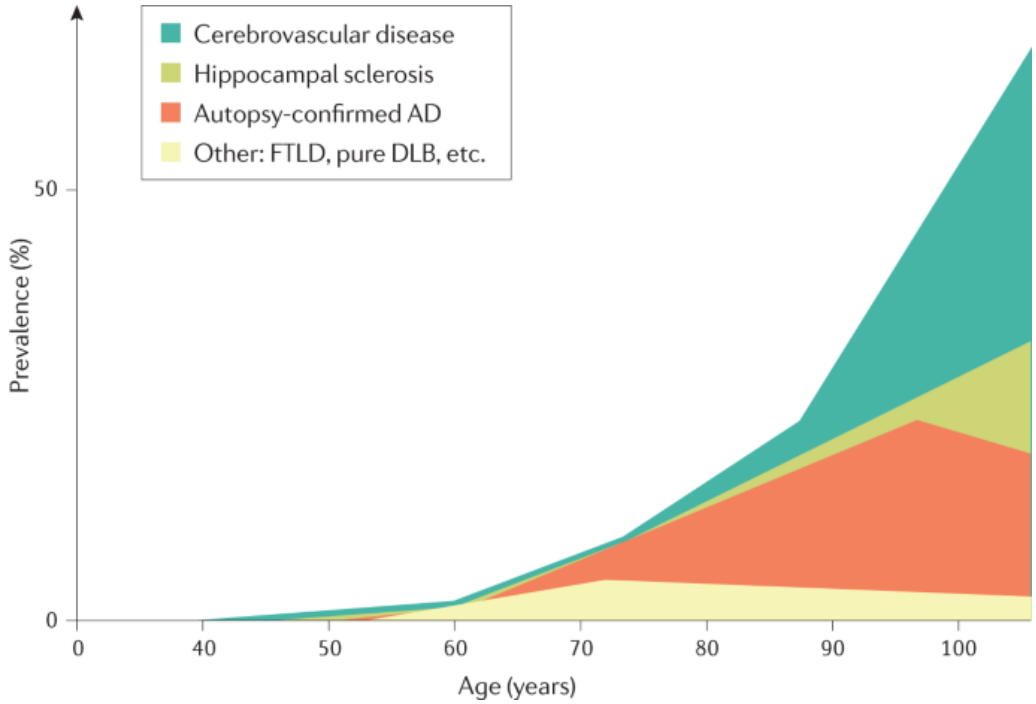

Figure 6. Age-related brain disease.

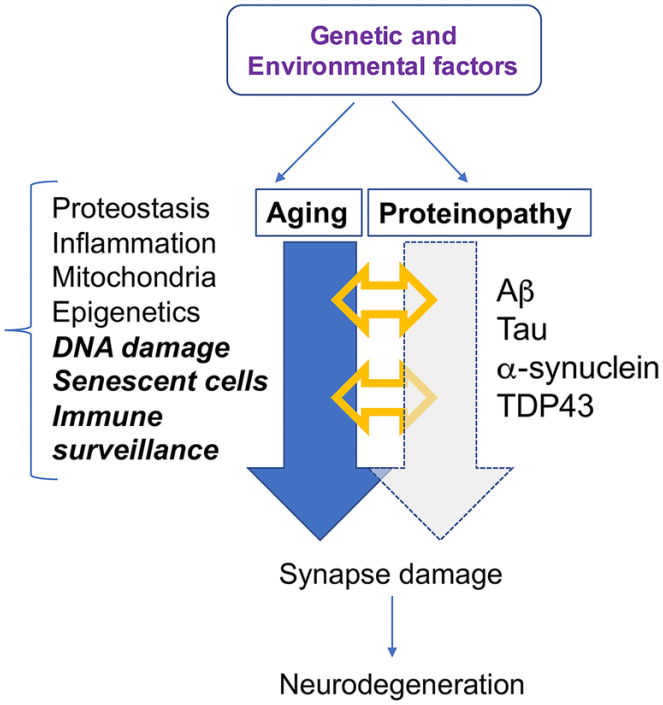

Figure 7. Proteinpathy and aging associated neurodegeneration.

1.3. The Role of Cell Types in Brain and the Changes with Aging

The brain is made up of various cell types, including neurons and glial cells, each playing unique roles. [Figure 8] Neurons are responsible for sending and receiving related to thinking, moving and sensory perception signals through synapses. Glial cells, involving oligodendrocytes, microglia and astrocytes, support and interact with neurons. Oligodendrocytes insulate neurons with the myelin sheath, while microglia act as immune cells in brain, cleaning debris and responding to damage. Astrocytes maintain the brain's environment to homeostasis state and support neural signaling. [32]

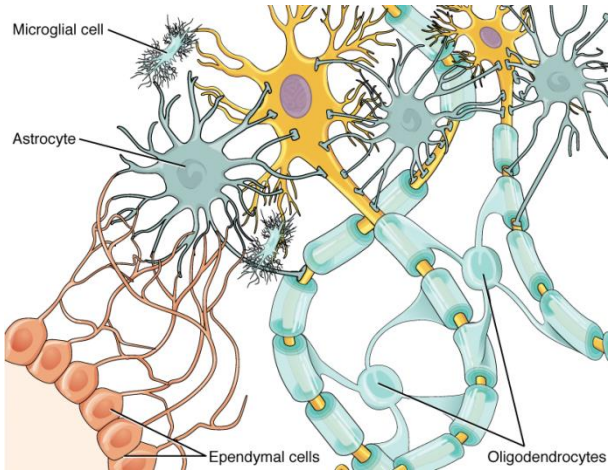

Figure 8. Anatomy of glial cells and neurons.

Understanding the age-associated alterations in different brain cell types, including neurons, glial cells, and the vasculature, may shed light on the early events that drive neurodegeneration. Microglia are the most extensively studied cells in the context of brain aging and neurodegeneration.[33] Advancements in scRNA-seq and snRNA-seq have enabled the identification of microglia in different states, deepening our understanding of their roles in Alzheimer's disease. In 5×FAD mice, Keren-Shaul et al. discovered disease-associated microglia (DAM) involved in clearing Aβ plaques, a finding confirmed in human AD brain samples. Additionally, snRNA-seq of the occipital and occipitotemporal cortex in AD patients revealed three microglial clusters: homeostatic microglia, AD1-microglia, and AD2-microglia.[34,35] These findings have advanced our understanding of how microglia contribute to age-related brain changes.

Oligodendrocytes are regarding demyelination. As the brain ages, the efficiency of myelin production and maintenance declines, affecting neural signal transmission. Park Hanseul et al found that aberrant Erk1/2 signaling is related with disease-associated oligodendrocytes(DAOs) and inhibition of Erk1/2 signaling in DAOs rescued axonal demyelination and reduced amyloid beta associated pathologies and cognitive decline in AD models.[36] Studies on oligodendrocyte function and the mechanisms behind

demyelination can provide insights into age-related cognitive decline and diseases like multiple sclerosis.

Astrocytes, previously considered merely as support cells, have now been recognized for their active role in central nervous system (CNS) health and disease. Recent discoveries of disease-associated astrocytes (DAA) and age-related astrocyte subtypes have highlighted their contributions to altered brain homeostasis, especially in the aging brain, where they may drive or exacerbate neurodegenerative processes.[37] Furthermore, astrocytes are increasingly implicated in the disruption of the blood-brain barrier (BBB) during inflammatory CNS conditions such as multiple sclerosis (MS). Astrocytic expression of VEGF-A has been identified as a key factor driving BBB permeability. In mouse models, inhibiting astrocytic VEGF-A expression was shown to reduce BBB breakdown, lymphocyte infiltration, and neuropathology, suggesting that targeting VEGF-A signaling in astrocytes could serve as a protective strategy against neuroinflammation and CNS disease progression. [38]

This knowledge can pave the way for the development of therapeutic strategies aimed at mitigating or delaying the onset of age-related diseases.

1.4. Single cell Sequencing Analysis and Multi-omics

A landmark study from the Ziesel and Hjerling (2015) labs conducted the first large-scale transcriptomic analysis of cells in the CNS, laying the foundation for understanding cellular diversity in the brain.[39] Recent technologies, including high-throughput transcriptomic, genomic, epigenomic, and spatiotranscriptomic sequencing in single-cell resolution methods, have provided unprecedented opportunities to study cellular heterogeneity and discover expression level and pathway changes implicated in brain aging.[40] [41,42] In this study, we used transcriptomics sequencing and open chromatin accessibility analysis. [Figure 9]

Transcriptomics

For detect cellular heterogeneity, droplet-based or plate-based single cell RNA sequencing methods have revolutionized transcriptomic analysis. Droplet based, such as Drop-seq, 10x Genomics Chromium, inDrop, and Seq-Well, allow high-throughput profiling of thousands of individual cells. Plate-based, such as Smart-seq and Smart-seq2, provide high sensitivity for detecting low-abundance transcripts. Enabling detailed gene expression analysis at the single cell level, researchers capture the transcriptomic profile of diverse cell populations within complex tissue like brain. These technologies are actively used to reveal specific gene expression patterns across interested cell types and uncover cellular changes in specific condition.

Open Chromatin accessibility

Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) and single-nucleus ATAC sequencing, which applies single cell technology to ATAC-seq, are widely used methods to assess chromatin accessibility at a genome-wide scale. [Figure.10] ATAC-seq captures open chromatin regions, often representing active regulatory elements or promoters. With snATAC-seq, it is possible to analyze individual nuclei epigenomic and gene regulatory changes. This approach is particularly valuable for studying how chromatin remodeling contribute to gene regulation in aging condition.

Single-cell transcriptomic technologies continuously upgrade its applification by combination with other types of '-omics' approaches such as proteomics(CITE-seq), epigenomics(10x Multiomics-Gene expression and ATAC-seq), and spatiotranscriptomics(Visium, Slide-seq). Multiple information allows for a more comprehensive understanding of the complex interactions and regulatory networks that drive aging processes. [43] [44] [45]

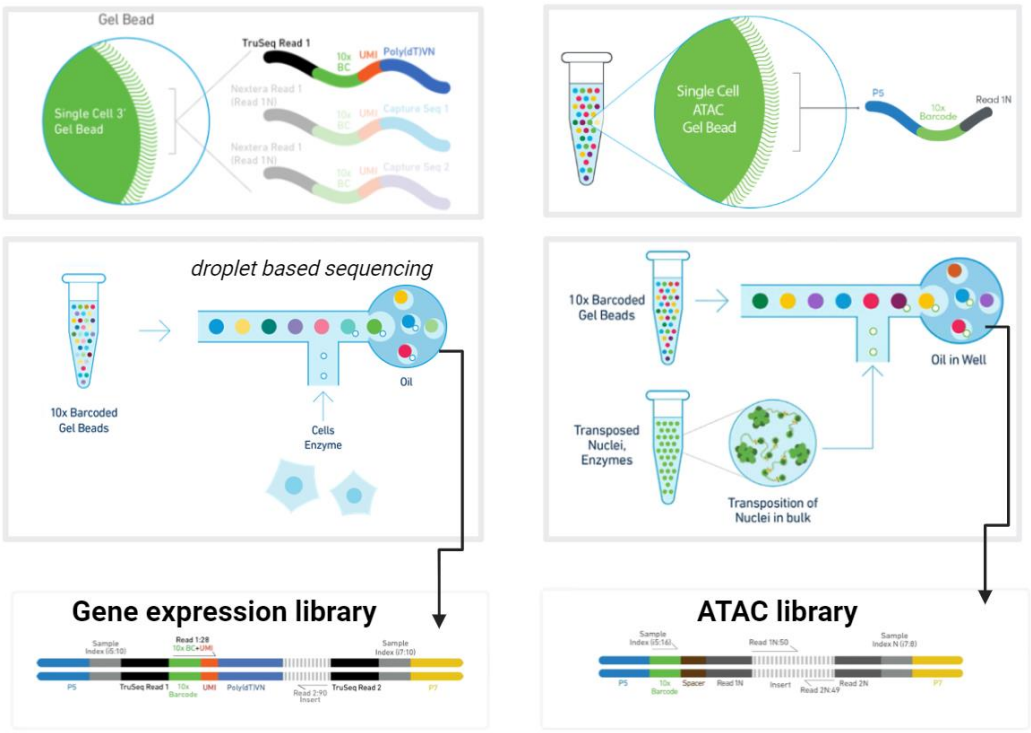

Figure 9. 10x Chromium single cell transcriptomic and epigenomic sequencing.

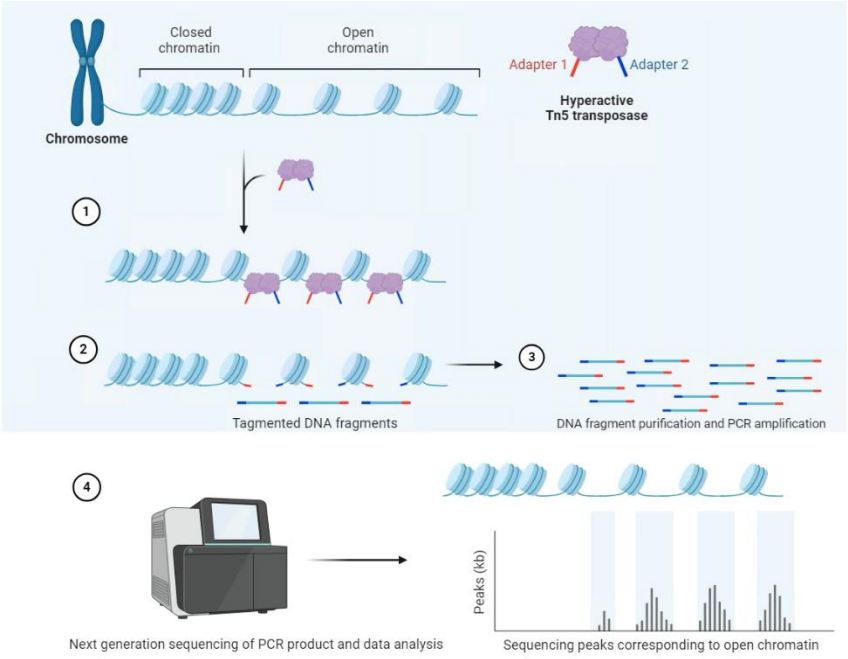

Figure 10. ATAC-seq.

2. Materials and Methods

2.1. Sample Collection and Preparation

Three young (4 months) and three old (21 month) C57/Bl6 female mice were obtained. At the defined day, mice were anaesthetized with 2.5–3% isoflurane and transcardially perfused with 10ml cold PBS to remove blood from brain. Following perfusion, the brain was dissected quickly, stored in media demonstrated for single cell isolation protocol, while immediately flash-frozen for 2 min on dry-ice and then moved to −80 °C for long term storage for single nuclei isolation.

2.2. Single-cell RNA sequencing

Single cells were isolated following a cell-isolation protocol provided by 10x Genomics. This method allowed cells to be ready for running on the commercially available high-throughput single-cell RNA sequencing technology - droplet-based 10X Chromium platform. [46] All GEMs generated were used for cDNA synthesis and library preparation using the Chromium Single Cell 3' Library Kit v3.1 (10X Genomics). We followed the manufacturer's instructions (User Guide, CG000315) for cell capture, barcoding, reverse transcription, cDNA amplification, and library construction. Libraries were quantified using an Agilent Bioanalyzer with a high sensitivity chip (Agilent) and were sequenced using the Illumina NovaSeq 6000 S4 platform, using 150-bp paired-end sequencing, using the following read lengths: 28 bp Read1, 10 bp I7 Index, 10bp I5 Index and 90 bp Read2. The 2 libraries of scRNAseq from young and old mouse brain were sequenced at 20,000 reads per cell, respectively. Single cell capture, library preparation, and sequencing were performed by Macrogen (www.macrogen.co.kr).

2.3. Single-nucleus RNA sequencing

We isolated nuclei from the cell suspension using a modified protocol provided by 10x Genomics (Isolation of Nuclei for Single Cell RNA Sequencing – CG000393). Single-nucleus suspensions were isolated from flash-frozen mouse brains stored at -80℃. Initially, the brains were minced on ice using a pre-chilled razor blade, and the chopped tissue was transferred to a tube containing a pre-cooled lysis buffer (LB; 10 mM Tris-HCl pH 7.4, 146 mM NaCl, 1mM $CaCl_2$, 21 mM $MgCl_2$, and 0.1% NP-40). The tissue was then incubated on ice for 15 minutes, during which it was triturated gently. At the end of the incubation time, wash buffer (WB; 10mM Tris, 146mM NaCl, 1mM $CaCl_2$, 21 mM $MgCl_2$), 2% BSA in PBS with 0.2 U/μl RNasin) was added to the mixture, which was then filtered through a 40-μm cell strainer and further digested mechanically for debris removal. The homogenized tissue was centrifuged at 700g for 10 minutes at 4℃. The supernatant was removed carefully to isolate the nuclei pellet.

A gradient centrifugation step was performed by mixing the nuclei suspension with 1.8M sucrose and layering it over a sucrose gradient before centrifuging at 13,000g for 45 min at 4℃. The nuclei were collected by removing the supernatant and washing the pellet in ST-SB buffer (2% BSA, 0.02% Tween-20, 10mM Tris, 146mM NaCl, 1mM $CaCl_2$, 21 mM $MgCl_2$). We counted the nuclei using a Countess II (Thermo Fisher Scientific).

This method allowed the nuclei to be ready for running on the 10x Chromium Single Cell 3′ v3 platform. All GEMs generated were used for cDNA synthesis and library preparation using the Chromium Single Cell 3' Library Kit v3.1 (10X Genomics). We followed the manufacturer's instructions (User Guide, CG000315) for cell capture, barcoding, reverse transcription, cDNA amplification, and library construction. Libraries were quantified using an Agilent Bioanalyzer with a high sensitivity chip (Agilent) and were sequenced using the Illumina NovaSeq 6000 S4 platform, using 150-bp paired-end sequencing, using the following read lengths: 28 bp Read1, 10 bp I7 Index, 10bp I5 Index and 90 bp Read2. The two scRNAseq libraries of young and old mouse brain were

sequenced at 20,000 reads per cell, respectively. Sequencing was processed by Macrogen.

2.4. Single-nucleus ATAC sequencing

For combination method of ATAC-seq and single cell sequencing, scATAC-seq(or snATAC-seq)[47], we isolated nuclei from the cell suspension following a brain nuclei isolation protocol in the reference of 10x Genomics protocol (Nuclei Isolation from Mouse Brain Tissue for Single Cell ATAC Sequencing, CG000212). The final nuclei were resuspended in Diluted Nuclei Buffer (Chromium Next GEM Single Cell ATAC Reagent Kits v2). The composition of the Tris-based Diluted Nuclei Buffer, including Magnesium concentration, is optimized for the transposition and barcoding steps in the Single Cell ATAC protocol, which is not used in RNA seq protocol.

After transposition step using transposition mix (Chromium Next GEM Single Cell ATAC Reagent Kits v2), we ran on the 10x Chromium Single Cell 3′ v3 platform, generated GEM, synthesized cDNA and constructed libraries. Libraries were quantified using an Agilent Bioanalyzer with a high sensitivity chip (Agilent) and were sequenced using the Illumina NovaSeq 6000 S4 platform, using 150-bp paired-end sequencing, using the following read lengths: 50 bp Read1, 8 bp I7 Index, 16bp I5 Index and 50 bp Read2. The two scRNAseq libraries of young and old mouse brain were sequenced at 25,000 reads per cell, respectively. Single-nucleus capture, library preparation, and sequencing were performed by Macrogen (www.macrogen.co.kr).

2.5. Transcriptomic data preprocessing

FASTQ files of raw reads from scRNA-seq and snRNA-seq were processed using the Cell Ranger software suite (v7.2.0, 10x Genomics Inc., USA)[46]. For gene annotations, reads were mapped to the mouse reference genome (GRCm38) with the Ensembl GRCm38 GTF file, resulting in production of gene-by-cell count matrices. With count matrices, Seurat objects were created by R package Seurat v5.1.0[48] and used for downstream analysis.

To filter out low-quality cells, cells with 6000< and 200> nFeature_RNA, 20000<

nCount_RNA, and 3%< percent.mt were removed. Each gene expression measurements in each cell was then normalized using the SCTransform[49] protocol to address variations in sequencing depths across cells. We use the top 2000 high variable genes as the default training parameter.

Cells associated with doublets were identified and removed using the scDblFinder [50] (v1.18.0) R package. To further remove cell-specific biases, cells were clustered using Seurat v5 and low-quality clusters visually inspecting outliers in the UMAP plot were annotated and excluded for downstream analysis. Using the Leiden algorithm in clustering analysis across multiple datasets. Cell embeddings are visualized in UMAP.

For annotating each cell types, we used label transfering in Seurat R package with transcriptomic reference, such as Azimuth[51] and Allen Institute Brain Atlas[42], and canonical marker genes of brain major cell types to fnalize manual annotation for each cell. Unassigned cells were reclassified based on expressing marker-genes and the major cell type of clusters to which they belong.

Cells were into 19 clusters using the FindClusters function on the first 30 PCs of high variable genes with resolution =1.2, and were visualized in the two-dimensional UMAP plot with the RunUMAP functions. For each cluster with >80% of the most abundant cell type, unassigned cells were classified into the cluster of major cell type and cells assigned to other minor cell types were removed as putative doublets.

2.6. Epigenomic data preprocessing

The paired-end sequence read fastq files were aligned to a mouse (mm10) combined reference genome (refdata-cellranger-atac-GRCh38-and-mm10-2020-A-2.0.0,10x Genomics) using Cell Ranger ATAC software (v2.0.0), including read filteration, alignment, barcode count, identification of Tn5 cut sites, detection of accessible chromatin peaks, cell calling and count matrix generation for peaks. Based on Signac(v1.14.0)[52] R package, we did peak-calling with mm10 genome reference and MACS2[53], annotation and quantifying per-cell counts in different genomic regions using EnsDb.Mmusculus.v79, and

calculating single-cell QC metrics, such as nucleosome signals, TSS enrichment, were processed on generated seurat objects.

For normalization, we ran term frequency inverse document frequency (TF-IDF) normalization on a matrix, and then ran LSI dimension reduction using RunSVD function. The first LSI component often captures sequencing depth rather than biological variation, so the component was removed from downstream analysis. Following dimension reduction such as UMAP and clustering utilized LSI space.

Creating gene activity matrix were done for annotation and validation of expressed canonical marker genes in major cell types. To annotate cells with pre annotated transcriptomic Seurat object, we utilized methods for cross-modality integration and label transfer using Signac package, referred as RNA anchor-based classification.

2.7. Data Integration

After creating gene-activity matrix of snATAC-seq dataset, we merged every transcriptomic and epigenomic dataset and performed integration with CCA integration by using Seurat v5 function to remove batch effect, such as sequencing depth and technological differences, for making possible to compare solely biological differences across aging. We processed clustering and dimension reduction again with integrated dataset to draw co-embedded UMAP plot of three modalities.

2.8. Downstream analysis

Differential expression analysis

Differential expression analysis was conducted using the FindAllMarkers function. Differentially expressed genes (DEGs) of aging whole brain and DEGs of aging in each cell type were identified, as well as DEGs of different clusters and major cell types. Wilcoxon Rank Sum test was used as a default to find DEGs and every DEGs were filtered with adjusted p-value<0.05 and |log2FC|>0.5 for significant differences in the number of cells.

Gene set enrichment analysis

Using the DEGs as input resources, we processed gene-set enrichment test (GSEA) and when the p-value < 0.05, a pathway is considered to be significantly associated. The enrichment analysis in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was determined based on the cell type specific aging related DEGs.

Single-cell trajectory analysis

Pseudo-time analysis is a great tool for understanding the dynamics and temporal trajectories of gene expression within cell types and cellular shifts during aging. Using Monocle2 [54], cell type-specific CellDataSets were created based on subset seurat objects. Next, we reduced dimensionality with default method named 'DDTree', a hybrid decision tree-deep neural network. Ordering cells on trajectory of the tree and finding the beginning point, we used orderCells function in monocle and specified state which contains most of the cells assessed to be time zero. Also, we plotted the expression levels of interested genes from DEG lists, all of which show significant changes as a funtion of differentiation, using the function plot_genens_in_pseudotime.

cis-regulatory network

For generating cis-regulatory networks and predicting novel cis-regulatory interactions from single-cell chromatin accessibility, we used run_cicero function in Cicero[55] R package which is provided by Monocle 3 and mouse.mm10.genome as a reference genome.

Motif analysis

We performed DNA sequence motif analysis using ChromVar[56] function by finding overrepresented motifs in a set of differentially accessible peaks or performing differential motif activity analysis based on JASPAR2020[57] motif position frequency information. We calculated a per-cell motif activity score and identified motifs associated with variability in chromatin accessibility between cells. Furthermore, footprinting analysis could be processed with added motif information.

GREAT analysis

We wanted to predict the overall functional changes of differential accessibile regions, both coding and non-coding regions. By using genomic regions enrichment of annotations tool(GREAT ver. 4.0.4) [58], the function and annotation of differentially open genomic regions were predicted by statistical enrichments for associations between genomic regions and annotations.

Analysis of intercellular communication

CellChat(v2)[59] is designed for inference and visualization of cell-cell interaction from single-cell expression data, and it also serves CellChatDB as a publicly available database of literature-supported receptor-ligand interactions. To infer the interactions between cell types observed exclusively in one dataset, cellchat was utilized on integrated transcriptomic dataset. To identify receptor-ligand interactions, we referred to the STRING database of protein-protein interactions.

3. Results

3.1. Multi-omics analysis of the aging mouse brain

We employed snRNA-seq, scRNA-seq and snATAC-seq[60] using the 10x Genomics Chromium platform version 3(10x v3). We applied both snRNA-seq and scRNA-seq for single-cell transcriptomic profiling because of the capture efficiency and the diversity of cell types in the mouse brain. Following the protocol from 10xGenomics and the lab-developed nuclei isolation protocol, we isolated cells and nuclei from the female C57BL/6 mouse whole brains at 4 months (n=3) for young age and 21 months (n=3) for old age. After quality filtering, we obtained 18,622 high-quality cells for scRNA-seq (8,989 and 9,633 cells from young and aged brain), 28,216 high-quality nuclei for snRNA-seq (19,574 and 8,642 nuclei from young and aged brain) and 22,915 high-quality nuclei for snATAC-seq (8,037 and 14,878 nuclei from young and aged brain).[Figure 11] Then we clustered and annotated with canonical marker genes. [Figure 12]

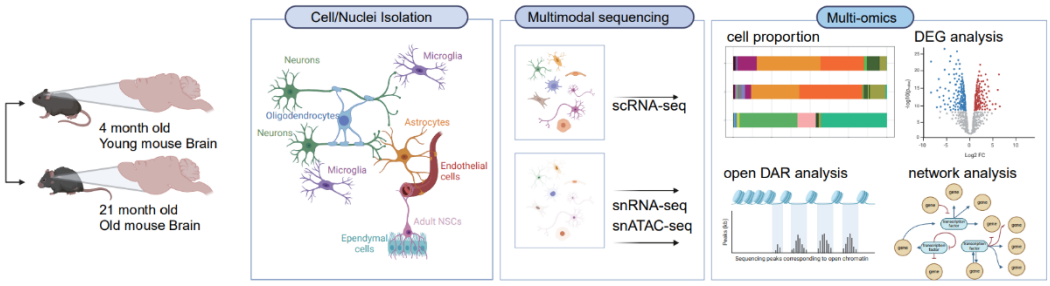

Figure 11. Schematic overview of experiment.

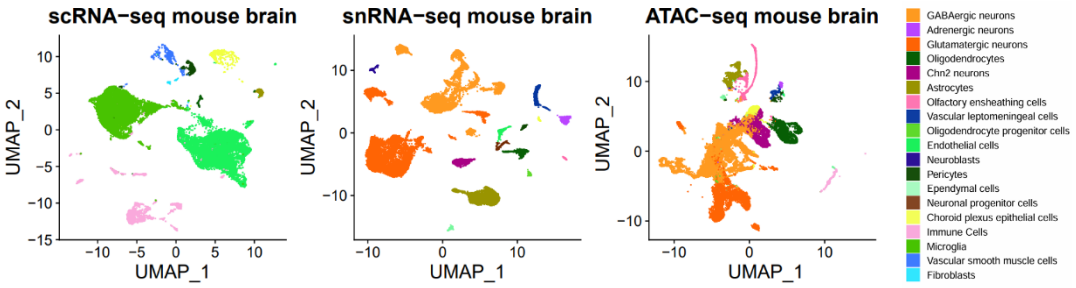

Figure 12. UMAP plot of each modality, color representing cell types.

After integrating both transcriptome dataset (scRNAseq and snRNAseq), we classified the clusters representing the major cell types of the mouse brain by assessing the expression level of canonical cell-type marker genes. [Figure 13] For example, microglia were defined by expression by *Tmem119*, endothelial cells defined by *Cldn5* and *Flt1*, oligodendrocytes by *Mbp* and *Plp1* expression, oligodendrocyte progenitor cell by *Pdgfra*, GABAergic neurons by *Gad1* and *Gad2*, Glutamatergic neurons by *Slc17a7* and adrenergic neurons by *Col25a1*. Less abundant cell types also observed such as immune cells (*Cd8a*, *Ccl5*), vascular leptomeningeal cells (*Apod*, *Slc6a13*), olfactory ensheathing cells (*Sash1*), neuroblasts (*Ntng1*), pericytes (*Abcc9*) ependymal cells (*Cfap44*), fibroblasts (*Col1a2*) and choroid plexus epithelial cells, however for erasing bias, we removed them.

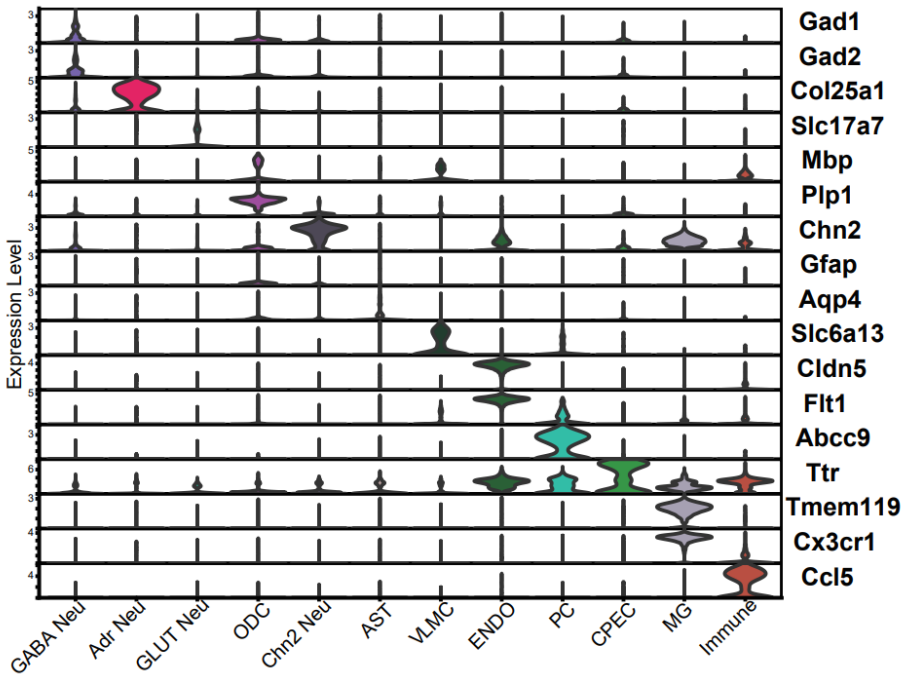

Figure 13. Violin plot depicting expression level of canonical cell type markers.

Next, we employed Seurat's label-transfer algorithm to annotate and gene-activity converged snATAC-seq dataset. we were able to check that main cell types in label-transfered snATAC were correctly labelled, based on the accessibility of the promoters of marker genes within each cell type. [Figure 14] Finally, we performed CCA-integration on three datasets to project all cells from three different modalities onto a unified reduced dimensional space, resulting in a coembedded UMAP plot. [Figure 15] Some neuronal cells, which are presumed to be GABAergic neurons, were located at the center and showed significant sparsity, likely due to the effect of the snATAC data.

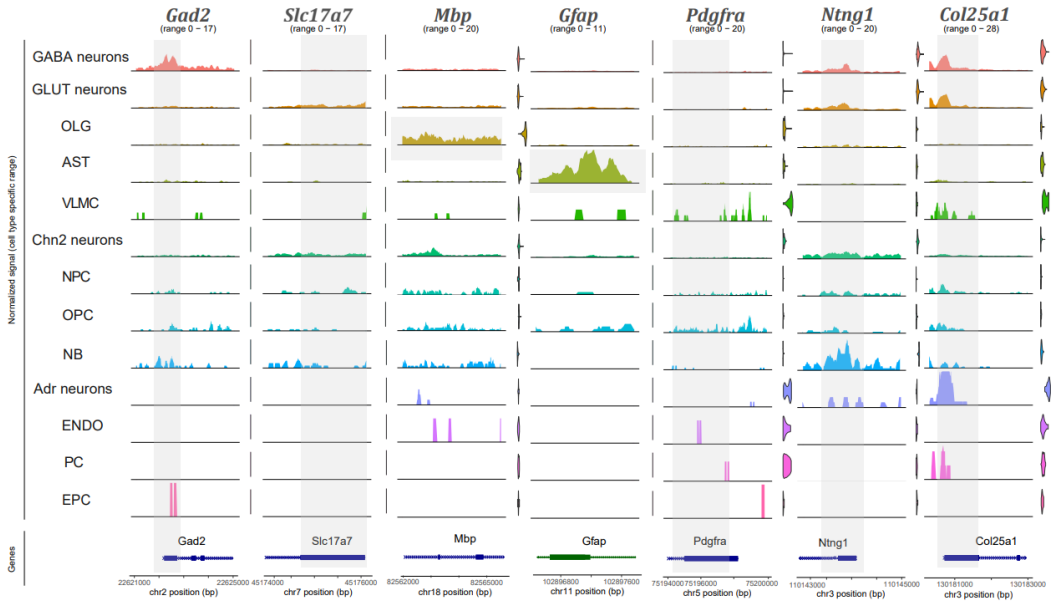

Figure 14. Accessibility of the promoters of cell type specific marker genes.

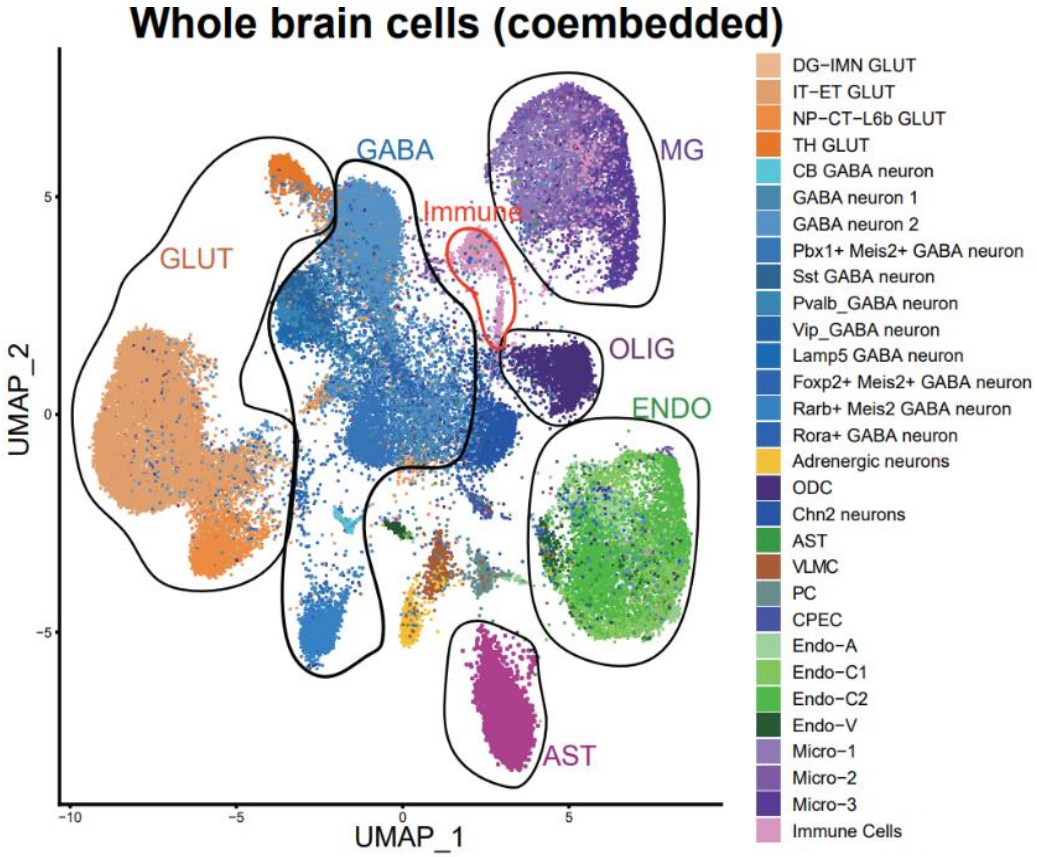

Figure 15. Coembedded UMAP of three different modalities of whole mouse brain.

3.2. Identification of cell-type composition in multi-modalities

Integrating transcriptome and chromatin accessibility profiles, we left 12 main cell types which contain more than 200 cells(nuclei) and further subclustered total 30 cell types for further analysis. [Figure 15] Some papers already showed that nuclei are relatively uniform in size and morphology, so it is possible to get more cell types from snRNA-seq rather than scRNA-seq, because some cell types are more vulnerable to the tissue dissociation process. snRNA-seq and scRNA-seq can capture different neuronal types, and our study also consistent to this. [45,61][Figure 16]

Two nucleus-derived sequencing method captured similar cell types when compared to the cell-derived sequencing method. [Figure 17] Validate correlation between the two nucleus-derived sequencing datasets, we assessed the relationship between average gene expression (snRNA-seq) and average gene activity (snATAC-seq). The plot shows a correlation with R=0.63 and p-value < 1e-05, indicating a positive relationship. [Figure 18]

Also, overall proportion of cell types were significant fluctuation between modalities, showing increases in some old genes and decreases in others, making it hard to compare between young and old brain. [Figure 19] This could be due to loss during the individual sample preparation process. Given the characteristics of the brain, it is sensitive to be isolated in single cell/nuclei resolution, compared to other tissue. Despite of these difficulty, we aimed to explore the potential biological significance from both sequencing behind these differences.

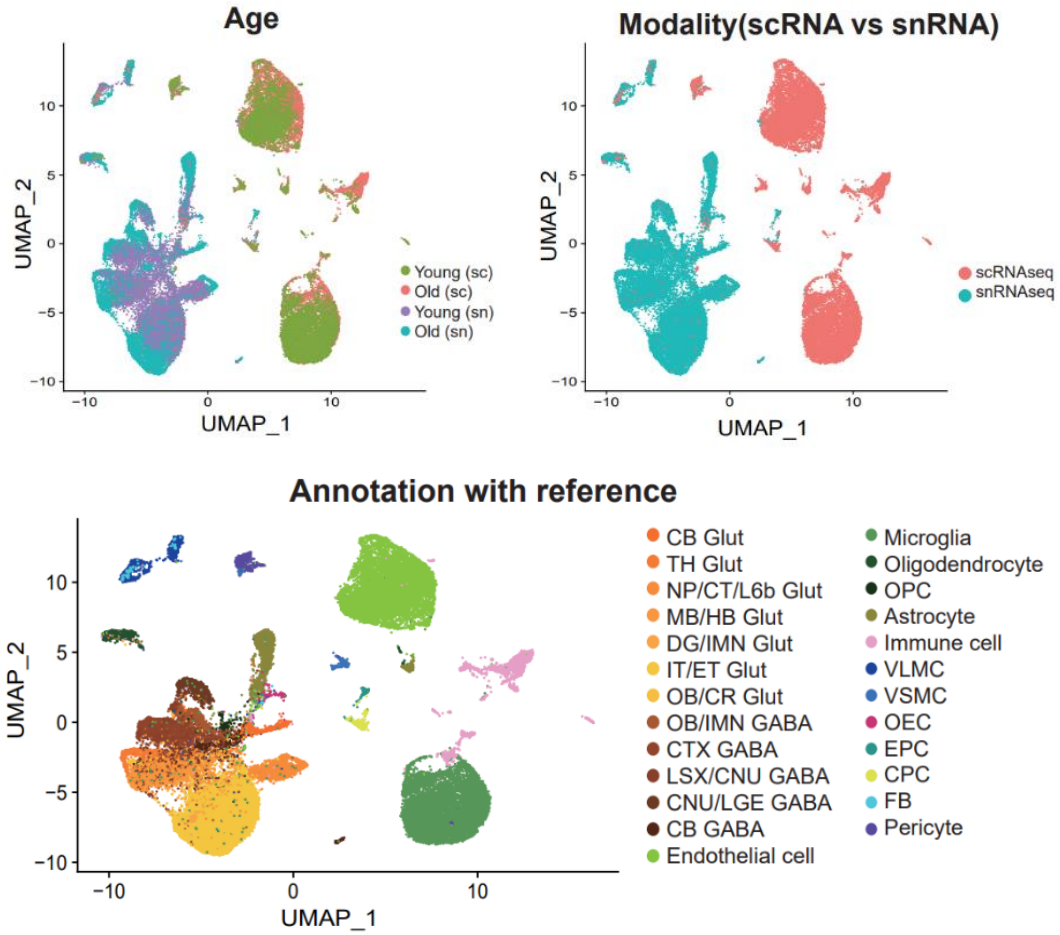

Figure 16. Integrated transcriptional datasests (snRNA-seq and scRNA-seq).

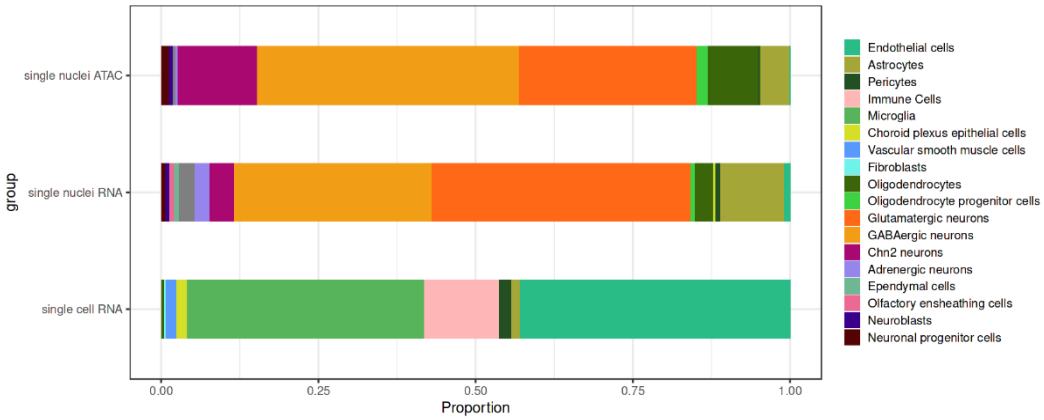

Figure 17. Comparing captured cell types in each modality.

Figure 18. Correlation between nuclei- gene expression and gene activity.

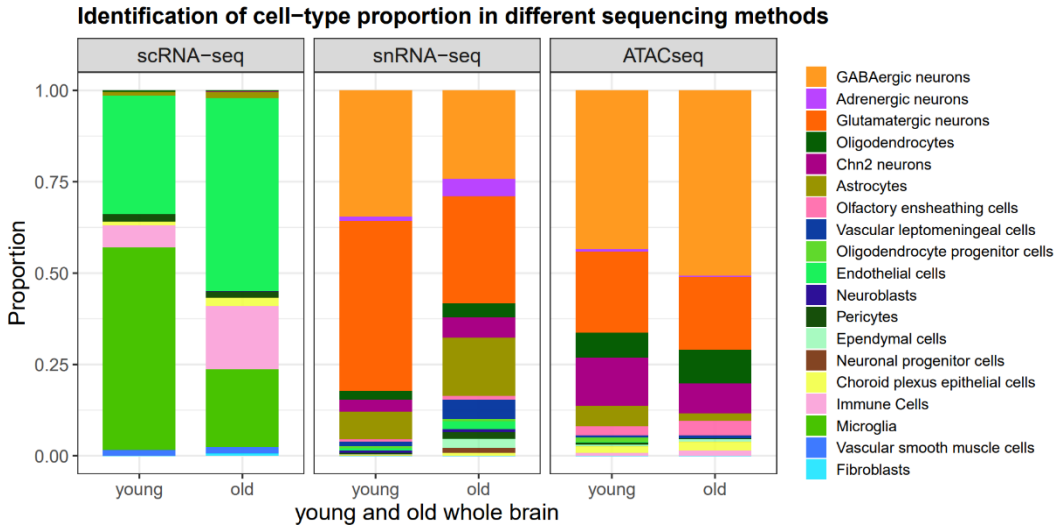

Figure 19. Comparing captured cell types in all situation (age, modality).

To put it briefly, Glutamatergic (excitatory, GLUT) neurons and GABAergic (inhibitory, GABA) neurons were divided 4 and 11 sub-clusters, respectively. GLUT neurons were further annotated using references from the Allen Brain Institute, while GABA neurons showed significant differences based on the expression of transcription factors, as seen in earlier brain-omics studies, and were annotated accordingly using relevant genes. Notably, for GABA neurons, we were able to identify well-known clusters such as Pvalb, Vip, Lamp4, and Sst expressing neurons, which are frequently reported in existing papers[62], and the decreased proportion of them with aging was aligned with other studies. [63] [Figure 20] We wanted to show the transcriptional and epigenomic modification in specific GABA neuron of aged brain for finding clues of aged-related neurodegeneration through hyper-excitable signaling.

Secondly, consistent to previous paper about aged brain endothelial cells' sequencing study[64], our data also clustered in venous(V), capillary(C), and arterial(A) cells but slightly significant proportion shift showed between young and old brain. Comparing capillary endothelial cell type 1(Endo-C1) and type 2(Endo-C2), more Endo-C2 was

positioned in aged brain and we assumed that these could be aged-endothelial cell. We made hypothesis that aged-endothelial cells make blood brain barrier (BBB) disruption and occur immune cells leakage to brain tissue, as our data showed increasing immune cells in old brain. Lastly, according to the data, microglia are sub-clustered into three groups (Micro-1, Micro-2, and Micro-3), with Micro-3 making up a larger proportion than Micro-1 in the aged brain. We thought that Micro-3 are aged microglia and wanted to explore their characteristics in more detail through further analysis

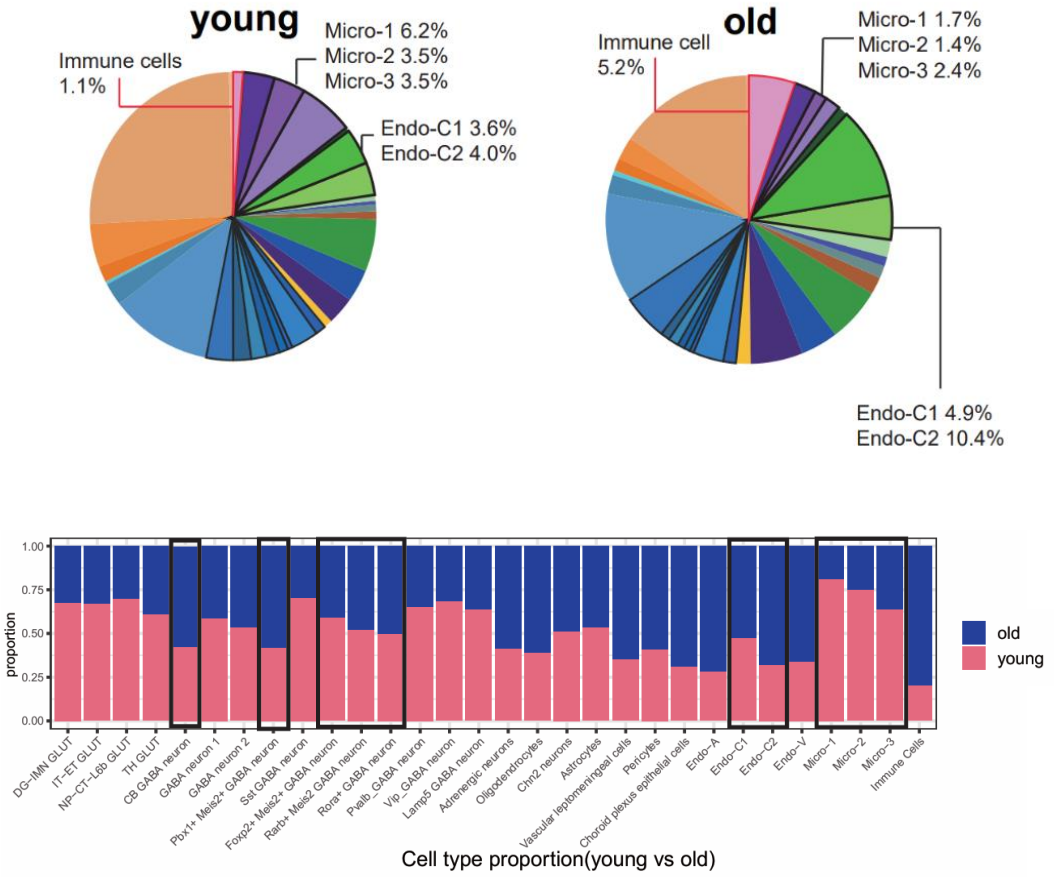

Figure 20. Cell type proportion comparing the young and old brain.

3.3. Gene expression alters in major cell types with aging

We next analyzed the differentially expressed genes (DEGs) with age in each major cell types of transcriptomic data. To globally understand how expression patterns changes with age, we performed differential gene expression analysis in all datasets of each modality (padj < 0.05, |log2FC| >0.4) and compared age-related up- or downregulated gene lists to age-related gene lists obtained from published studies. [Figure 21] Using this method, in both scRNA-seq and snRNA-seq gene lists, the *P2ry12* gene, which is a marker gene of homeostatic microglia, was consistently down-regulated in our gene expression data as well as in public datasets. Additionally, ten genes, including *Slc16a1*, *Vim, Gsn, B2m, Cldn5, Id1, Klf2, Klf4*, *Cxcl12*, and *Cdkn1a*, were found to be commonly up-regulated across three gene lists from snRNA-seq, scRNA-seq, and public data. Interestingly, Vim and Gsn are related to cytoskeletal remodeling or cellular movement and claudin-5(*Cldn5*) is known as an endothelial cell marker gene that roles as a tight junction protein to maintain the blood-brain barrier. Additionally, *Cxcl12* is a chemokine that plays a role in immune cell migration and is involved in stem cell niches. Cdkn1a, also known as p21, is a cell cycle regulator involved in mediating cell cycle arrest and is one of the famous senescence markers. To find different enriched pathways between single cell and nucleus sequencing, we analyzed gene-set enrichment analysis with upregulated genes with aging in each modality [Figure 22]. Single-cell based dataset showed enriched ‘antigen-presenting’ pathway, while single-nucleus dataset showed upregulated ‘neurogenesis’ pathway. This result make sense when we match the yielded cell types and enriched pathways in each modality.

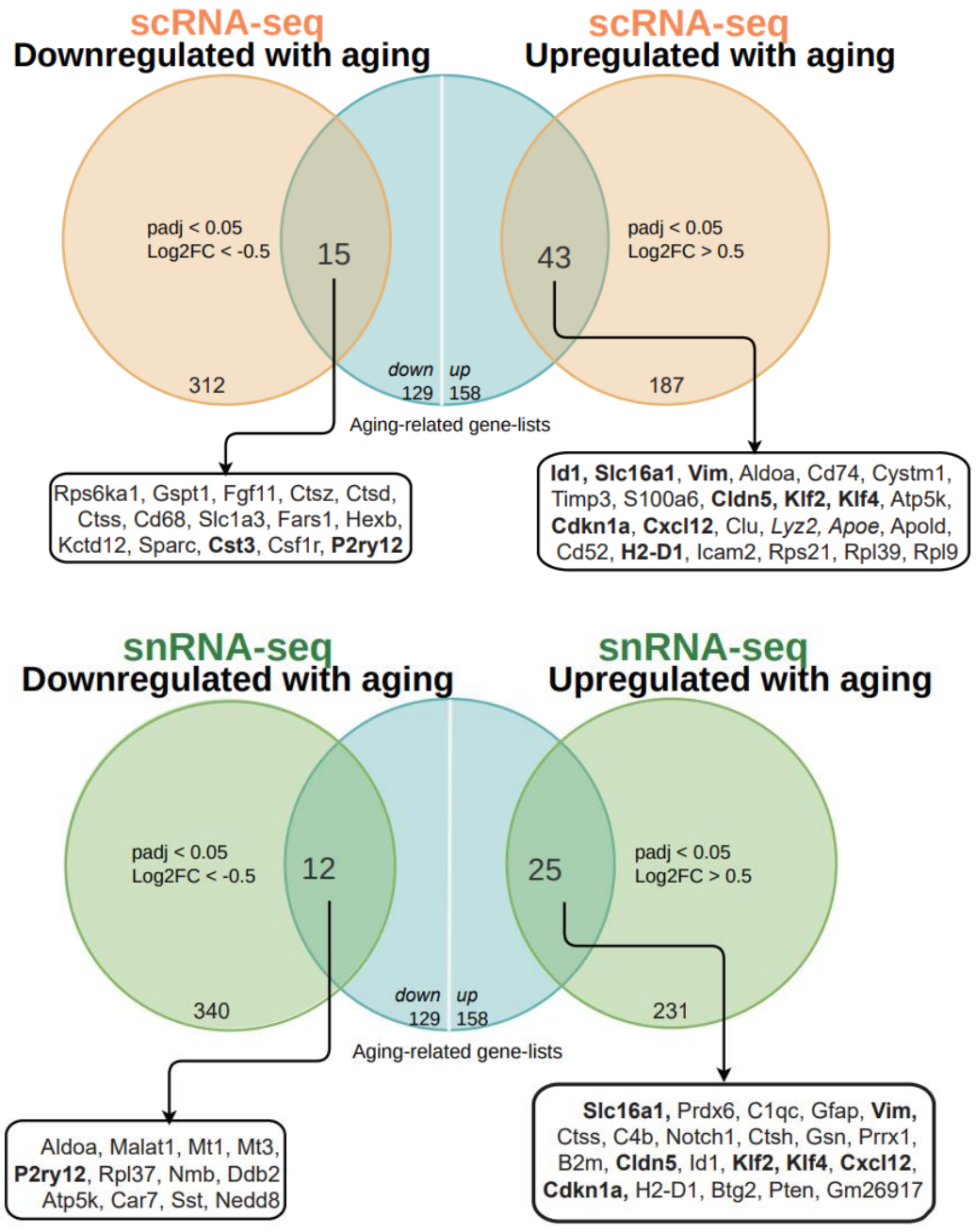

Figure 21. Overlaps of DEGs with public age-related up/down regulated genes.

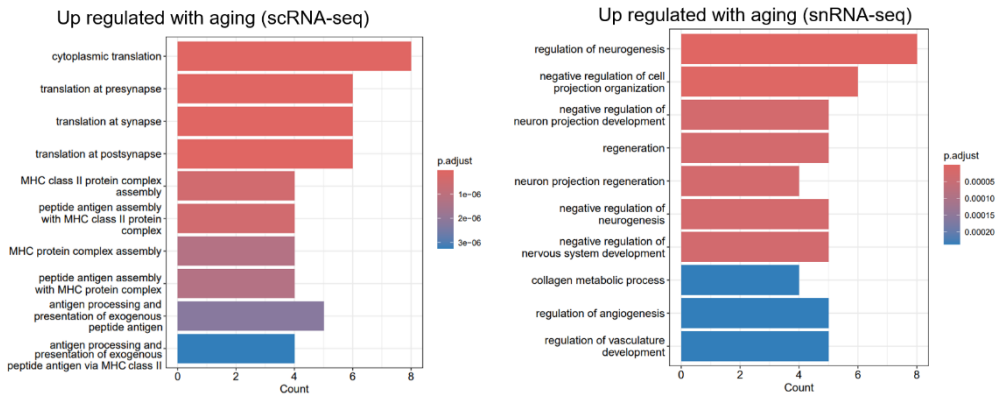

Figure 22. Enrichment analysis with each modality, pathways upregulated with aging.

Next we investigated differentially expressed genes in each major cell types in integrated dataset. [Figure 23] Neurons (GABA, GLUT, adrenergic neurons, Chn2 high expressing neurons), oligodendrocytes (ODC), astrocytes (AST), and VLMC (vascular leptomeningeal cells) showed the greatest amount of differentially up-regulated genes with age. We had a question why *Meis2* gene was up-regulated in GABAergic neurons in old mouse brain, because the *Meis2* coded protein act as an important transcription factor in early brain development and had been studied on differentiation of neuronal cells into specific neuronal fates. Recently *Meis2* expressing GABAergic neurons were discovered in the brains of patients with Alzheimer's disease by snRNA-seq.[65] This founding led us to take an interest in the shift of these GABAergic neurons. In the case of endothelial cells, the differentially expressed gene lists overlapped significantly with the previously mentioned list, including genes like *Cdkn1a*, *B2m*, *Klf4*, *Slc16a1*, and *Cxcl12*. As we expected, *P2ry12* was significantly decreased in microglia, while neurodegenerative disease related gene *Apoe* extremely up-regulated in microglia. However immune cells infiltrated into old brain showed less inflammatory features than young brain. [Figure 20,23] To demonstrate the gene lists functions, we performed Gene Set Enrichment Analysis (GSEA) using the enrich GO. Each cell types showed unique signatures of aging. [Figure 24] To sum up, in the case of microglia, they can be explained by the aged

microglia seen in previously known disease models, but for GABAergic neurons or endothelial cells, there would be additional explanations to consider.

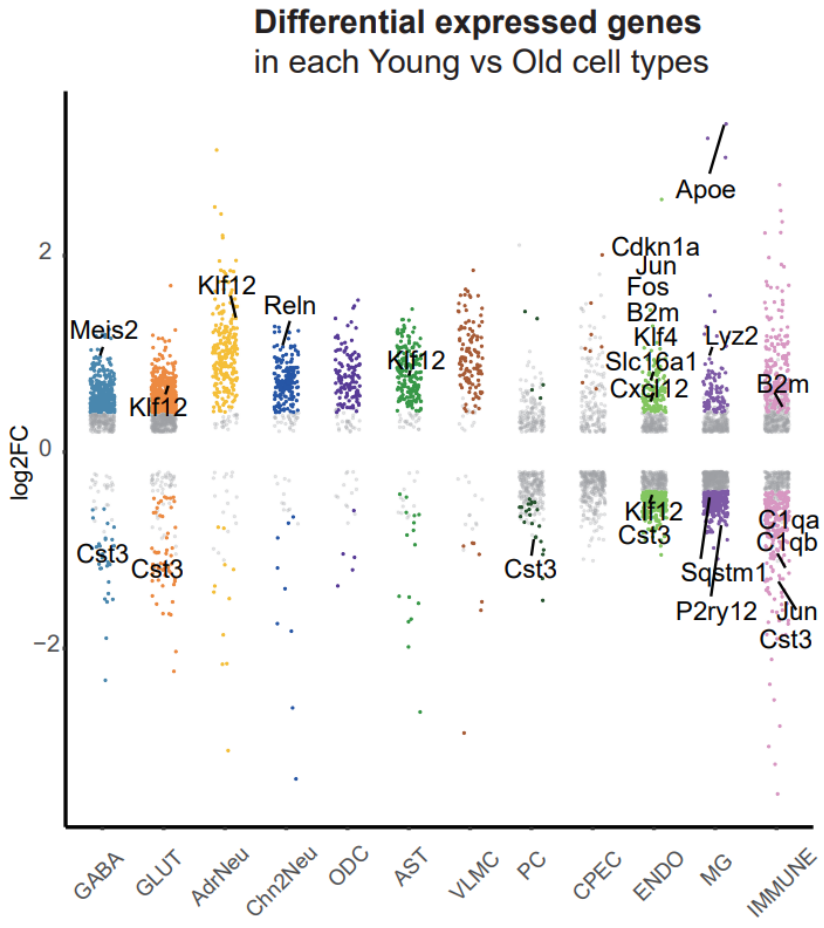

Figure 23. DEGs of aging in each major cell types.

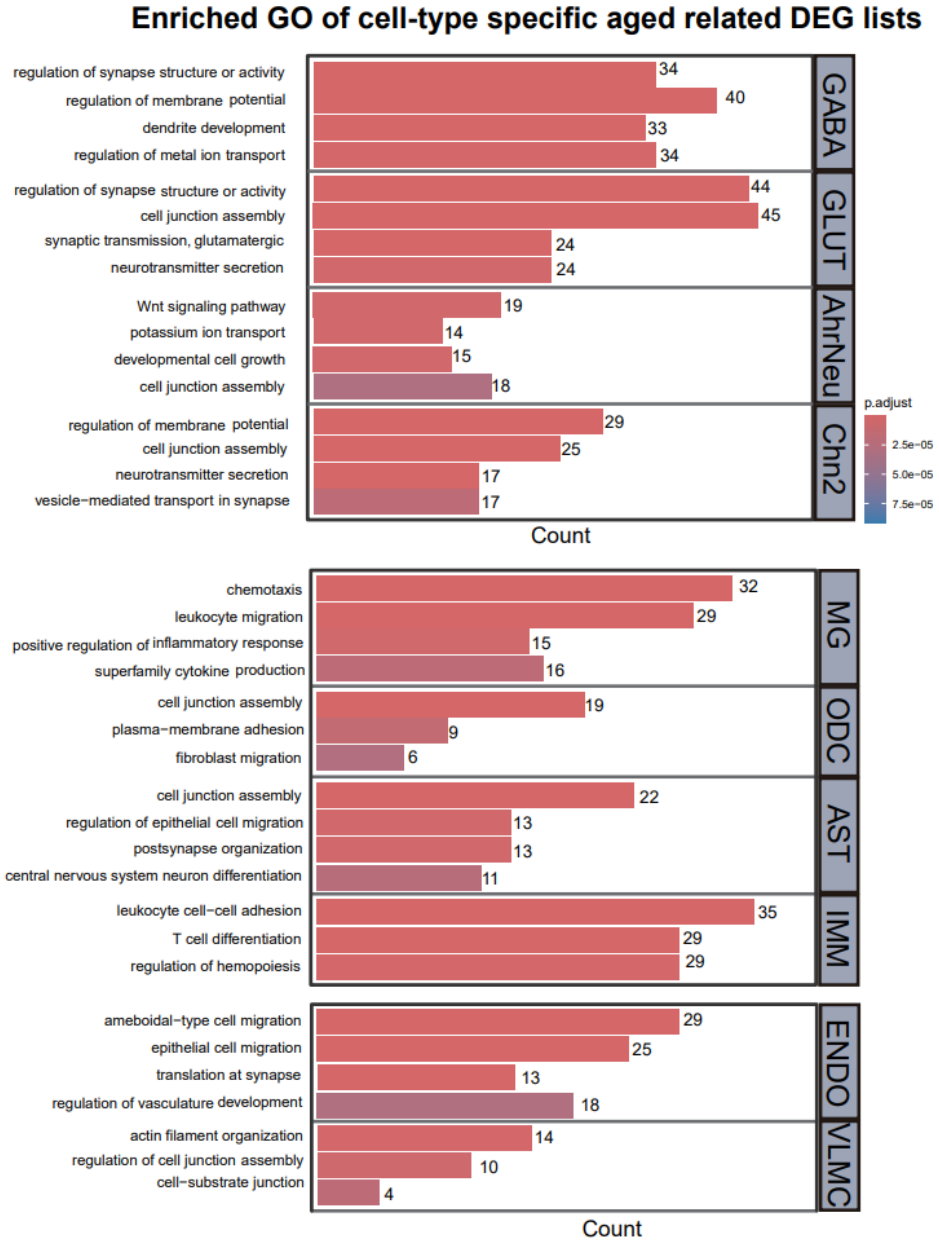

Figure 24. Gene set enrichment analysis(GO) of aging signals in each major cell types.

3.4. Aging microglia associate with inflammatory response

To reveal heterogeneity in microglia, we grouped microglia into three classes (Micro-1, -2, and -3) and compared the ratio of microglia subclasses, as mentioned previously. [Figure 25] To identify transcriptional patterns of the subclasses, we conducted differentially expressed gene analysis by using the FindAllMarkers funtion in Seurat and comparing expression levels of DAM marker genes. [Figure 26-27] According to published database[66], Micro-1 shared some signatures with homeostatic features, such as high level of *Tmem119, P2ry12, Hexb,* and *Cx3cr1*, while Micro-3 highly expressed inflammatory genes such as *Lyz2* and DAM-associated gene, including *B2m, Apoe, Lpl, Cst7, Axl, Itgax, Spp1, Cd9, Ccl6* and *Csf1*. Furthermore, in aged brain, these expression pattern showed more significantly. Interestingly, Micro-2 in our dataset highly expressed Trem2, referred as a signaling marker at stage 1 DAM in previous mentioned paper, and it down-expressed in Micro-3 as in Micro-1. We raised the question of why Miro-2 and Miro-3 show different signaling patterns, even though Miro-2 has diffused between Miro-1 and Miro-3.

To capture the molecular dynamics in microglia, we applied trajectory inference analysis with monocle2 package. [Figure 28] Along with a MST algorithm, we confirmed the lineage transitions between Micro-1 and Micro-3 and intermediated state Micro-2. Also Micro-3 formed a distinct branch, representing terminally differentiated stated that diverges significantly from Micro-1 and Micro-2. It might be due to unique environmental signals or specific gene regulatory networks that push Micro-3 into a distinct functional role. To figure out expression dynamics of well-known genes, *Apoe* and *Lyz2* seemed to increase with pseudotime, potentially contributing to the distinct identity of Micro-3. [Figure 29] Taken together, we identified three microglia subclasses through transcriptomic analysis, revealing that Micro-1 exhibited homeostatic characteristics, Micro-3 expressed inflammatory and disease-associated markers and Micro-2 acted as an intermediate state between two with trajectory analysis. A notable observation was the high expression of Trem2 in Micro-2, resembling stage 1 DAM, while *Apoe* and *Lyz2* increased over time in Micro-3 especially in aged-mouse brain, solidifying the unique, terminally differentiated

identity of Micro-3, like stage 2 DAM. This suggests that unique environmental signals and gene regulation could drive the specialization of Micro-3 into its inflammatory role.

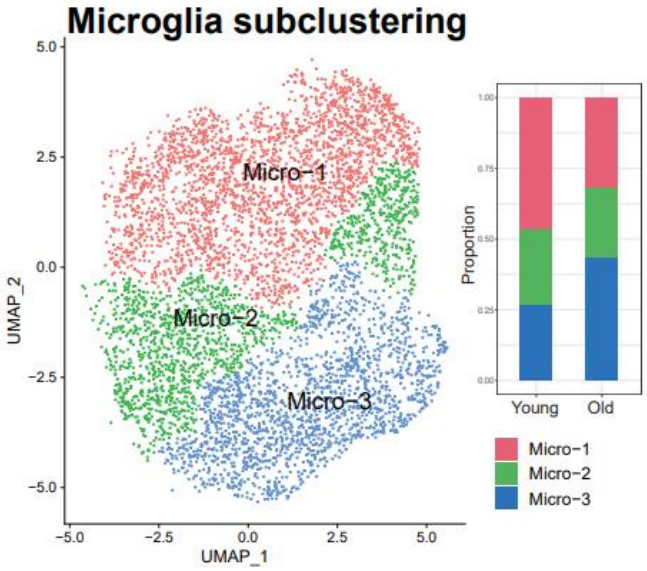

Figure 25. Subclustering of microglia.

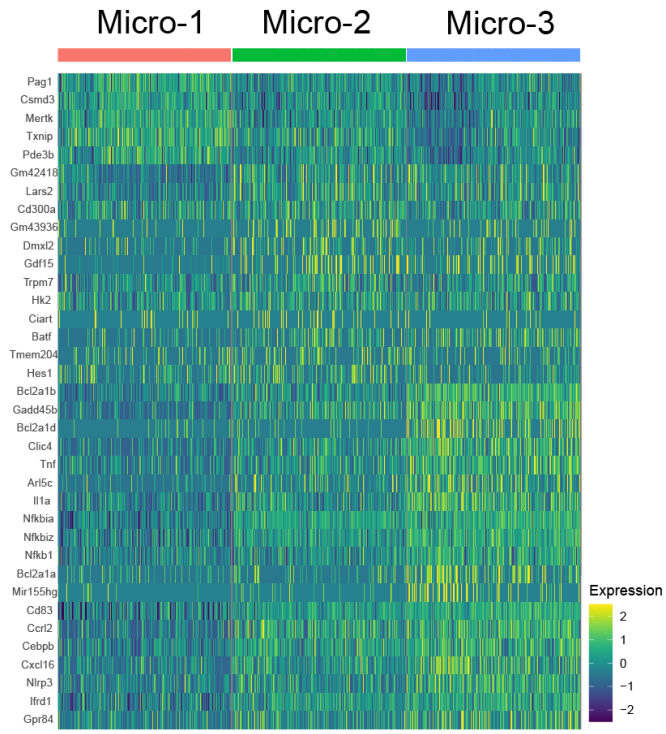

Figure 26. Marker genes of three microglia subtypes.

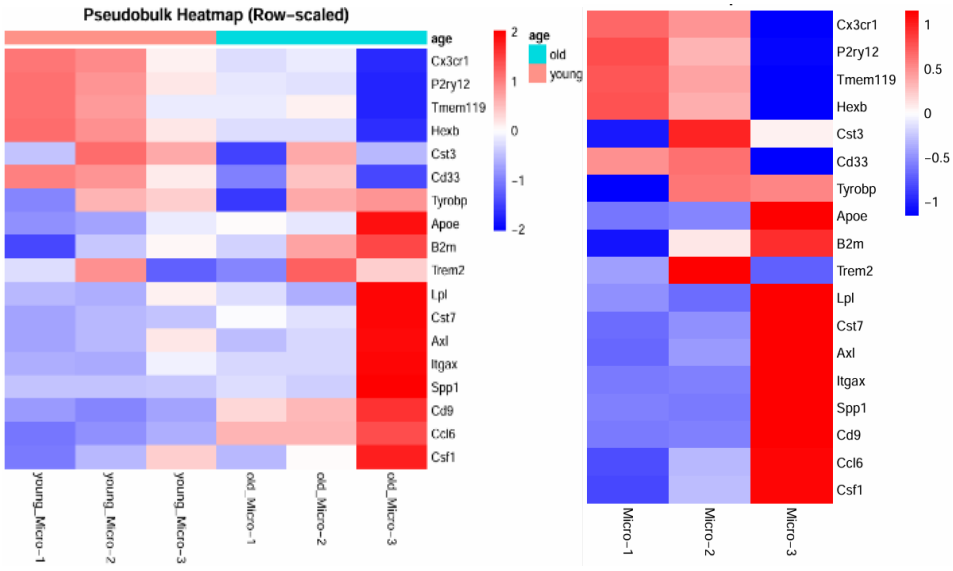

Figure 27. Row-scaled pseudobulk expression of genes in microglia subtypes.

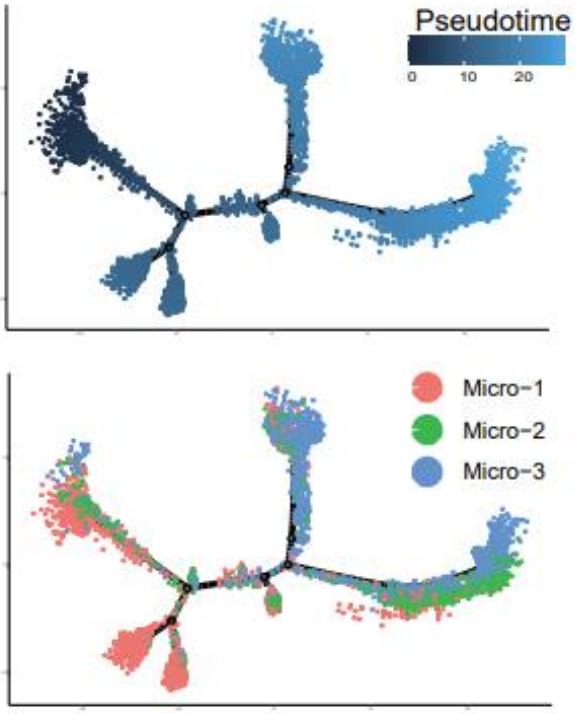

Figure 28. Pseudotime trajectory of microglia.

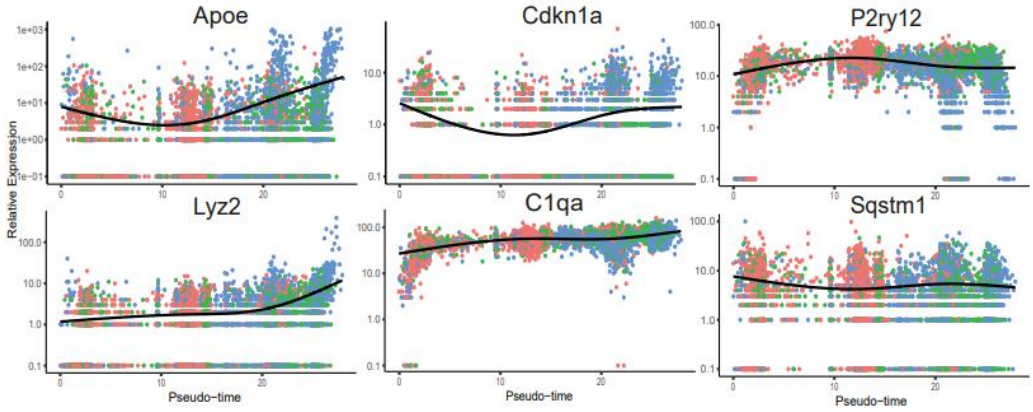

Figure 29. Pseudotemporal gene expression of microglia.

3.5. Capillary endothelial cells show high senescent signatures

As previously mentioned, endothelial cells in mouse brains are organized into distinct clusters of venous, capillary and arterial cells, with capillary endothelial cells showing a continuous and diffuse pattern between the other two types. To explore the heterogeneity of endothelial cells, we classified all subset endothelial cells into four subtypes (Endo-A, Endo-C1, Endo-C2, and Endo-V) with marker genes of *Bmx* (arterial), *Slc16a1* (capillary), *Nr2f2* (venous), and *Vcam1* (arterial and venous) and compared the proportion of these subtypes across age groups.[Figure 30.31] It was intriguing to observe that Endo-C split into two distinct types (Endo-C1 and Endo-C2). In the previous transcriptomic analysis of aging brain endothelial cells(BEC) paper[63]. It was visually evident that there were two distinct groups. We discovered that the proportion of Endo-C2 increased slightly with aging.

We performed DEG analysis with single cell dataset and pseudobulk dataset, as same way of microglia analysis, to confirm the annotation and characterize the transcriptional patterns of each subtype. With aging, more *Vcam1* in venous endothelial cells was expressed. More interestingly, we discovered that *Actg1* and *Hspb1* are marker genes of Endo-C2 which were also lightly increased with aging. Actin gamma is part of the actin cytoskeleton network which makes up the structural framework inside cells. *Hspb1* is related to hypoxia and stress pathway. Consistent to the previous paper, capillary endothelial cells all expressed higher *Cxcl12, Ifi27, Acvlr1, B2m* and *Jun* with aging, and their expression level were significantly higher in Endo-C2 than Endo-C1. [Figure 32-33] Endo-C2 upregulated innated immunity (*Cxcl12, Ifi27*), antigen processing(*B2m*), and TGF-b signaling(*Acvrl1*), suggesting that aged capillary endothelial cells show significant modifications in upregulating innate immunity, antigen processing, TGF-b signaling and oxidative stress response pathways than other vessel segment.

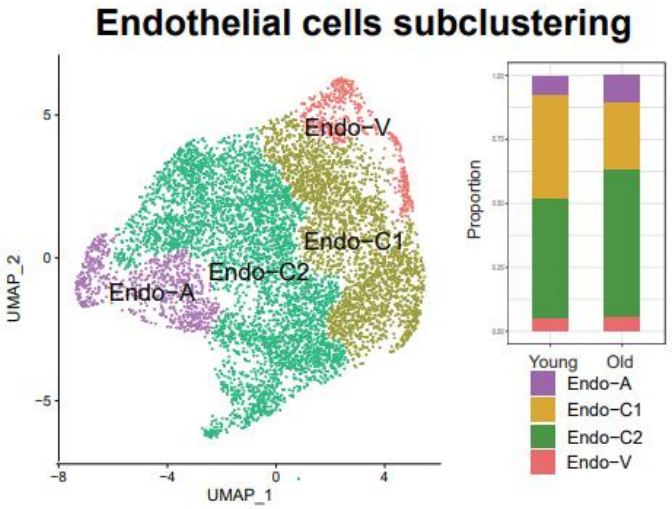

Figure 30. Subclustering of endothelial cells.

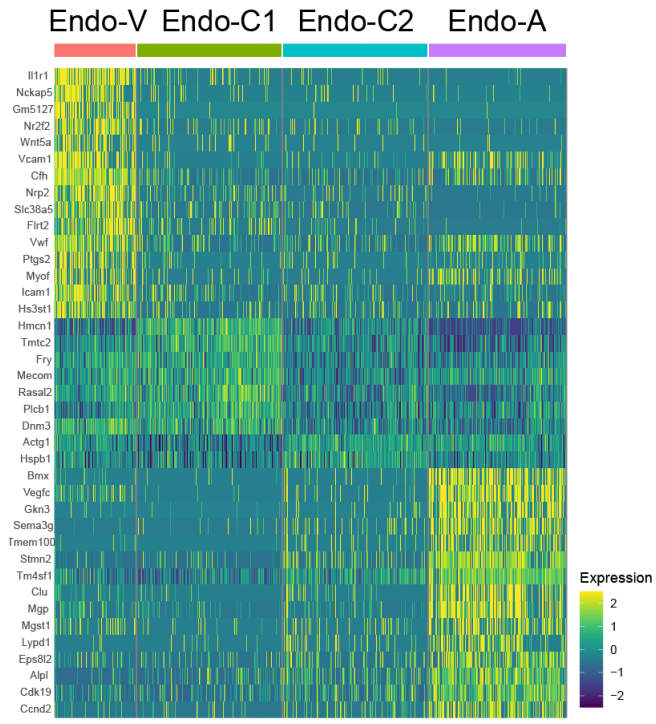

Figure 31. Marker genes of four endothelial subtypes.

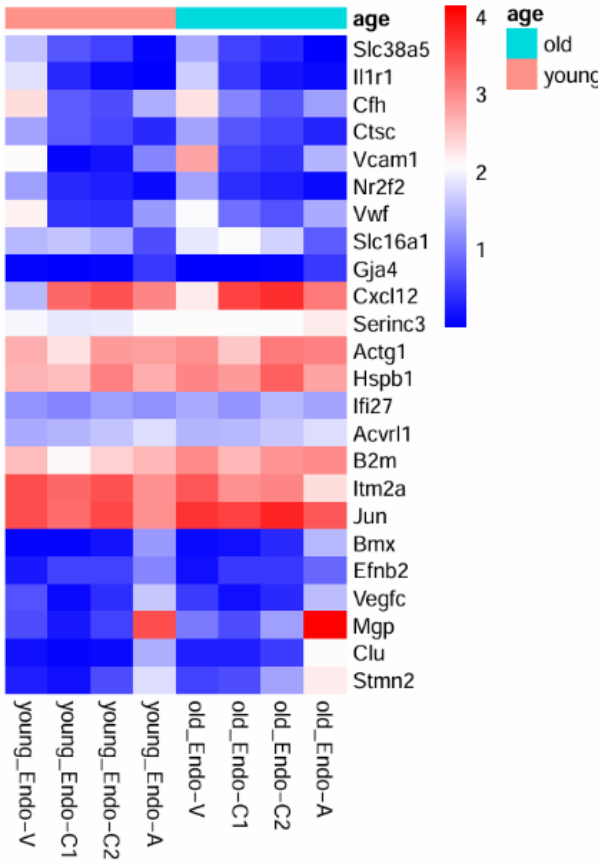

Figure 32. Normalized pseudobulk expression of genes in endothelial cell subclusters.

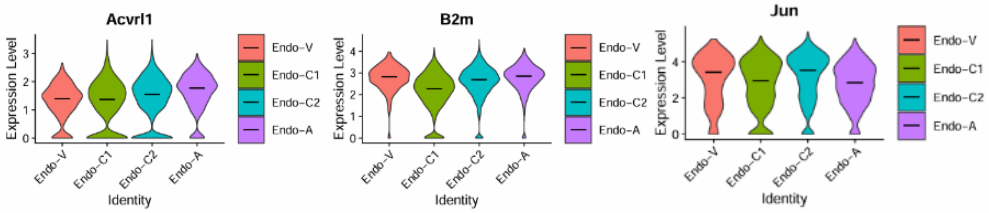

Figure 33. Normalized gene expression level of genes in endothelial cell subclusters.

Next we traced the pseudotemporal dynamics of mentioned gene expression from Endo-C1 to Endo-C2. [Figure 34] Marker genes of Endo-C1 such as *Fry*, *Hmcn1*, *Mecom* and *Tmtc2* were gradually down-regulated with trajectory, while marker genes of Endo-C2(*Actg1*, *Hspb1*) and publicly mentioned up-regulated genes of aged capillaries(*Junb*, *B2m*) all gradually up-regulated with trajectory. [Figure 35] In summary, our analysis revealed considerable diversity within endothelial cells, with Endo-C2 appears to be more stressed and related to immune response, particularly in aged samples.

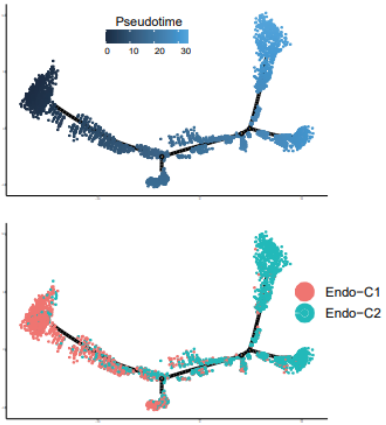

Figure 34. Pseudotime trajectory of capillary endothelial cells.

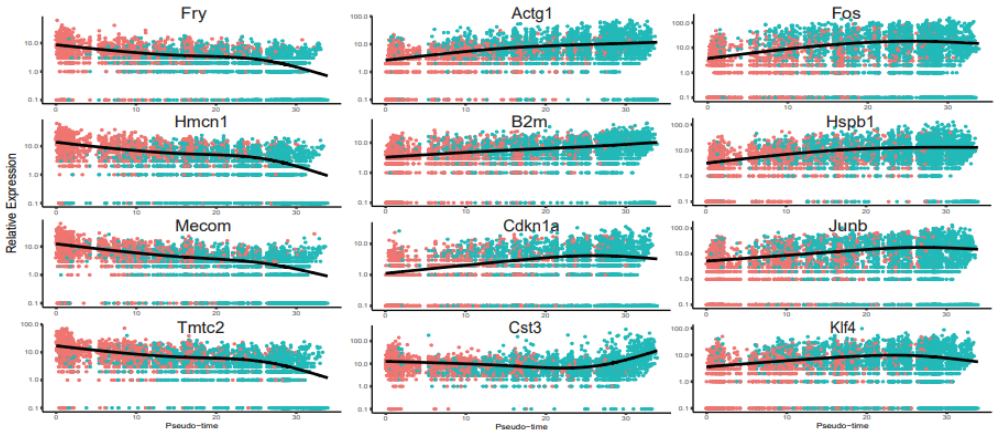

Figure 35. Pseudotemporal gene expression of capillary endothelial cells.

3.6. Aging-related Meis2-high expressing GABAergic neurons

By systematically classifying the genes that enriched in the various GABAergic neuron clusters, we were able to distinguish subtypes based on gene expression profiles except GABA neuron 1 and GABA neuron 2 which had no specific marker genes and difficult to distinguish strictly. [Figure 36] The GABAergic neurons were divided into three main groups based on marker genes: Meis2-high expressing neurons, highlighted in the black-boxed clusters, Sst/Lamp5/Vip/Pvalb neurons, representing specific subtypes of GABAergic neurons marked by these genes (as seen in the red-boxed clusters), and Meis2-low expressing neurons, represented in the remaining clusters.

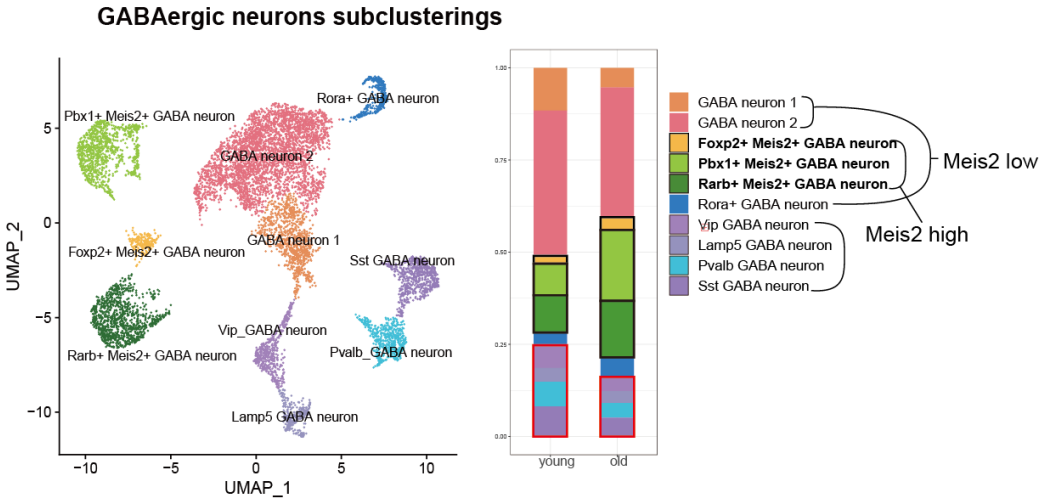

Figure 36. Subclustering of GABAergic neurons and shifts in composition with aging.

As shown in the heatmap in [Figure 37], we observed distinct gene expression patterns in these subgroups. Meis2-high neurons were marked by enriched expression of *Meis2* and some part with *Rarb*, which is highly associated with developmental and regenerative processes. On the other hand, Sst/Lamp5/Vip/Pvalb neurons represented well-known subtypes of inhibitory neurons, each expressing their respective markers, such as *Sst* (Somatostatin), *Lamp5*, *Vip*, and *Pvalb* (Parvalbumin). The Meis2-low neurons, which comprised the remaining clusters, were characterized by reduced *Meis2* expression and transcriptionally have no marker genes comparing to other groups.

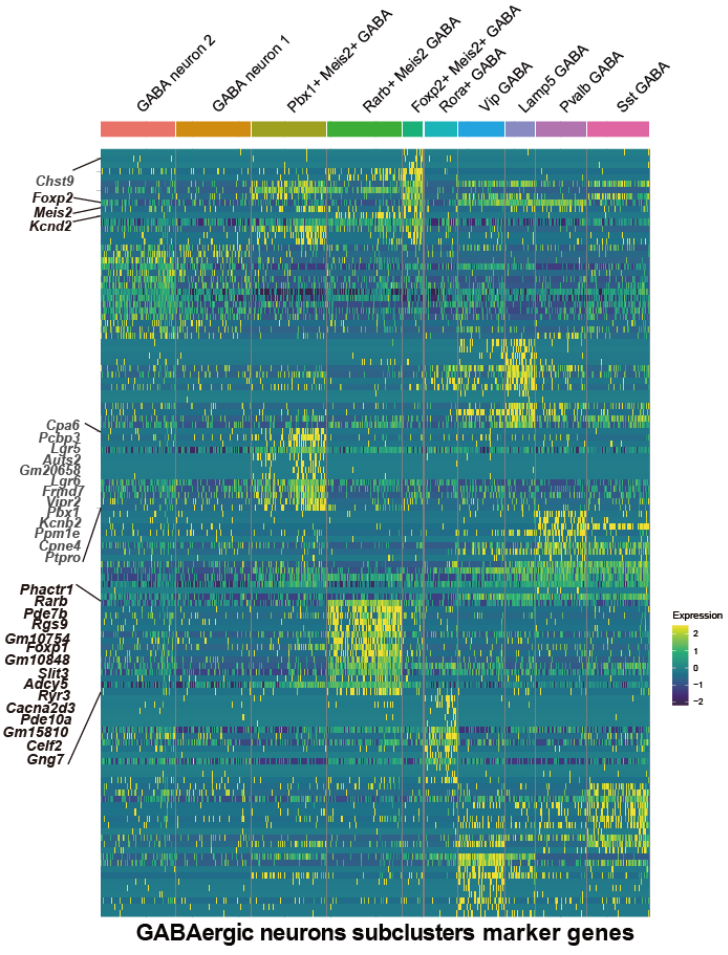

Figure 37. Marker genes of ten GABAergic neuron subclusters.

Through the proportional analysis of these neuron types in young and aged brains, we found a notable shift with aging. [Figure 38] *Meis2*-high neurons increased significantly in aged samples, indicating that this neuronal subtype may play a more prominent role in the aging brain. In contrast, *Sst/Lamp5/Vip/Pvalb* neurons decreased with age, suggesting that the functions of these specific inhibitory neurons might decline in the aging brain. It is consistent with other aging related papers.[62,65] The proportion of *Meis2*-low neurons remained relatively stable but, comparing with overall ratio of GABA neuron, it showed a slight decrease. This overall reduction in the ratio of Meis2-low neurons, along with the decline in *Sst/Lamp5/Vip/Pvalb* neurons, can be interpreted as a general decrease in GABAergic neuron populations with aging, potentially contributing to reduced inhibitory signaling in the aged brain.

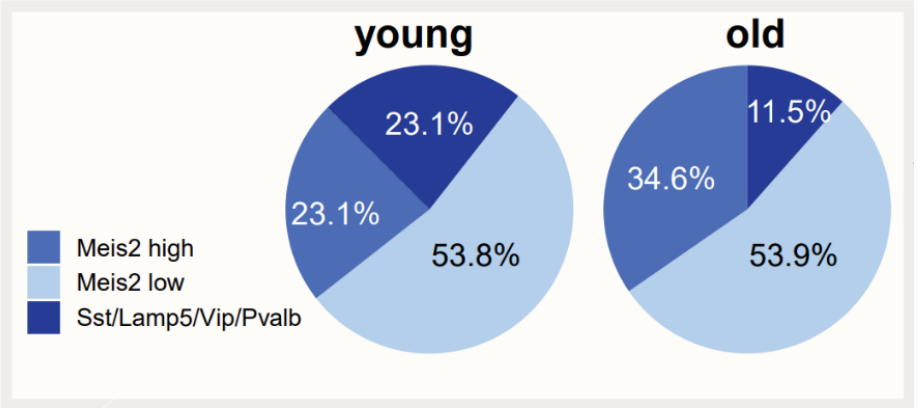

Figure 38. Proportional changes in GABAergic neurons.

We further performed pseudotemporal ordering of the transcriptional dynamics of the GABAergic neuron clusters to capture the molecular trajectory of *Meis2*-high expressing neurons during aging.[Figure 39] With MST analysis, we observed that the *Meis2*-high GABAergic neurons occupied distinct regions along the trajectory, forming two separate branches, suggesting that these neurons undergo divergent developmental or functional

changes. Specifically, these branches may reflect transitional states leading toward different functional subtypes of *Meis2*-high neurons . In the context of aging, we observed that Meis2-high neurons exhibited increased proportions in aged brains, indicating their potential role in the aging process. Additionally, we observed that these neurons showed pseudo-temporally increasing expression of genes regulating neuron differentiation, including *Foxp2*, *Pbx1*, and *Phactr1*, further supporting their involvement in age-related neural dynamics. Foxp2 is important for neurogenesis by increasing dendrite length and synaptic plasticity and for vocal behaviors, learning and motor function.[66] Pre-B-cell leukemia homeobox 1(Pbx1) is known to control midbrain dopaminergic neuron(mDAn) development and related to Parkinson's disease.[67] Phosphatase actin regulator-1(Phactr1) encodes a synaptic protein regulating signaling and cell adhesion.

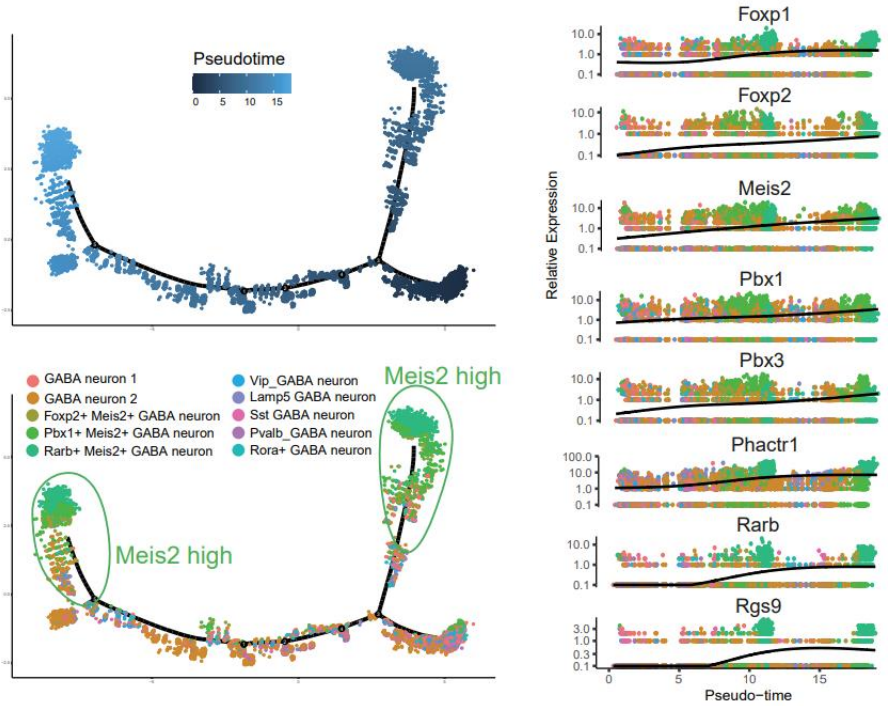

Figure 39. Trajectory and pseudotemporal gene expression in GABA neurons.

Taken together, our results suggest that *Meis2*-high GABAergic neurons follow a distinct molecular trajectory with aging, characterized by increased expression of developmental and aging-associated genes, as well as shifts in their functional roles. These findings point to the importance of *Meis2*-high neurons in maintaining inhibitory neural networks during the aging process and suggest they may contribute to the broader reorganization of GABAergic circuits in aged brains.

3.7. Meis2+ Rarb+ GABAergic neurons associated with cognition

We wondered if neurons express high Rarb gene exist in public brain atlas, so we mapped with reference. Interestingly Rarb+ was highly expressed in CNU-LGE GABAergic neuron clusters, which contains striatal and pallidal GABAergic neurons. [Figure 40] As concsistent, gene Drd1 and Drd2 also expressed highly in this cluster, which are marker genes of dopaminergic striatal projecting neurons(SPN-D1 and D2). Same as public data, our Rarb+ high GABA population contained Drd1 and Drd2 high cells. [Figure 41,42]

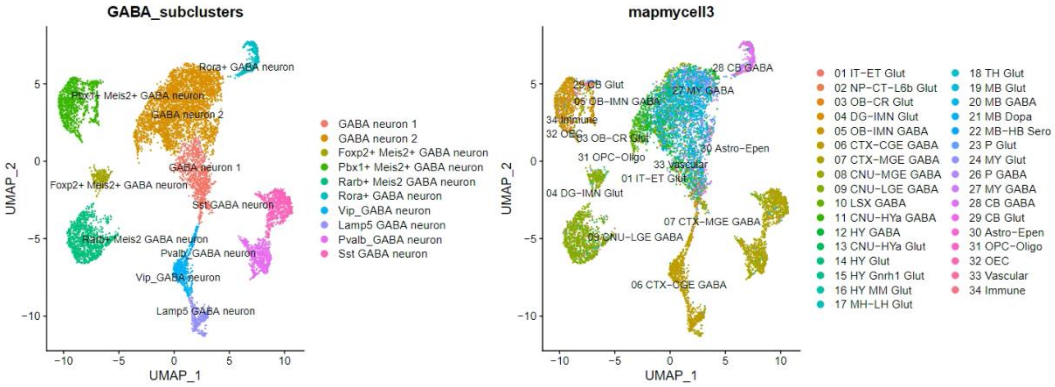

Figure 40. UMAP plot as Figure 36 (left) and ABC label transfered UMAP (right).

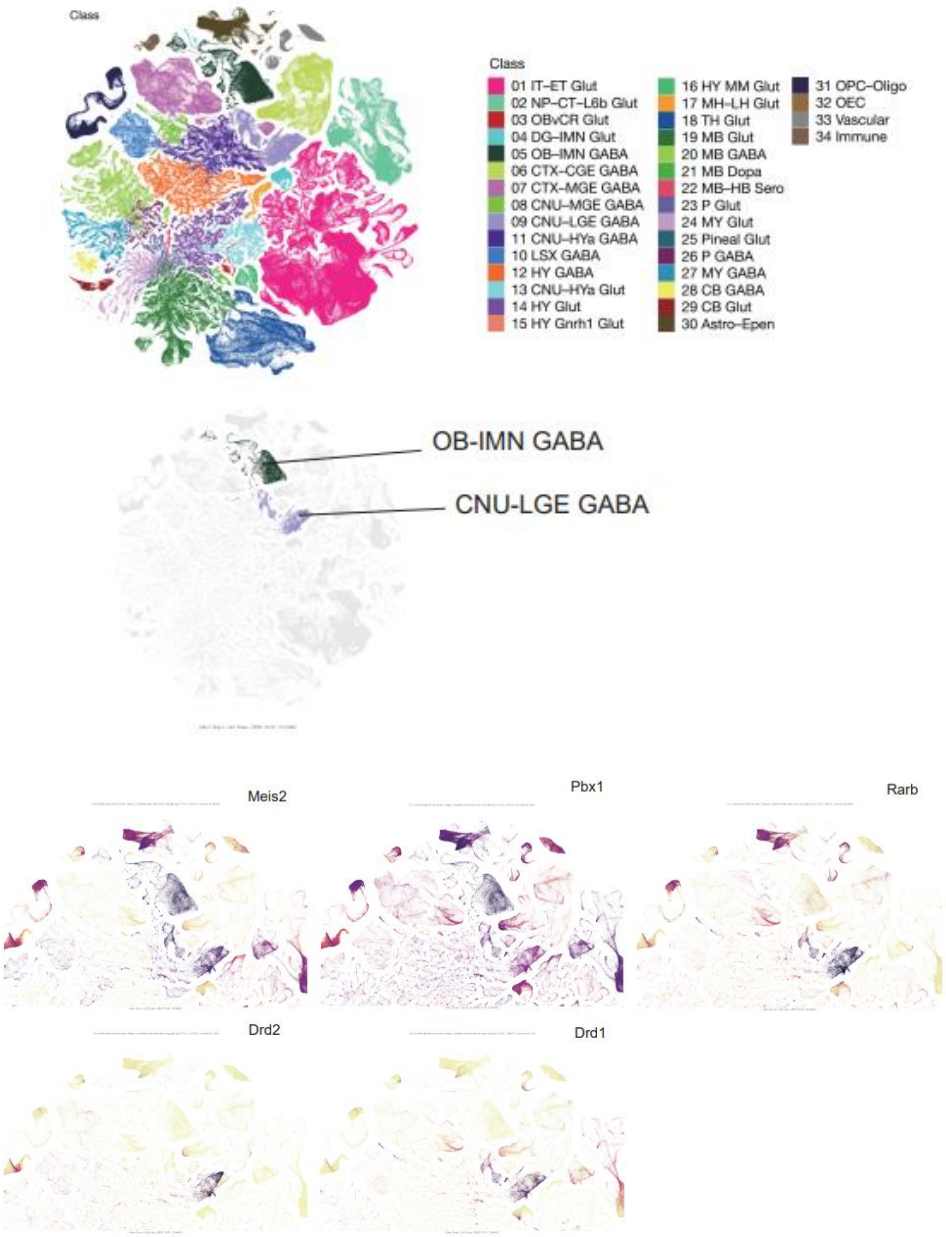

Figure 41. Allen brain cell (ABC) atlas and marker genes of Meis2 high population.

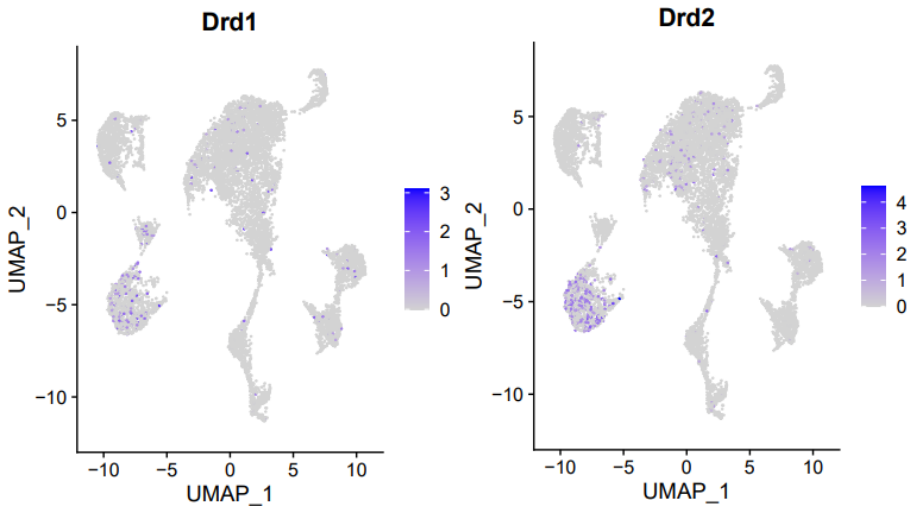

Figure 42. Expression level of SPN marker genes(*Drd1* and *Drd2*).

To complement our gene expression analysis, we analyzed label transfered snATAC-seq to identify cell-type specific transcription factor (TF) motifs in GABAergic neurons. Transcription factors (TFs) play a crucial role in regulating cell fate and function, particularly in neural development and aging-related processes. First, we calculate differentially accessible regions (DARs) in our pre-annotated ATAC-seq data of GABAergic neurons with FindAllMarkers in Seurat package. Next, we scanned motif enrichment within these regions for known TF binding motifs, by using ATAC-seq analysis pipelines. With this analysis, we indirectly sought to investigate the regulatory elements that might influence the distinct molecular and transcriptional identities of all GABAergic neurons. The TF motif enrichment analysis revealved distinct transcriptional regulators associated with developmental and functional roles in these unique population. [Figure 43]

To find epigenetic changes of aged-GABAergic neurons, we focused on *Meis2* high GABAergic neurons, characterized by expression of key marker genes such as *Pbx1*, *Foxp2*, and *Rarb*. The most noteworthy finding was the differential regulatory signatures within the Rarb+ Meis2 high GABAergic neurons. Rarb+ Meis2 high GABAergic

neurons, displayed low enrichment of NEUROD1, CTCF, and NEUROG2, similar to Foxp2+ neurons, but showed a high enrichment of EGR, FOS, JUN, PKNOX1, GLI2, PBX3, TBX18, KLF9, and KLF17 motifs. The presence of EGR, FOS, and JUN further implicates this subpopulation in synaptic plasticity and stress response. Moreover, the enrichment of PKNOX1, GLI2, and PBX3 suggests additional roles in neuronal differentiation and transcriptional regulation of neural identity. The KLF9 and KLF17 motifs are particularly interesting, as these factors are involved in regulating neuronal development and plasticity, possibly linking this subpopulation to cognitive processes and adaptation in response to brain aging

To confirm the funtional feature of Rarb+ Meis2+ high GABA neuron, we analyzed GSEA(KEGG and GO) analysis with top marker genes of transcriptomic data [Figure 44] and top differential open regions [Figure 45]. Interestingly Rarb+ Meis2 high neurons were significantly enriched in pathways related to cognitive functions(learning and memory), and synaptic organization(synaptic structure and activity). It revealed that Rarb+ Meis2+ subpopulation is strongly associated with maintaining neural connectivity and functionality, particullary in the context of aging. Also, it is crucial to play role in compensating for cognitive decline associated with aging, by maintaining or reorganizing inhibitory circuits in the brain. All results reinforce the idea that this subpopulation of neurons could be central to adaptive responses in the aging brain, offering potential therapeutic targets for addressing cognitive deficits in neurodegenerative diseases.

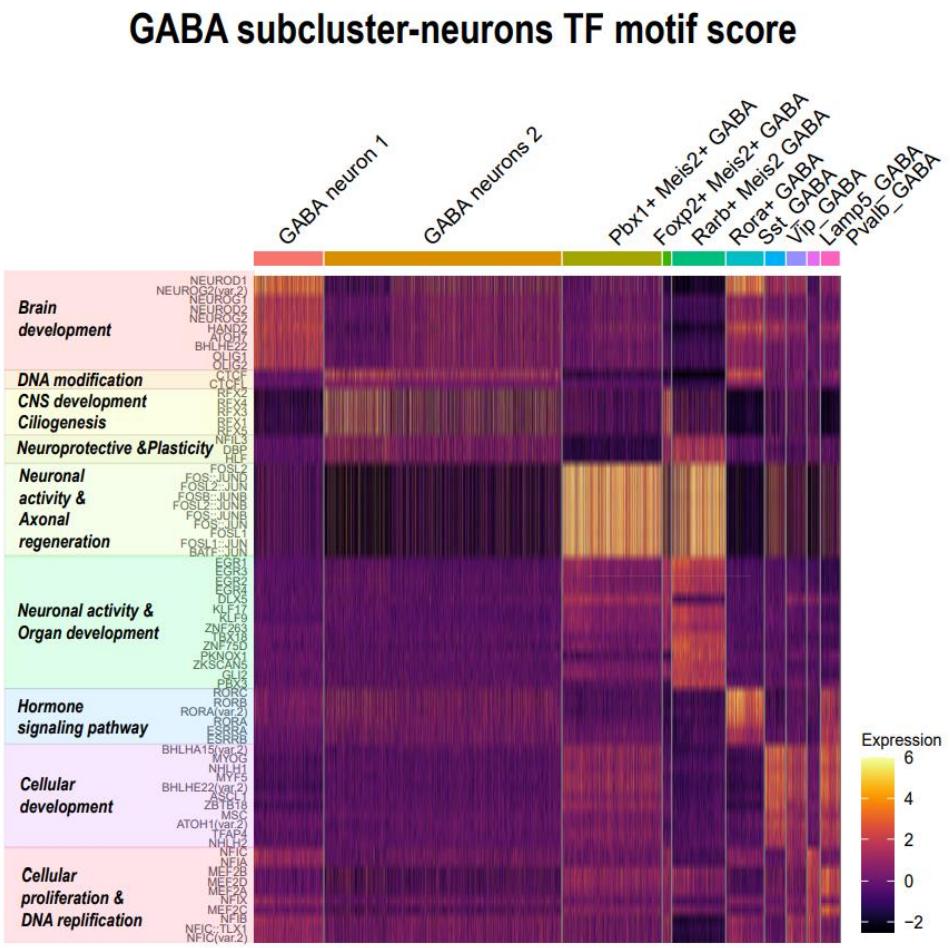

Figure 43. Differential motif scores of GABA clusters based on chromatin accessibility.

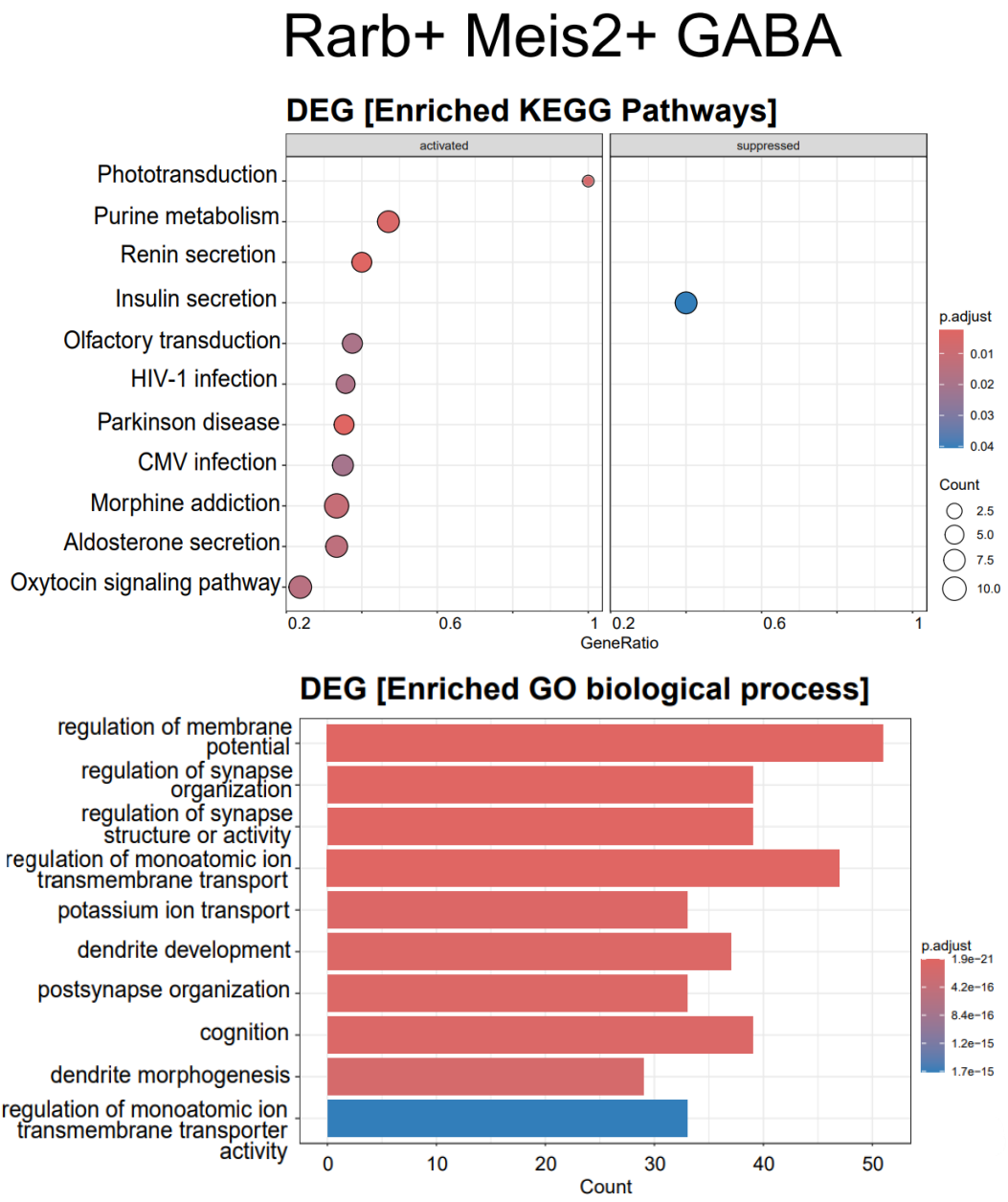

Figure 44. KEGG and GO analysis of Rarb+Meis2+ GABAergic neurons.

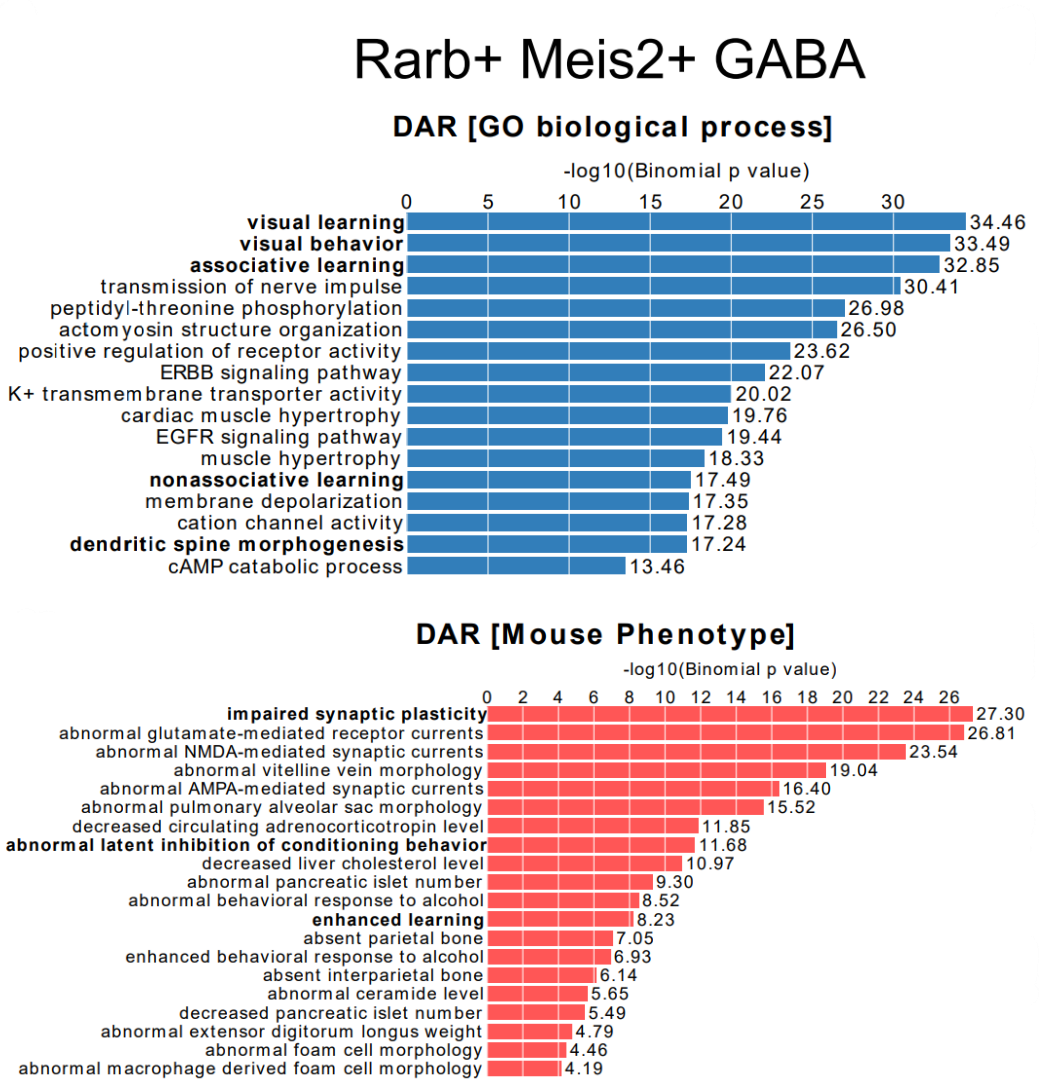

Figure 45. Enriched pathways and phenotype in Rarb+Meis2+ GABAneurons.

3.8. Epigenetic and Transcriptional Signatures of GABAergic neurons

Following the detailed analysis of Section 3.7, expanded our investigation to examine other Meis2 high GABAergic neuron subtypes, such as Pbx1+ and Foxp2+ neurons, as well as the Meis2 low subpopulations, including GABA neuron 1, GABA neuron 2, and Rora+ GABAergic neurons. In Pbx1+ Meis2 high GABAergic neurons, we observed low enrichment of NAFIL3, DBP, and HLF, but a high enrichment of FOSL2, FOS::JUNB, BATF::JUN, and DLX5 motifs. This suggests that these neurons are more involved in transcriptional programs related to neuronal activity and regeneration, given the high expression of FOS-JUN family members, which are known to regulate responses to neural activity and synaptic plasticity. DLX5, a TF involved in GABAergic neuron development, further supports the developmental aspect of this subpopulation.

In contrast, Foxp2+ Meis2 high GABAergic neurons exhibited low levels of NEUROD1, CTCF, and NEUROG2 motifs, typically associated with neurodevelopment and CNS specification. Instead, they showed a high enrichment of RFX (RFX1-5) family motifs, potentially maintaining the sensory ciliary structures necessary for cellular signaling.

Meanwhile, in the Meis2 low GABAergic neuron subtypes, GABA neuron 1, GABA neuron 2, and Rora+ GABAergic neurons, distinct transcriptional regulation was observed. Despite the absence of specific marker genes, GABA neuron 1 and GABA neuron 2 exhibited strong enrichment for TF motifs such as NEUROD1, NEUROG2, OLIG1/2, and RFX (RFX1-5) family. The presence of NEUROD1 and NEUROG2, key regulators of brain development and CNS differentiation, suggests that these neurons may represent more immature or plastic populations. The enrichment of OLIG1/2, which are traditionally linked to oligodendrocyte development, suggests a potentially novel role in neuronal differentiation, particularly in maintaining neuronal plasticity and homeostasis

within the CNS[68]. Additionally, RFX motifs, which regulate ciliary gene expression, may indicate a role in neuronal ciliogenesis and the broader regulation of inhibitory signaling pathways.

Rora+ GABAergic neurons, another Meis2 low subtype, displayed high enrichment for RORA, RORB, ESRRA, and ESRRB motifs, reflecting their known roles in neuronal activity regulation and circadian rhythm. The presence of RORA and RORB in these neurons suggests a key function in coordinating GABAergic signaling in response to environmental cues such as circadian cycles.

Overall, these findings highlight the transcriptional and epigenetic diversity between Meis2 high and Meis2 low GABAergic neurons. Across different subclusters, we identified key TF motifs associated with differentially open chromatin regions.

3.9. Interaction shifts with microglia can lead pathology phenotype

Recently, it has revealed that specific disease-associated or aging-related cells, such as DAM (disease-associated microglia), DAO (disease-associated oligodendrocytes), or DAA (disease-associated astrocytes), are implicated in the development of neurodegenerative diseases. Even though they were discovered in aged or knockout mouse, systematic interactions within the entire aged brain remain underexplored. To better understand the potential pathological features associated with these interactions, we conducted an cell-cell interaction (CCI) analysis between these mentioned aging related-cell populations.

A comprehensive analysis of cell-cell communication in both young and aged brains using transcriptional data with cellchat [Figure.46] showed novel signaling patterns in aged brain, involving IFN-γ (Interferon-gamma), FASLG (Fas ligand), IL-1 (Interleukin-1), PD-L1 (Programmed death-ligand 1), BAFF (B-cell activating factor), NKG2D (Natural killer group 2, member D), L1CAM (L1 Cell adhesion molecule), NECTIN and CLDN (Claudins). These signals are associated with inflammaging (IFN-γ, IL-1), regulating apoptosis and killing aged cells (FASLG, NKG2D) and associating BBB or cell junction assembly (CLDN, NECTIN). Some pathways including CSPG4, Opioid, and SELL were only active in young brain, contributing to neuroprotection, cell mobility, and immune response regulation. The decline of these pathways with aging suggests a reduced capacity for neural regeneration, immune surveillance, and inflammation control, which may contribute to increased susceptibility to neurodegenerative diseases.

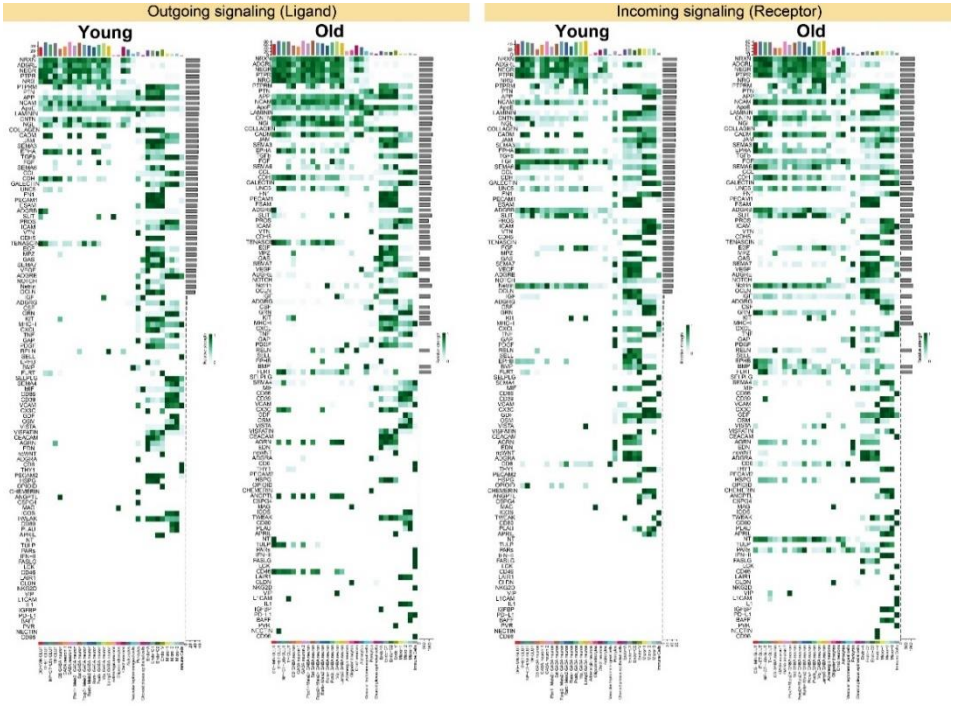

Figure 46. Outgoing and incoming signals from both young and old brains.

In-depth analysis of the GRN (Granulin) and RELN (Reelin) pathways, both linked to neurodegenerative diseases[67,69], reveals significant changes in their signaling dynamics with aging. [Figure 47] In the young brain, GRN signaling primarily interacted between microglia and brain endothelial cells, maintaining immune regulation. However, in the aged brain, GRN signaling shifted, with increased interactions directed toward neurons, both GABAergic and Glutamatergic neurons, alongside an upregulation of *Sort1* expression [Figure 48-50], indicating enhanced neuronal involvement in the inflammatory response. This suggests a growing role for neuronal GRN signaling in driving neurodegeneration through both immune and neural pathways.

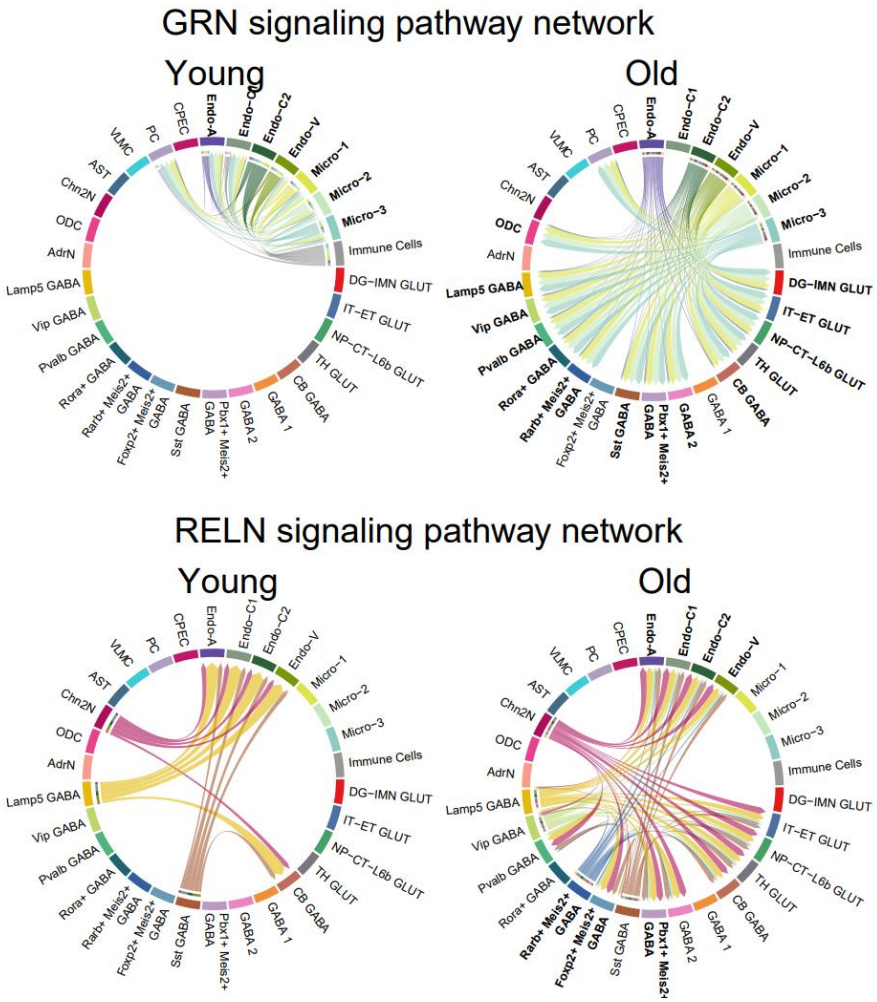

Figure 47. GRN and RELN signaling pathways in young and old mouse brain.

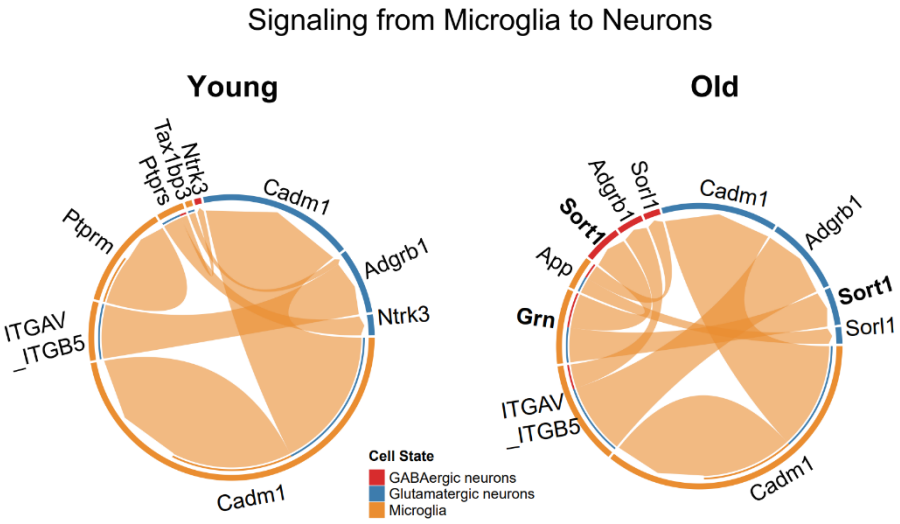

Figure 48. Signaling from microglia to neurons in young and old brains.

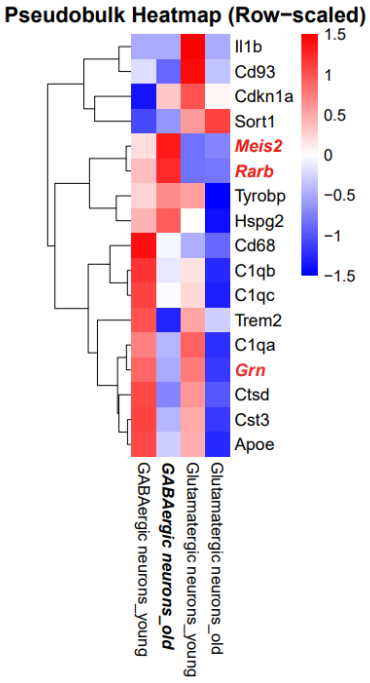

Figure 49. Row-scaled pseudobulk gene expression in GABA and GLUT neurons.

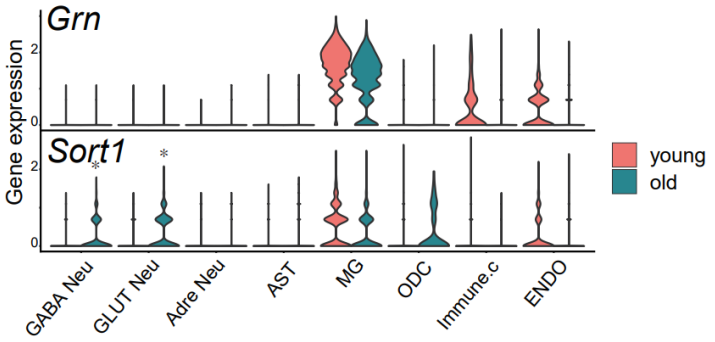

Figure 50. Comparing gene expression of Grn (ligand) and Sort1 (receptor).

For the RELN pathway, a notable shift occured with the emergence of Rarb+ Meis2 high GABA and Vip GABA neurons as novel signaling sources in the aged brain, while endothelial cells showed a reduction in RELN signaling reception, corresponding with decreased expression of Itgb1 and Itga3. It is interesting that Endo-C2 has weaker RELN incoming signaling than Endo-C1 that refers trajectory of endothelial cells also explain the same direction. These changes imply a decline in endothelial function and an increased role for neurons in RELN-mediated processes, potentially contributing to synaptic disorganization and cognitive decline. [Figure 47,51]

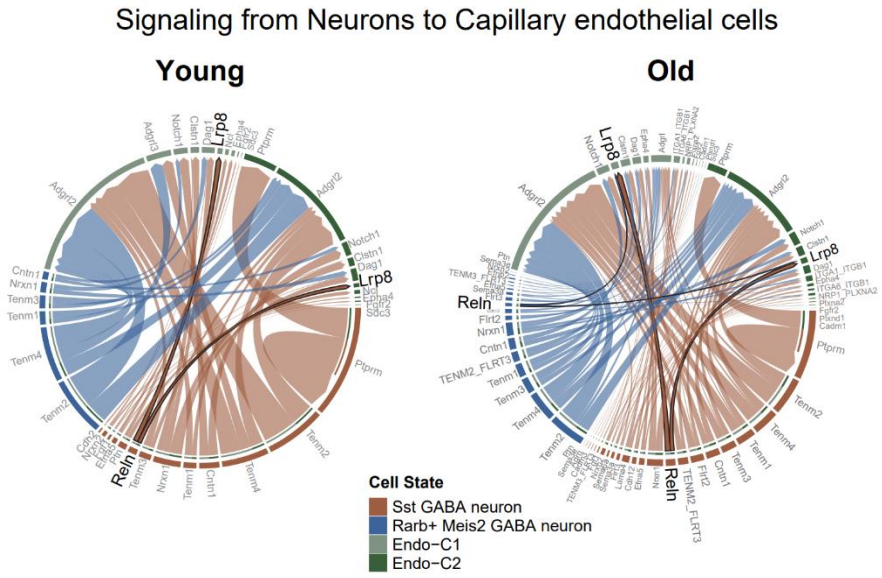

Figure 51. Signaling from neurons to capillary endothelial cells.

4. Discussion

In this study, we employed multi-omics analysis to comprehensively characterize the transcriptional, epigenomic, and cell-cell interaction dynamics of the aging mouse brain. Through integrating snRNA-seq, scRNA-seq, and snATAC-seq, we elucidated several key shifts in cellular populations, signaling pathways, and gene regulation that underpin aging-related processes in the brain. Our analysis revealed alterations in some brain cell types, highlighting their potential roles in age-associated neurodegeneration and suggesting a interplay between transcriptional and epigenetic modifications in aging.

One of our major findings was the identification of three microglia subtypes (Micro-1, Micro-2, and Micro-3), with Micro-3, which showed increased abundance in aged brains, representing a pro-inflammatory and disease-associated state. Our pseudotemporal analysis suggests that Micro-3 acts as a terminally differentiated form of microglia, diverging from the homeostatic Micro-1 via an intermediate Micro-2 state. The trajectory analysis, combined with upregulated markers such as *Apoe* and *Lyz2*, provides insight into how Micro-3 could contribute to neuroinflammation and chronic immune activation, consistent with disease-associated microglia (DAM) identified in models of neurodegenerative diseases. The increased presence of Micro-3 in the aged brain suggests an exacerbation of inflammatory processes that may promote neuronal damage and contribute to the overall decline in brain health with aging.

Endothelial cells in the brain, which are integral components of the blood-brain barrier (BBB), also showed significant age-associated changes. We observed distinct clustering of endothelial cells into arterial, venous, and capillary subtypes, with a specific increase in the aged capillary endothelial subtype (Endo-C2). Endo-C2 exhibited transcriptional signatures indicative of increased stress and immune activity, including upregulation of genes such as *Hspb1* and *Actg1*. Our pseudotemporal analysis of endothelial cells revealed that Endo-C2 is likely a senescent-like state, and its enrichment in aged brains

suggests that endothelial dysfunction and BBB disruption may be significant contributors to age-related neuroinflammation. The increased expression of inflammatory cytokines and adhesion molecules further implies that Endo-C2 endothelial cells may facilitate the infiltration of peripheral immune cells into the brain parenchyma, thus contributing to brain immune dysregulation and neurodegenerative processes.

Our analysis of GABAergic neurons uncovered a novel subpopulation of Meis2-high expressing neurons, which showed significant increases in abundance in aged samples. The Meis2-high subtype, particularly Rarb-positive neurons, displayed gene expression patterns associated with developmental and plasticity-related processes, including increased expression of transcription factors such as *Foxp2*, *Pbx1*, and *Phactr1*. These findings suggest that Meis2-high GABAergic neurons might play a compensatory role in maintaining neural circuitry during aging, potentially counteracting the decline of other GABAergic neuron populations, such as those expressing *Sst*, *Lamp5*, *Vip*, and which decreased in abundance. Furthermore, label-transfer analysis of snATAC-seq data revealed enrichment of transcription factor motifs such as EGR, FOS, and PKNOX1 in the Meis2-high neurons, suggesting that epigenomic remodeling in these neurons may underlie their plasticity and contribute to the reorganization of inhibitory circuits in the aged brain.

Cell-cell interaction analysis provided insight into the systematic changes in intercellular communication occurring during aging. We observed novel signaling patterns in aged brains, involving pathways such as IFN-γ, FASLG, and IL-1, all of which are associated with inflammaging. The emergence of pro-inflammatory signaling, particularly through GRN and RELN pathways, highlights shifts in interactions between microglia, endothelial cells, and neurons, which could contribute to synaptic dysfunction and neuronal loss. The increased involvement of neurons in GRN signaling, particularly GABAergic neurons, suggests a growing neuronal role in perpetuating inflammatory

responses in the aged brain, which may exacerbate neurodegenerative processes.

Notably, the RELN pathway showed a shift in its signaling dynamics, with Rarb+ Meis2-high GABAergic neurons emerging as a novel source of RELN signaling in the aged brain. This shift in signaling dynamics, coupled with decreased endothelial reception, implies a decline in endothelial function and increased neuronal contribution to RELN-mediated processes, potentially leading to impaired synaptic stability and cognitive function. These findings suggest that aging leads to both cellular and systemic shifts in intercellular signaling that may collectively drive the progression of neurodegeneration.

While our study provides valuable insights into the aging brain, it is important to acknowledge certain limitations. The relatively small sample size (n=3 per age group) limits the statistical power of our findings, and future studies with larger sample sizes are needed to validate our observations. Despite this limitation, our integrative approach enabled us to capture broad transcriptional and epigenetic changes that would be challenging to observe using traditional methods, such as FACS sorting, which may not effectively capture the full diversity of cellular states and interactions present in the aging brain.

In summary, our study reveals significant transcriptional, epigenetic, and cell-cell interaction changes in the aging mouse brain, highlighting the roles of specific cell subtypes in age-associated pathology. Microglia, endothelial cells, and GABAergic neurons undergo distinct shifts, with aged microglia exhibiting enhanced pro-inflammatory states, capillary endothelial cells showing signs of senescence, and Meis2-high GABAergic neurons potentially acting as compensatory regulators of inhibitory signaling. These findings provide insight into the cellular and molecular underpinnings of brain aging and highlight potential therapeutic targets to mitigate aging-related neurodegeneration.

5. Conclusion

This study utilized an integrative multi-omics approach to investigate the aging mouse brain, revealing complex transcriptional, epigenomic, and intercellular changes across different brain cell populations. We identified significant age-related shifts in microglia, endothelial cells, and GABAergic neurons, which contribute to neuroinflammatory and neurodegenerative processes. Specifically, we characterized the transition of microglia into a pro-inflammatory and disease-associated state (Micro-3), which suggests a role in promoting chronic inflammation in the aged brain. We also described the emergence of an aged-specific capillary endothelial cell subtype (Endo-C2), which displayed stress-related and immune activation signatures, indicating potential blood-brain barrier dysfunction and facilitation of immune cell infiltration.

Our findings in GABAergic neurons highlight a novel Meis2-high subtype that increases in abundance with aging, potentially compensating for the decline of other inhibitory neurons. The Meis2-high neurons, particularly those expressing Rarb, showed enriched pathways related to plasticity, suggesting they may help maintain inhibitory network stability in the aged brain. The shifts in RELN and GRN signaling pathways observed in aged brains indicate altered intercellular communication that may drive neurodegeneration through both inflammatory and synaptic pathways.

Overall, our study provides a comprehensive view of the aging brain, identifying key cellular and molecular mechanisms that contribute to neurodegeneration. The identification of specific cell populations, such as Micro-3 microglia, Endo-C2 endothelial cells, and Meis2-high GABAergic neurons, as well as their associated signaling pathways, offers potential therapeutic targets for mitigating age-related cognitive decline and neurodegenerative diseases. Future studies should focus on elucidating the functional roles of these cell populations and their interactions to better understand the mechanisms of aging and develop strategies for intervention.

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Abstract in Korean

전사체 및 후성유전체 데이터 통합 분석을 통한 마우스 뇌에서의 노화연구

노화는 뇌의 세포 구성과 신호 경로에 변화를 일으키며 알츠하이머와 같은 신경퇴행성 질환의 위험을 증가시킨다. 최근에는 많은 연구자들이 차세대 시퀀싱 기술을 뇌 연구에 활용하면서 그동안 밝히기 어려웠던 뇌에 서 중요한 역할을 하는 다양한 세포 이질성 분석에 크게 기여하고 있다. 특정 노화 세포 유형이나 뇌의 특정 영역에서의 변화와 관련된 위험요소는 연구되어 왔으나, 전체 마우스 뇌의 노화 내재적 특성에 대해서는 아직 많이 알려지지 않았다.

본 연구에서 단일 세포 및 단일 핵 전사체 시퀀싱(scRNA seq, snRNA seq)과 단일 핵 ATAC 시퀀싱(snATAC seq)을 통합 분석하고자 하였으며 뇌에서의 전사, 후성유전적, 세포 간 상호작용의 동태를 알아보고자 하였다. 분석 결과, 뇌 내 여러 주요 세포 유형에서 노화 관련 주요 변화를 확인하였다. 특히 미세아교세포의 염증 유발 및 질병 연관 상태로의 전이, 노화된 모세혈관 내피세포의 스트레스 및 면역 활성 증가, Meis2 발현이 높은 GABA성 뉴런의 보상적 역할이 주요 결과로 도출되었다. 세포 간 상호작용 분석을 통해 노화된 뇌에서 신경세포를 향하는 염증성 신호 경로의 활성화가 관찰되었으며, 이러한 변화들이 신경 기능 저하와 관련될 가능성을 시사하였다.

본 연구는 노화된 뇌의 세포적 및 분자적 기전을 규명하고, 염증성 미세아교세포와 신경세포 및 내피세포 간 신호가 노화 관련 신경퇴행에 중요한 역할을 한다는 것을 강조하며 인지 기능 저하와 뇌 질환의 잠재적 표지자로서의 가능성을 시사한다. 나아가 노화 관련 신경퇴행성 질환을 완화하기 위한 잠재적 치료 표적을 제시한다.

핵심되는 말: 노화, 다중 오믹스 분석, 단일세포 전사체, 단일세포 후성유전체, 통합분석, 미세아교세포, 내피세포, GABA 뉴런, 신경퇴행성질환, 뇌