Decoupling of mRNA and protein expression in aging brains reveals the age-dependent adaptation of specific gene subsets

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Abstract

During aging, changes in gene expression are associated with decline in physical and cognitive abilities. Here, we investigated the connection between changes of mRNA and protein expression in the brain by comparing the transcriptome and proteome of the mouse cortex during aging. Our transcriptomic analysis revealed that aging mainly triggers gene activation in the cortex. We showed that increase of mRNA expression correlates with protein expression, specifically in the anterior cingulate cortex where we also observed an increase of cortical thickness during aging. Genes exhibiting an aging-dependent increase of mRNA and protein levels are involved in sensory perception and immune functions. Our proteomic analysis also identified changes in protein abundance in the aging cortex and highlighted a subset of proteins that were differentially enriched but exhibited stable mRNA levels during aging, implying the contribution of aging-related post transcriptional and post-translational mechanisms. These specific genes were associated with general biological processes such as translation, ribosome assembly and protein degradation, but also important brain functions related to neuroplasticity. By decoupling mRNA and protein expression, we have thus characterized distinct subsets of genes that differentially adjust to cellular aging in the cerebral cortex.

Introduction

Aging is a gradual process of natural changes leading to the progressive loss of physiological integrity and the impairment of essential biological functions. This deterioration of physiological functions with advancing age is the primary risk factor for developing chronic diseases and increasing vulnerability to death. The cellular and tissue changes are sustained by molecular aberrations at different regulatory layers: DNA and chromatin, RNA and proteins. During aging, DNA integrity is challenged by genomic instability and telomere attrition. Protective and repair mechanisms fail to address the damages inflicted to DNA and genetic damages tend to accumulate (Moskalev et al. 2013), leading to deleterious cellular effects such as senescence or apoptosis (Hewitt et al. 2012). Age-related changes in gene expression are linked to epigenetic alterations. During aging, specific epigenetic alterations, such as the increase of H4K16ac, H4K20me3 and H3K4me3 histone marks (activation), the decrease of H3K9me and H3K27me3 histone marks (repression), or the global increase of DNA methylation level (repression), can directly impact the expression of the genes (Horvath 2013; Horvath and Raj 2018; Fraga and Esteller 2007; Han and Brunet 2012). Finally, the ability to preserve protein homeostasis, also called proteostasis, under normal and stress conditions is gradually compromised with age (Kaushik and Cuervo 2015; Taylor and Dillin 2011). These primary hallmarks of cellular aging are conserved across evolution and can lead to loss of important cellular functions (Lemoine 2021). During aging, the overall mRNA translation rate decreases as the translation machinery becomes less efficient. This decrease is due to the deregulation of the stoichiometry of translation components leading to decreased abundance and activity of ribosomes (Dhondt et al. 2017; Janssens et al. 2015) and the possible sequestration of mRNAs in stress granules where they cannot be translated (Anisimova et al. 2020). Therefore, the mRNA/protein expression ratio for a specific gene might be strongly affected during aging.

In a cell, the average transcription rate is about two mRNA molecules per hour, with a range between 0.1 and 100 mRNA molecules per hour depending on the given locus (Schwanhausser et al. 2011). The median translation rate is about 140 proteins per mRNA per hour, with a maximum rate of 1,000 proteins per mRNA per hour (McManus, Cheng, and Vogel 2015; Hausser et al. 2019; Schwanhausser et al. 2011). Moreover, proteins are on average 5 times more stable than mRNAs (Schwanhausser et al. 2011). Therefore, it is easier to change the abundance of mRNAs to have an impact on protein level. The mRNA level is mostly important for newly synthesized proteins since their initial concentration is minimal whereas changes at the protein level are needed for pre-existing proteins with basic cellular functions (Jovanovic et al. 2015). Even though higher concentration of mRNA molecules has more chances to be transcribed into many more proteins, there are several regulatory mechanisms in place modulating the level of protein expression: the translation rate, the rate of degradation but also the protein synthesis delay and transport (McManus, Cheng, and Vogel 2015). Translation rate is primarily modulated by the mRNA sequence itself - codon composition, upstream Open Reading Frames (uORFs), presence of internal ribosome entry sites (IRES) (Locker, Chamond, and Sargueil 2011; Wethmar 2014). The translation rate can also be modulated by the availability of the ribosomes, and the binding of different molecules to the mRNA such as micro-RNA or long noncoding RNA (lncRNA). Therefore, two groups of genes exhibiting different types of regulation have been described: i) genes that mainly rely on transcriptional regulation, with change of mRNA level correlating with that of proteins, and ii) genes that depend on a translational regulation where RNA dynamic is not reflected into protein levels because of post-transcriptional mechanisms (Liu and Aebersold 2016; Liu, Beyer, and Aebersold 2016). In steady state, mRNA levels primarily explain protein levels as their respective average remains stable over time even when cells undergo long-term dynamic

processes such as proliferation or differentiation (Hsieh et al. 2012; Kristensen, Gsponer, and Foster 2013; Li et al. 2014). For short-term adaptation, post transcriptional processes are more important than initial levels of mRNA, as the regulation of transcript levels alone would be too slow. The increase of translation for already existing transcripts or the increase of protein degradation are faster and more efficient mechanisms. There is a poor mRNA-protein correlation during stress as it causes significant change in translation rate and protein misfolding (Cheng et al. 2016; Lackner et al. 2012). During aging, mRNA and protein expression levels can drastically change (Anisimova et al. 2020; Takemon et al. 2021; Waldera-Lupa et al. 2014; Wei et al. 2015). The age-dependent association between mRNA and protein levels remains mainly unexplored and it is not known whether cellular aging impacts specific groups of genes.

In mice, transcriptomic studies on different tissues with bulk RNA-seq experiments have shown that most genes age with the same pattern in different tissues but differ in amplitude and onset of expression depending on the organ (Schaum et al. 2020; Ori et al. 2015). The brain is one of the organs that ages the fastest (Ibrayeva et al. 2021). Here we have investigated the connection between changes of mRNA and protein expression in the aging brain by comparing the transcriptome and proteome of the cerebral cortex during aging. Our transcriptomic analysis showed that aging mainly triggers gene activation in the cortex, and that genes with increased of mRNA and protein expression are associated with sensory perception and immune functions. Our proteomic analysis revealed changes in protein abundance in the aging cortex and highlighted a subset of proteins that were differentially enriched but exhibited stable mRNA levels during aging. These genes targeted by aging-dependent post transcriptional mechanisms were associated with general biological processes (i.e., translation, ribosome assembly, protein degradation) but also functions related to neuroplasticity (i.e., neurogenesis, dendrite outgrowth, synapse formation). By

decoupling mRNA and protein expression, we have characterized distinct subsets of genes that differentially adjust to cellular aging in the cerebral cortex.

Material et Methods

Mice

Male C57BL/6J mice were purchased from the Réseau Québécois de Recherche sur le Vieillissement (RQRV, Québec City, Canada). All mice received a standard diet i.e., NIH-31 modified open formula mouse/ rat sterilized diet (Teklad 2019, Harlan Laboratories) and were kept on a 12:12 day light cycle at 22-23°C with access to a small house and tissues for nesting. Adequate measures were taken to minimize pain and discomfort. Animal care and handling was performed in accordance with the Animal Care Committee of Université Laval (CPAUL) and the guidelines of the Canadian Council on Animal Care and the Université Laval institutional policy.

The weight of each mouse was taken before euthanasia. Mice were placed under anesthesia (isoflurane) and then a cervical dislocation was performed. The brain was first removed from the cranial cavity, washed in Phosphate-buffer-saline (PBS) and then weighted before the microdissection of the cerebral cortex. If mice presented visible tumors in any major organs (for example the liver), they were discarded from the study.

Histology

Whole brains from 6- and 24-month-old mice were put in a plastic cassette, submerged in paraformaldehyde (PFA) 4% for 16h at 4°C. Then, several washes of fresh Ethanol 70% were performed 3 x 5 min followed by incubating in ethanol 70% for 12h at 4°C. Fixed brains were sent to the Histology Platform of the Université de Sherbrooke (Canada) to be embedded in paraffin and cut in a 4µm coronal cross-section (around Bregma 0). The cortical thickness and the surface area of the cortical layer were measured on brain slides stained with hematoxylin and eosin. The

measurement was blind for the age group and performed on 2 slides per brain for 6 mice per age group.

RNA and protein extraction

Each frozen sample of cortex was crushed and powdered using the BioSqueezer Snap Freeze Press system (Biospec), to allow homogenization and equal fractionation for both transcriptomic and proteomic studies. Equipment was previously cooled using dry ice. The subsequent powder was then fractionated and stored at -80°C in multiple eppendorf tubes for RNA or protein extraction.

To isolate total RNA from the tissue powder, a hybrid technique combining the TRIzol reagent protocol (Invitrogen, Thermo Fisher Scientific) and the Quick-RNA™ Miniprep Kit (Zymo Research) was used. Cortex powder was first homogenized in 500µL of cold TRIzol and then 500µL of chloroform:isoamyl alcohol 24:1 (Sigma Aldrich) were added. Samples were vortexed for 20 seconds and incubated for 2 min at room temperature. The samples were then centrifuged at 10,000g for 18 min at 4°C. The aqueous phase, that contains RNAs, was transferred in a new Eppendorf tube. An equal volume of 100% ethanol was added and the mixture was then loaded into a Zymo-Spin™ IIICG Column from the Quick-RNA™ Miniprep Kit. Further steps were conducted according to the manufacturer's protocol. The RNA concentration in ng/µL was determined using a Nanodrop system (Thermo Fisher Sscientific).

To isolate proteins, tissue powder was resuspended in white Laemmli 1X (62,5 mM TRIS pH 6.8, 10% v:v glycerol, 2% v:v SDS) and boiled 5 min at 95°C. Samples were then sonicated by burst of 5 sec at 12% amplitude then 10 sec on ice, for a total of 5 times. Absolute protein quantification was performed by Bicinchoninic acid assay (BCA) using the Pierce™ BCA Protein Assay Kit according to the manufacturer's parameters. The absorbance was read at 562 nm using a spectrophotometer and the absolute quantification was calculated.

RNA-Seq library preparation

RNA-seq libraries were prepared using NEBNext® single cell/low input RNA library preparation Kit (New England Biolabs). Library preparation was initiated with 10ng of total RNA. RNA along with all the other reagents were thawed on ice. Further steps were conducted according to the manufacturer's protocol until the final library amplification. Each final library was then purified using the MinElute PCR Purification Kit (Qiagen) and then quantified on a Qubit system (Thermo Fisher Scientific). Multiplexed libraries were pooled in approximately equimolar ratios and were then run in a 2% agarose gel. DNA between 300 and 700 base pairs was then cut and purified using the Qiagen Gel Extraction Kit (Qiagen). Library sequencing was performed at the RNomics platform of the Université de Sherbrooke (Canada). Single-End sequencing was performed on all the samples with a 75bp sequencing depth on a NextSeq machine. The raw data have been deposited at the Gene Expression Omnibus (GEO) under the subseries entry GSE218637.

Data analysis

Quality control of RNA-seq data was performed using FastQC (version 0.11.8) with default parameters. Trimming of the reads was achieved using TrimGalore (version 0.6.4) with default parameters except for: --illumina --max_n 5 --clip_R1 3 --three_prime_clip_R1 3. Quality of the data was also evaluated using FastQC after trimming of adapters and low-quality regions. Reads were then mapped to the mouse genome (GRCm38, GENCODE vM23, primary assembly) with STAR (version 2.7.3a). STAR was used with default parameters except for: --outFilterMultimapNmax 10 --outSAMtype BAM SortedByCoordinate --quantMode GeneCounts --outSAMprimaryFlag AllBestScore --outFilterMismatchNmax 5. The evaluation of gene expression (CPM and TPM) was done using FeatureCounts from the SubRead package (version 2.0.0). FeatureCounts was used with default

parameters except for: -M --fraction -Q 15 --fracOverlap 0.25 --largestOverlap --minOverlap 15. Exons were set as features (-t exon) and grouped by gene (-g gene_id). Statistical Analysis was performed using the edgeR package (version 3.24.3) from R (version 3.5.3) and significant changes in gene expression (1% FDR) were evaluated after filtration using the genefilter package (version 3.15) as previously done (Bourgon, Gentleman, and Huber 2010). Data visualization was done using python (version 3.7.3).

Quantitative PCR

Total RNA (500ng) was used to generate cDNA using the Biorad iscript RT supermix RT-qPCR kit (Biorad) following the manufacturer's instructions. The expression of different target genes was validated by quantitative PCR (qPCR), using the Azur Cielo 3™ system (Azure Biosystems). The specific primers used for the amplification are listed below:

mRPLP0 F: CTGAGATTCGGGATATGCTGTTGGCC

mRPLP0 R: CGGGTCCTAGACCAGTGTTCTGAG

mCYP26B1 F: CCAATTCCATTGGCGACATCCACCG

mCYP26B1 R: GGCAGGTAGCTCTCAAGTGCCTC

mSERPINB9B F: CACGTGGGTCTCCAAACAGACTGAAG

mSERPINB9B R: CATCATCTGCACTGGCCTTTTCTCATC

mLILRB4 F: GACCTCATGATCTCAGAAACCAAGGAC

mLILRB4 R: GTTCTCAGATTGTGTGTTCTTCACAGAAGC

mKLF10 F: CTCCAGCAAGCTTCGGAGGGGAAA

mKLF10 R: GGGGCTGTAAGGTGGCGTTAAACA

mCCL8 F: CCTGTCAGCCCAGAGAAGCTGACTG

mCCL8 R: CAGAGAGACATACCCTGCTTGGTCTGGA

mGADD45a F: GCAGGATCCTTCCATTGTGATGAATGTGG

mGADD45a R: CTAGCTGAGCTGCTGCTACTGGAGAAC

mKCNG1 F: ATGAGGAGGCTGCGTGACATGGTGGA

mKCNG1 R: GGATGAAGCGCAGCAGGAACTCTAG

mEgr2 F: GTCTGGTTTCTAGGTGCAGAGATGGG

mEgr2 R: CCTTTGACCAGATGAACGGAGTGGC

mCXCL13 F: CAGGCAGAATGAGGCTCAGCACAG

mCXCL13 R: CTTCATCTTGGTCCAGATCACAACTTCAG

mBC1 F: CCTGGGTTCGGTCCTCAGTCTGGA

mBC1 R: GGTTGTGTGTGCCAGTTACCTTG

mGAP43 F: CCTCCAACGGAGACTGCAGAAAGC

mGAP43 R: TCAGGCATGTTCTTGGTCAGCCTCG

The reactions were performed with the PerfeCta SYBR Green SuperMix (QuantaBio) as recommended by the manufacturer. Real-time PCR was performed with a hot start step 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20 s. Analysis was performed using Azur Cielo software (Azure Biosystems). The relative expression of the genes was normalized by that of RPLP0.

Sample preparation for mass spectrometry

Reduction, Alkylation of Proteins. All buffers used in this stage were prepared with MS-grade water. The protein reduction step was carried out by incubating the protein in Laemmli with 10 mM DTT for 30 min at 60°C. The alkylation of the proteins was carried out by adding 15 mM iodoacetamide (Sigma Aldrich) for 30 min at room temperature away from light. In order to increase the depth of discovery, the proteins were separated by size on a SDS-PAGE gel. 20 µg of

proteins were loaded onto a 4-20% PROTEAN® TGX™ Precast Protein Gels (Biorad). The gel was stained with Invitrogen™ SimplyBlue™ SafeStain (Thermo Fisher Scientific) according to the manufacturer's instructions. The gel was then cut in 4 fractions ranging from 250-75 kDa, 75-37 kDa, 37-20 kDa, and 20-10 kDa. Each fraction was diced into 1mm cubes and then the gel pieces were destained. All incubation steps were performed at 30°C at 800 RPM in a thermoshaker. The pieces were first washed in MS-grade H20 for 30 min. Then we added the same volume of CH3CN for a final concentration of 50% v:v CH3CN:H20, incubated for 15 min. The supernatant was removed, and the pieces were resuspended in 20 mM NH4CO3 for 15 min. Then the supernatant was removed and the pieces were resuspended in 50% v:v CH3CN, 10 mM NH4CO3 for 15 min. The last two steps were repeated until no trace of blue was visible in the gel pieces. The proteins were digested by adding 1 µg Pierce MS-grade trypsin (Thermo Fisher Scientific) diluted in 20 mM NH4CO3 and incubated overnight at 37°C.

Peptide retrieval. The following incubation steps were all performed at 30°C at 850 RPM in a thermoshaker. An equal volume of CH3CN (equivalent to that of the trypsin NH4CO3) was added onto the gel pieces and incubated 30 min. The supernatant containing the peptides was transferred into a low-bind Eppendorf™ microcentrifuge tubes. An equal volume of 1% (v:v) Formic Acid (FA) (Thermo Fisher Scientific) was added to the gel pieces and the sample incubated for 20 min. This second supernatant was transferred in the same tube with the first supernatant. The steps of adding FA and transferring the supernatant was repeated once. Then 150µL of CH3CN were added and the mixture was incubated for 15 min. Again, the supernatant, containing the last peptides, is transferred to the tube. These samples were thereafter concentrated by a centrifugal evaporator at 65°C until complete drying (4-6h), and resuspended in 30 µl of 0.1% trifluoroacetic acid (TFA) buffer.

Purification and Desalting of the Peptides on C18 Columns. The peptides were purified with ZipTip 10-l micropipette tips containing a C18 column (EMD Millipore). The ZipTip was first moistened by suctioning 10 µl of 100% ACN solution three times, then equilibrated by suctioning 10 µl of 0.1% TFA buffer three times. Each peptide sample was passed on the balanced ZipTip by ten up-and-downs of 10 µl of the sample. This step was performed three times to pass the entire sample on the column. The ZipTip was then washed with 10 µl of 0.1% TFA buffer three times.

Data dependent acquisition (DDA) mass spectrometry

Mass spectrometry. Trypsin-digested peptides were separated using a Dionex Ultimate 3000 Binary RSLCnano for High Performance Liquid Chromatography (HPLC). 1.5 µg of peptides per fraction were loaded onto an OrbiTrap mass spectrometer. With a constant flow of 4 µl/min the samples were loaded and separated onto a nanoHPLC system (Dionex Ultimate 3000) with a trap column (Acclaim PepMap100 C18 nano column, 0.3 mm id x 5 mm, Dionex Cormporation, Sunnyvale, CA). Peptides were then eluted off towards an analytical column heated to 40°C (PepMap C18 column (75 µm x 50cm)) with a linear gradient of 5-45% of solvent B (80% acetonitrile with 0.1% formic acid) over a 4h gradient at a constant flow of 450 nl/min. Peptides were analyzed using an OrbiTrap QExactive (Thermo Fischer Scientific) using an EasySpray source at a voltage of 2.0kV and the temperature of the column set to 40°C. Acquisition of the full scan MS survey spectra (*m/z* 350-1600) in profile mode was performed in the orbitrap at a resolution of 70,000 using 1,000,000 ions. The ten most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy 35% and resolution of 17,500) after the accumulation of 50,000 ions with maximum filling times of 250 ms for the full scans and 60 ms for the MS/MS scans. All unassigned charge states as well as species with single, seven or eight charge for precursor ion were rejected. A dynamic exclusion

list was set to 500 entries with a retention time of 40 s (10 ppm mass window). Data acquisition was done using Xcalibur (version 4.2). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2022) with the dataset identifier PXD038179.

Data analysis. Data analysis was performed using MaxQuant software (Cox and Mann 2008) (1.6.17). For statistical analysis Prostar software package (Wieczorek et al. 2017) (Prostar 1.26.3; DAPAR 1.26.1) was used with the following settings. Potential contaminants as well as reverse sequences identified were removed and only proteins with at least two unique peptides detected in all replicates of at least one condition were kept. Partially observed values (POV) were imputed using SLSA (Structured Least Square Adaptative) regression-based imputation method while values missing in the entire condition (MEC) using DetQuantile with a low deterministic value. Hypothesis testing was performed using Limma test. Log2 Fold Change threshold was set at 1 and –log10 p-value threshold at 1.3. Results were visualized on a volcano plot using VolcaNoseR tool (Goedhart and Luijsterburg 2020). From the significantly different proteins heatmap was generated using Heatmapper tool (Babicki et al. 2016) with average linkage clustering and Pearson distance measurement. Peak list files were searched from the Openprot mouse proteome (openprot.org; version 1.6).

Parallel reaction monitoring (PRM) mass spectrometry

Protein quantification was performed using label-free PRM method. For quantification on an OrbiTrap mass spectrometer, the 3 fractions containing the peptides of interest were pooled in equal concentration and were loaded and separated onto a nanoHPLC system (Dionex Ultimate 3000) with a constant flow of 4 µl/min onto a trap column (Acclaim PepMap100 C18 nano column, 0.3 mm id x 5 mm, Dionex Cormporation). Peptides were then eluted off towards an analytical column

heated to 40°C (PepMap C18 column; 75 µm x 50cm) with a linear gradient of 5-45% of solvent B (80% acetonitrile with 0.1% formic acid) over a 4-h gradient at a constant flow of 450 nl/min. Eluted peptides were analyzed on an OrbiTrap QExactive (Thermo Fischer Scientific) using an EasySpray source at a voltage of 2.0kV. Acquisition of the MS/MS spectra (*m/z* 350-1600) was performed in the Orbitrap. An inclusion list containing the *m/z* values corresponding to the monoisotopic form of the selected peptides was generated. The collision energy was set at 28% at 140 000 for 1 000 000 ions with maximum filling times of 250 ms with an insulation width of 0.6. Data acquisition was done using Xcalibur (version 4.2). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al. 2022) with the dataset identifier PXD038179.

Identification and quantification of peptides was performed on Skyline software (21.1.0.146). The relative amount of proteins was calculated using peptide peak areas extracted from retention time points for all fragment ions (if possible the 3-5 most abundant ions were chosen for quantification for each peptide) and normalized against peptides of endogenous proteins whose abundance did not change in the samples. Normalized values were then used to calculate ratios between the two experimental groups (24-month/6-month) and values of technical replicates were used for data visualization.

Immunoblotting

The proteins were resuspended in 1x Laemmli (62.5 mM Tris pH 6.8, 25% v/v glycerol, 2% m/v SDS, 0.01% m/v bromophenol blue, 10% v/v 1M DDT) and then boiled for 5 minutes at 95°C. 20 µg of each sample were then analyzed on a 10% SDS-PAGE gel in 1x running buffer at 100V for around 1h30. Proteins were transferred on a nitrocellulose membrane (Whatman) with 1× Tris-glycine buffer at 100 V for 1 h. The membranes were blocked with 5% m/v powdered milk

dissolved in 1x PBS - 0.1% tween for 30 min, and then washed 3 times for 5 min in 1x PBS - 0.1% tween with agitation at room temperature. Membranes were incubated overnight at 4°C with primary antibody. Next day, membranes were washed 3 times for 5 min in 1X PBS-tween at room temperature and incubated for 1h at room temperature with the horseradish peroxidase (HRP)-conjugated secondary antibody. After this incubation, the membranes were washed again 3 times for 5 min with 1x PBS - 0.1% tween. Results were visualized on a iBright machine (Thermo Fisher Scientific).

Antibody	Company	Reference	Dilution
Anti-Actin	Sigma Aldrich	A5441	1:20,000
Anti-GAP43	Abclonal	A19055	1:1,000
Anti-mouse HRP	Biorad	170-6516	1:5,000
Anti-rabbit HRP	Biorad	170-6515	1:5,000

Immunofluorescence

Brain slides were first incubated for 20 min in Citrisolv (1x) for deparaffinization. For rehydration, brain slides were washed two times with EtOH 100% for 3 min, and then sequentially washed with EtOH 95%, EtOH 80% and water for 1 min. Slides were then incubated into boiling sodium citrate (temperature around 95°C) for 20 min and then cooled in same solution for 20 min without heat. Slides were then washed 2 times with PBS 1x. For permeabilization and blocking, slides were incubated with a blocking solution (PBS 1X + 0,1% Triton + 4% BSA) at room temperature for 2h minimum in closed humidified chamber. The primary antibody was then added to the brain sections and incubated overnight at room temperature. The next day, slides were washed 3 times for 10 min with PBS 1x and a fresh secondary antibody solution (dilution 1:1000) was added for 45 min in dark at room temperature. Slides were then washed 3 times with PBS 1x for 10 min each. A drop

of ProLong Glass Antifade Mountant (DAPI, Thermo Fisher Scientific) was finally added on the center-left of the tissue with a coverslip on top. All brain slides were analyzed on the Axioscope 5/7/Vario system (Zeiss) with the same acquisition settings. The acquisition and analysis of images was blind for the age group.

Antibody	**Company**	**Cat n°**	**Dilution**
Anti-GAP43	Abclonal	A19055	1:50
Anti-CCL8	Abclonal	A6977	1:100
Alexa Fluor 488 anti-rabbit	Invitrogen	A-11017	1:1,000

Results

Cortical thickness increases during aging

Throughout this study, two age groups of C57BL/6J mice were used to investigate the cerebral cortex aging: young adults and old adults. Aging studies typically used 3-month and 18-month-old mice to respectively represent these two populations, however important morphological modifications occur up to 4 months of age such as the myelination (Hammelrath et al. 2016; Semple et al. 2013). To ensure that molecular differences with older mice do not arise from developmental and physiochemical changes related to the developmental stages, we used 6-month-old mice for representing the age group of young adults. In the same way, 18 months of age in mice (equivalent of 55 years in humans) does not represent the older stages of life. Hence, we used 24-month-old mice (equivalent of 75 years in humans) for the age group of old adults because biomarkers of old age are prominently detected in animals of that age (Dutta and Sengupta 2016).

We first investigate the anatomical changes of the brain in young adult (6-month-old) and old adult (24-month-old) mice (n=6 per age group; 3 males and 3 females). We first measured the cortical thickness on brain slides stained with hematoxylin and eosin (**Supplemental Figure 1A**). Since the cortical thickness varies on a coronal brain cross-section, the top layer being thicker than the side one, we measured the thickness of the "top" region (in blue in **Figure 1A**) and the "bottom" region (orange in **Figure 1A**), with measurements made in several locations as indicated in **Figure 1A** (respectively 4 and 5 measurements for the top and bottom regions). We observed that the top cortical layer, corresponding to the anterior cingulate, motor and primary somatosensory areas of the cortex, was significantly thicker in 24-month-old brains (1.41 ± 0.25 mm) compared to 6-month-old brains (1.19 ± 0.18 mm) while we did not find any significant difference for the bottom region (0.75 ± 0.23 mm vs 0.65 ± 0.24 mm) (**Figure 1B**). We also measured the total surface area

of the cortical layer and showed that the overall cortex area was not significantly different between the young and old adult groups (respectively 9.37 ± 0.94 mm^2 vs 10.28 ± 1.09 mm^2) (**Supplemental Figure 1B**). We observed a significant age-dependent increase of the body weight for females (22.87 ± 1.37 g vs 26.73 ± 3.24 g) but not for the males (34.65 ± 3.73 g vs 33.9 ± 4.44 g) (**Supplemental Figure 1C**). However, we showed that males and females exhibited the same wet brain weight throughout lifespan (489 ± 13 mg at 6 months of age vs 490 ± 19 mg at 24 months of age) (**Figure 1C**). It indicated that the increase of cortical thickness was not associated with an age-related change in wet brain weight.

Aging mainly triggers activation of gene expression in the cortex

We next investigated changes in mRNA expression in the aging cortex. We microdissected the cerebral cortex of 6- and 24-month-old male mice (n=3 for each age group), extracted total RNAs from the tissue and then performed a transcriptomic analysis (**Supplemental Table 1**). First, the principal component analysis (PCA) showed that the global gene expression pattern of 6-month-old cortices was very similar (**Figure 2A**). The global gene expression pattern of 24-month-old cortices was distinct from that of 6-month-old cortices as suggested by the average position of the samples (cross) on the x-axis which represents the first principal component responsible for 80% of the variance (**Figure 2A**). We also noted that the gene expression within the 24-month-old group was more variable as suggested by the position of each sample regarding the second principal component only responsible for 5% of the total variance. Together, these results indicated that 6- and 24-month-old cortices exhibited a distinct global gene expression signature.

We next analyzed each individual gene and their differential expression between 6 and 24 months of age and represented them in a volcano plot (**Figure 2B**). Our analysis showed that 1,586 genes are differentially expressed with a false discovery rate (FDR) at 1% and a p-value inferior to 10^{-3}.

Among those differentially expressed genes (DEG), the majority was upregulated during aging, sometimes up to 7 times, while few DEG were downregulated with a minor reduction of their expression (**Figure 2B**). We next selected DEG with a stringent FDR of 0.1%. The expression pattern of these genes at 6 and 24 months was represented in a heatmap (**Figure 2C**). With these parameters, we showed that 427 genes were differentially expressed in the cortex between 6 and 24 months of age. As shown in **Figure 2B**, the majority of these DEG were upregulated (n = 400) while only 27 genes were downregulated (**Figure 2C**; **Supplemental Table 2**). Interestingly, we found that genes whose expression was upregulated during aging were significantly overrepresented in a 6 Mb window of several chromosomes (chromosomes 2, 5, 9, 10, 17, 19) compared to the density of genes in the background (**Supplemental Figure 2A**). We thus identified 8 clusters of genes that were statistically enriched in specific regions of chromosomes. We did not observe a similar distribution for the downregulated genes (**Supplemental Figure 2B**).

We finally performed a gene ontology (GO) analysis to identify in which specific pathways or functions those genes were involved. Using ShinyGO, a tool for in-depth analysis of gene sets (Ge, Jung, and Yao 2020), we showed that upregulated genes (n=400) were mainly involved in biological process of sensory perception while the 27 down-regulated genes were mostly involved in neuron-associated functions such as neuron development and differentiation, or metal ion transport (**Figure 2D-E**). Gene-set enrichment analyses with the BioPlanet database, that catalogues 1,658 pathways with their healthy and disease state annotations, also revealed multiple pathways enriched for the up- and down-regulated genes. We found that genes whose expression was increased in the cortex during aging were mainly involved in the cell surface interaction or adhesion of multiple immune cells (i.e., granulocytes, monocytes) (**Figure 2F**). Many upregulated genes encode proteins implicated in the immune response such as cell adhesion molecules (Cd244a, Cd19), receptors (Cd207, Ccr4, Lilbr4) or inflammatory chemokines (Ccl8, Cxcl13). A similar

analysis for the down-regulated genes revealed that they were mostly involved in the BDNF signaling pathway, transmission across synapses or glutamate receptor trafficking, all pathways mainly linked to neuron functions (**Figure 2G**). Together, these results showed that, in the cortex, aging mainly triggered the expression of genes related to sensory perception and immune response.

Increased mRNA expression correlates with higher level of proteins

To validate our transcriptomic results, we then performed quantitative PCR (qPCR) on more cortex samples (n=8 per age group, 4 males and 4 females). Among the 1,586 DEG with a 1% FDR (**Figure 2B**; **Supplemental Table 1**), we selected candidates with an overall good mRNA expression and/or with a biological relevance for the functions of interest e.g., immune response. We picked 5 downregulated genes (*Klf10*, *GADD45a*, *KCNG1*, *Cyp26b1*, *Egr2*) and up-regulated genes (*CXCL13*, *BC1*, *Serpinb9b*, *Lilrb4* and *Ccl8*). Our qPCR results confirmed the differentially expression of all genes in the aging cortex with, for instance, the expression of Cyp26b1 decreased by 57% (1.00 ± 0.35 at 6 months vs 0.43 ± 0.26 at 24 months) or the expression of Lilrb4 increased by 155% (1.00 ± 0.40 at 6 months vs 2.25 ± 0.90 at 24 months) (**Figure 3A**). Even higher levels of mRNA have more chances to produce more proteins, age-dependent regulatory mechanisms could modulate the level of protein expression. We then investigated whether changes of mRNA level correlated with those of proteins. We first performed a mass spectrometry (MS) analysis on cortex samples of 6- and 24-month-old male mice (n=2 for each age group) using a bottom-up MS technique also known as “shotgun” in Data-Dependent-Acquisition (DDA) mode. To compare more precisely mRNA and protein levels, we used proteins extracted from the exact same cortex samples employed for the transcriptomic study. For 6- and 24-month-old cortices, we respectively extracted 101.9 ± 23.5 μg of proteins per mg of tissue and 119 ± 30.9 μg of proteins per mg of

tissue (**Supplemental Figure 3A**), suggesting that the total protein quantity remains quite stable in the aging cortex. Before the initial filtering steps (i.e., removal of contaminants and reverse sequences, maintenance of proteins with at least two unique peptides in all replicates), we identified a total of 2,471 proteins in both young and old adult cortices (**Supplemental Table 3**). We overlapped DEG identified by RNA-seq (n=427) with the list of proteins identified by DDA MS but unfortunately few DEG (n=13) were detected at protein level by MS (**Supplemental Figure 3B**). We showed that proteins detected by MS mainly originated from the most expressed transcripts (**Supplemental Figure 3C**) and that transcripts found differentially expressed in the aging cortex are not among the most expressed in general (**Supplemental Figure 3D**; **Supplemental Table 2**). Among these 13 DEG detected by MS, the majority (10/13) were downregulated during aging and involved in neuronal functions as shown in **Figure 2E**. We previously showed that genes upregulated in the aging cortex were mainly coding for proteins implicated in the immune response (**Figure 2F**) and since the immune cell population is in minority in the cerebral cortex, it might hence be difficult to detect these proteins by DDA MS as further discussed. To override this technical limitation and confirm that increase of mRNA expression led to more protein expression in the aging cortex, we next performed immunofluorescence on 6- and 24-month-old brain slides. Among our candidate genes, the *Ccl8* gene was selected because its mRNA expression fold change was one of the highest (**Figure 3A**), and Ccl8 antibodies were commercially available and already tested. Ccl8 immunofluorescence on coronal cuts of 6-month-old brains showed a cytoplasmic staining for cells mainly located in the motor area (MO) of the cortex (**Figure 3B-C**; top panel). Interestingly, the anterior cingulate area (ACA) of the cortex did not exhibit any significant Ccl8 immunostaining at 6 months of age, but cells in this specific cortex area displayed a strong increase of Ccl8 signal at 24 months of age (**Figure 3C-D**). We did not observe any significant increase of Ccl8 signal in the motor area of the cortex during aging (**Figure**

3C). Together these results showed that, in the aged cortex, increased Ccl8 mRNA expression correlated with higher level of proteins, specifically in the ACA.

A subset of genes depends on translation regulation in the aging cortex

With the results of DDA MS on 6- and 24-month-old cortices, we next investigated whether some genes exhibited differential abundance in protein level during aging. The distribution of proteins showing significant differential enrichment in the aging cortex was represented by a volcano plot and a heatmap (**Figure 4A-B**). The volcano plot displayed an extreme distribution for some proteins that were originally not identified in one age group (**Figure 4A**; **Supplemental Table 4**). We identified 164 proteins enriched in the old adult cortex and 42 proteins specifically enriched in the young adult cortex (**Figure 4A**; **Supplemental Table 5**). Using ShinyGO, we showed that proteins upregulated in the aging cortex were mainly involved in multiple catabolic and metabolic processes (including the proteasome complex), fatty acid beta-oxidation, organelle organization or mRNA nonsense-mediated decay (**Figure 4C**). Interestingly, we found a significant enrichment for members of the PSMD and PSMC families (PSMD3, PSMD5, PSMD6, PSMD11, PSMD13, PSMC1, PSMC4, PSMC5), both encoding proteasome 26S subunits involved in the degradation of damaged, misfolded, abnormal, and foreign proteins (Kao et al. 2021; Xuan et al. 2021) (**Supplemental Table 5**). A similar analysis for proteins downregulated during aging revealed that they were mostly involved in translation, mitochondrial membrane organization, or ribosome assembly (**Figure 4D**). For instance, we found a significant enrichment for ribosomal proteins (RPL18, RPL19, RPL23a, RPL38, RPL7a, RPL8, RPL9, RPS10, RPS26) (**Supplemental Table 5**). We next examined whether genes encoding proteins with significant differential enrichment during aging exhibited changes in mRNA expression. We showed that genes encoding differentially enriched proteins during aging had a stable mRNA expression in the cortex between

6 and 24 months of age (**Figure 4E**), suggesting that this subset of genes was the target of post transcriptional mechanisms during aging.

To confirm our findings, we carried out a precise quantification of protein abundance using parallel reaction monitoring (PRM) MS (**Supplemental Figure 3E**). This technique requires to select beforehand the protein and unique peptide(s) of interest to be targeted by MS. The technical limitations of the Q Exactive mass spectrometer allowed the quantification of approximately 20 proteins with two quantotypic peptides per protein (i.e., unique peptides that reflect the stoichiometry of the protein). We selected 42 unique peptides from 22 different proteins including two housekeeping proteins for normalization (Atp5b, Got 2) (**Supplemental Table 6**). For each sample, the same peptide solution used for the DDA method was re-injected into the mass spectrometer with the list of the m/z values for these 42 peptides to allow their systematic detection (**Supplemental Table 6**). We analyzed the enrichment of each peptide in 6- and 24-month-old cortex samples compared to control peptides and represented the results as a heatmap of the ratio of these peptides between each age group (**Figure 4F**). We confirmed with the PRM method that most selected peptides exhibited a change in intensity ratio, with some peptides showing a strong increase (Aldh2, Aldoc, Ctbp1, Mapk1, Psmd11, Tomm70a) and others a strong decrease (Apoa1, Apoe, App, Atp2b2, Cadm2, Cntn1, Ndufb10, Phf2, Prkcb, Prrt2) (**Figure 4F**). Another way to represent the results is to graph the area relative to the internal standard. When represented by a graph rather than a heatmap, we were able to confirm a decrease for Apoe, Atp2b2, Apoa1, and Prrt2 peptides, and an increase for Syn2 peptide (**Figure 4G**). Together, our findings showed that, in the aging cortex, a subset of genes was regulated at post-transcriptional level and that mRNA dynamic was not reflected into protein levels.

Aging increases protein expression of genes associated with neuroplasticity

Among the 165 proteins enriched in the old mouse cortex, we observed that many proteins also played a significant role in neurogenesis, axonal guidance, dendrite outgrowth, and synapse formation such as Camk2d, Cacnb3, Dlgap1, Dpysl5, Gabra1 or Gap-43 (Casoli et al. 1996; Jeanne et al. 2021; Kool et al. 2019; Li et al. 2012; Rasmussen, Rasmussen, and Silahtaroglu 2017; Pallotto and Deprez 2014) (**Supplemental Table 5**). Evidence suggested that older brains had a substantial plasticity as illustrated with patients with stroke showing dramatic recovery with sustained therapy (Ramanathan, Conner, and Tuszynski 2006). We next investigated the age-related changes in neuroplasticity considering Gap-43 (Growth associated protein 43) as a marker of brain adaptive capabilities. Indeed, Gap-43 is expressed in axonal growth cones during development and regeneration (Rosskothen-Kuhl and Illing 2014). We first examined Gap-43 expression by immunoblotting on 6- and 24-month-old cortex samples, and observed that Gap-43 protein was indeed significantly increased during aging (1 ± 0.07 at 6 months vs 2.17 ± 0.14 at 24 months) (**Figure 5A**; **Supplemental Figure 4A**). We confirmed by qPCR that the mRNA expression level was stable between 6 and 24 months of age (1 ± 0.01 at 6 months vs 0.96 ± 0.04 at 24 months) (**Figure 5B**). We finally performed immunofluorescence on 6- and 24-month-old brain tissue. Gap-43 immunostaining on coronal cuts of 6-month-old brains showed a good and specific signal in the motor and anterior cingulate cortices (**Figure 5C**, top panel; **Supplemental Figure 4B**). We observed a strong and significant increase of signal in the same brain areas at 24 months of age (4285 ± 944 AU at 6 months vs 7181 ± 593 AU at 24 months) (**Figure 5C**, bottom panel). The results confirmed that Gap-43 expression increased at protein level in the aging cortex while its mRNA expression level remained stable, suggesting that post-transcriptional regulatory mechanisms are involved during aging.

Discussion

As we age, the volume of the brain reduces at the rate of around 5% per decade starting at the age of 40 (Svennerholm, Bostrom, and Jungbjer 1997). In middle-aged adults, a wide-spread cortical thinning has been observed, reflecting significant global atrophy (Salat et al. 2004). However, older age was associated with greater cortical thickness in the dorsolateral and anterior cingulate cortices (Dotson et al. 2015). Our findings support this observation as we described an increase of the cortical thickness between 6 and 24 months of age (**Figure 1B**). There is evidence to suggest that the relationship between age and cortical thickness is not a linear model, particularly for the anterior cingulate cortex (ACC). Indeed, ACC thickness is reduced until the age of 60 but is thereafter greater at older ages (Fjell et al. 2014). One hypothesis to explain the "reversal" of cortical thinning could be the increase of neuroplasticity (Draganski et al., 2006; Engvig et al., 2010). When the brain is damaged, older adults can maintain cognitive and body functions by activating effective compensatory resources in response to this degradation (Pauwels, Chalavi, and Swinnen 2018). Our results support this neuroplasticity as we observed in the aging cortex an enrichment of proteins playing an important role in neurogenesis, axonal guidance, dendrite outgrowth, and synapse formation (e.g., Camk2d, Cacnb3, Dlgap1, Dpysl5, Gabra1, Gap-43) (**Supplemental Table 5**). We particularly showed that expression of Gap-43, important for axonal growth cones and marker of neuroplasticity (Rosskothen-Kuhl and Illing 2014; Casoli et al. 1996), was higher in the aging cortex (**Figure 5C**). Interestingly, Gap-43 expression only increased at protein level as its mRNA expression level remained stable in the aging cortex (**Figure 5A-B**), suggesting that this neuroplasticity could occur at translational level rather than through a transcriptional regulation. However, cortical thickness might not only be supported by neuroplasticity. Inflammation increases with age and could cause an age-dependent related thickness. Cortical thinning was

globally associated with level of systemic inflammation in older persons without dementia (Fleischman et al. 2010) but other studies showed a positive relationship between inflammatory markers and cortical thickness, specifically for the ACC (Krishnadas et al. 2013; Poletti et al. 2019; Dotson et al. 2015; Sörös 2010; Fjell et al. 2014). For instance, TNF-α, interleukine-8, and CCL2 correlated positively with ACC thickness (Poletti et al. 2019). Interestingly, we showed that genes whose expression was increased at RNA level in the cortex during aging were mainly involved in the immune response, including genes coding for inflammatory chemokines (Ccl8, Cxcl13) (**Figure 2**). We specifically showed that Ccl8 expression was significantly and specifically increased in the ACC during aging (**Figure 3C-D**). These results support that inflammation increases in the aging cortex and could potentially be implicated in the cortical thickening. It is important to note that this aging-dependent regulation occurs at the transcriptional level.

We performed RNA-seq and MS analyses on 6- and 24-month-old cortices. By decoupling mRNA and protein expression in the same cortex samples, we were able to identify distinct subsets of genes with different age-dependent regulations: i) genes regulated at the transcriptional level, with change in mRNA level, and ii) genes regulated at a translational level with stable mRNA expression during aging. Our RNA-seq analysis indicated that aging mainly triggers gene activation in the cortex as 93% of the differentially expressed genes showed an increase in their mRNA level (**Figure 2B-C**). We also found that a significant number of these genes were related to sensory functions but also immune cells' functions (**Figure 2F**). We were not able to confirm these transcriptomic changes at the proteomic level since only a minority of proteins encoded by DEG were detected by MS (**Supplemental Figure 3B-C**). This matter was not due to post-mortem issues since cortex samples were homogenized and equally fractionated at the same time for both transcriptomic and proteomic studies. In lieu, the immune cell population being in minority in the cerebral cortex could logically explain the absence of detection of these proteins by MS. We also

showed that proteins detected by MS mainly originated from the most expressed transcripts which did not include upregulated genes related to the immune response (**Supplemental Table 2**; **Supplemental Figure 3C-D**). Moreover, transcriptomics and proteomics methods have very different levels of sensitivity and measurement, and it is still easier to detect mRNAs than their respective proteins. For instance, the GTEx Consortium performed a quantitative analysis of proteins from more than 12,000 genes across 32 normal human tissues (Jiang et al. 2020). They reported that many low-abundance transcripts were often underdetected at the protein level, and that 16% of protein-coding genes with good RNA abundance (TPM>32) were not detected at the protein level (Jiang et al. 2020). Immunostainings on brain slides were performed to override this limitation and confirm that genes upregulated at the transcriptional level have indeed higher level of proteins (**Figure 3**). It is interesting to note that genes whose expression was upregulated during aging were statistically enriched in specific regions of chromosomes (**Supplemental Figure 2A**). This observation suggests that major epigenetic chromatin remodeling events occur in the cortex. It has been proposed that the loss of heterochromatin, which is characteristic of aging, can lead to changes in the genome architecture and hence increased expression of genes residing in these regions (Pal and Tyler 2016). It also might explain why we only identified 27 downregulated genes in the aging cortex whose functions are mainly linked to neuron functions (**Figure 2E, 2G**). Together, our results support previously published transcriptomic data showing that functions affected in the human brain by the aging process were for the upregulated genes, response to stress, glial cells and immune response, and for the downregulated genes, synapse, neurotransmission, and calcium signaling pathway (Gonzalez-Velasco et al. 2020; Erraji-Benchekroun et al. 2005; French et al. 2017).

Using MS, we also identified genes coding proteins that were differentially enriched in the aging cortex and exhibited stable mRNA levels during aging, suggesting that this specific subset of genes

depends on an aging-dependent translational regulation. We identified 208 proteins differentially enriched in the aging cortex (**Figure 4A**) and showed that 79% of these proteins (164/208) were involved in catabolic and metabolic processes such as the proteasome complex (**Figure 4C**). PSMD and PSMC proteins, which are proteasome 26S subunits involved in the degradation of misfolded and abnormal proteins (Kao et al. 2021; Xuan et al. 2021), were specifically enriched in this subset (**Supplemental Table 5**). It was reported that damaged and misfolded proteins tend to accumulate during the brain aging process, and that the proteasome is responsible for the degradation of these proteins (Carrard et al. 2002; Keller, Gee, and Ding 2002; Saez and Vilchez 2014). Age-related alterations in proteasome structure and activity contribute to brain aging by increasing the level of protein aggregation, and potentially leading to the development of age-related neuropathologies such as Alzheimer's disease with the aggregation of β-amyloid proteins. The enrichment of specific proteasome subunits in the aging cortex might modify proteasome biology, favor a decline of proteasome activity upon aging, and hence reduce protein degradation. We also showed that 21% of proteins (42/208) less enriched in the aging cortex, including ribosomal proteins, were mostly involved in translation and ribosome assembly (**Figure 4D**; **Supplemental Table 5**). During aging, the global translation rate decreases as the translation machinery becomes less efficient. This decrease is due to the deregulation of the stoichiometry of translation components that affects the abundance of ribosomes (Dhondt et al. 2017; Janssens et al. 2015) but also the rate and accuracy of translation (Gonskikh and Polacek 2017; Skariah and Todd 2021). Therefore, the age-dependent enrichment of ribosome components might affect protein homeostasis and promote a loss of proteostasis, one of the primary hallmarks of aging. Indeed, flawed translation has been described to lead to premature aging (Shcherbakov et al. 2022) and even promote early symptoms of Alzheimer's disease in aging mice (Brilkova et al. 2022).

By decoupling mRNA and protein expression, we thus identified distinct subsets of genes that differentially adjust to cellular aging in the cerebral cortex. In the future, it will be important to determine for the first subset of genes the precise mechanisms of epigenetic and transcriptional regulation, and for the second subset of genes, the role for post-transcriptional, translational and degradation regulation in adjusting protein abundance. Single-cell methods either at RNA or protein level have made significant advances over the last few years and could help to further discriminate which cell population (e.g., immune cells, glial cells or neurons) is targeted by these age-dependent mechanisms. As recently tackled (Tasaki et al. 2022), assessing the discordance between mRNA and protein abundance in neurodegenerative diseases could also reveal potential pathological protein drivers and foster new ways to investigate diseases that currently do not have any therapeutic options like Alzheimer's disease.

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Figure legends

Figure 1. Anatomical characteristics of the aging brain.

(A) Schematic depicting a coronal brain cut with the two areas where cortical thickness was measured. The blue area represents the "top" of the cerebral cortex comprising of the anterior cingulate area, the motor area, and the primary somatosensory area (limbs). The orange area represents the "bottom" of the cerebral cortex with the supplemental somatosensory area (barrel and nose), the visceral area, the gustatory area and the piriform area. The thickness of the top and bottom areas was measured at different locations as indicated by numbered arrows. **(B)** Coronal cuts of whole brain of 6 and 24-month-old mice (respectively grey and red; n=6 per age group; 3 males and 3 females) were stained with hematoxylin and eosin, and the thickness of the top and bottom areas was measured. (*) $p < 0{,}0001$ in one-way ANOVA; ns: not significant. **(C)** Graph representing the wet weight of the brain after dissection (n=6 per age group; 3 males and 3 females). ns: not significant from unpaired t-test with equal means.

Figure 2. Age-related changes in the transcriptome signature of the cerebral cortex.

(A) Principal component analysis (PCA, explaining 85% of the variance in total) for the gene expression of 6-month-old (red) and 24-month-old (blue) cortices (n=3 per age group). The cross represents the centroid for each sample set. **(B)** Volcano plot of differentially expressed genes, mapping the 400 upregulated genes (red) and 27 downregulated genes (blue) at a 1% FDR. FC, fold change. **(C)** Hierarchical clustering of genes showing significant differential expression between 6 and 24-month-old cortices. Red and blue indicate relatively higher and lower expression, with genes independently scaled to a mean of zero. **(D-E)** Gene Ontology analysis of upregulated (**D**) and downregulated (**E**) genes using the ShinyGO tool (version 0.76.1). The pathway database

used was GO Biological process. Parameters were set at a 0.05 FDR cut off with no redundancy in GO terms. **(F-G)** Pathways enriched among upregulated **(F)** and downregulated **(G)** genes using the BioPlanet 2019 pathway database. Pathways are sorted by p-value ranking.

Figure 3. Validation of candidate genes differentially expressed in the aging cortex.

(A) mRNA levels of Klf10, GADD45a, KCNG1, Cyp26b1, Egr2, CXCL13, BC1, Ccl8, Lilrb4b and Serpina9b were measured in cortices of 6-month-old (grey) and 24-month-old (red) mice by quantitative PCR and normalized to that of RPLP0 (n=8 per age group; 4 males and 4 females). Results are indicated as mean ± SD. (*) $p < 0.04$ (**) $p < 0.005$ (***) $p < 0.001$ (****) $p < 0.0001$ from unpaired t-test with equal means. **(B)** Schematic representation of the cortex area analyzed by immunofluorescence. Motor area (MO), Anterior Cingulate Area (ACA), Corpus Callosum (CC), Lateral Ventricle (LV). **(C-D)** Ccl8 immunofluorescence on coronal cuts of 6-month-old and 24-month-old brains. Images illustrate the representative staining in the whole top cortex area **(C)** and in the anterior cingulate area **(D)** with the Ccl8 antibody (green) and DAPI (blue). (n=3 per age group). Scale bar: 50 μm.

Figure 4. Age-related changes in the proteome of the cerebral cortex.

(A) Volcano plot of the distribution of differentially enriched proteins, mapping the 164 downregulated proteins (red) and 42 upregulated proteins (blue). Data analysis was performed using Prostar software (log2 Fold Change threshold: 1, –log10 p-value: 1.3) (n=2). **(B)** Hierarchical clustering of proteins showing significant differential enrichment in the aging cortex, based on the ratio of the area below the curve. Green and red indicate relatively high and low enrichment, with a ratio independently scaled to a mean of zero. **(C-D)** Gene Ontology analysis of proteins

upregulated (**C**) and downregulated (**D**) in the aging cortex using the ShinyGO tool. The pathway database used was GO Biological process. Parameters were set at a 0.05 FDR cut off with no redundancy in GO terms. **(E)** Heatmap of RNA transcript expression for each gene encoding differentially enriched proteins in 6 and 24-month-old cortices. Samples were independently scaled to a mean of zero. Green and red side bars indicate respectively up- and down-regulated proteins during cortex aging **(F)** Heatmap of peptides quantified by PRM mass spectrometry. Each horizontal line represents one peptide. Green and red respectively indicate high and low expression. Values were based on the ratio of the area below the curve for 6 and 24-month-old cortex samples (n=2 per age group), compared to that of the two Atp5b and Got2 control peptides. **(G)** Peptide quantification by PRM mass spectrometry. The area of each peptide was normalized by that of Got2 for 6-month (grey) and 24-month-old (red) cortex samples (n=2 per age group; three technical replicates using two unique peptides). Results are indicated as mean ± SD.

Figure 5. Analysis of GAP43 expression in the aging cortex.

(A) Protein levels of GAP43 within the 6-month-old (grey) and 24-month-old (red) cortex were analyzed by western-blot (left panel). Actin was used as a loading control. On the right panel, quantification of GAP43 signal (normalized by actin signal) (n=4 per age group). Results are indicated as mean ± SD. (****) $p < 0.001$ from unpaired t-test for equal means. **(B)** mRNA levels of GAP43 were measured by quantitative PCR and normalized to that of RPLP0 (n=8 per age group). Results are indicated as mean ± SD. **(C)** GAP43 immunofluorescence on coronal cuts of 6-month-old and 24-month-old brains. Images illustrate the representative staining in the cortex motor area with the GAP-43 antibody (green) and DAPI (blue). On the left, quantitative

comparative analysis of GAP43 expression (n=6 per age group). (****) $p < 0.001$ from unpaired t-test for equal means. Scale bar: 100 μm.

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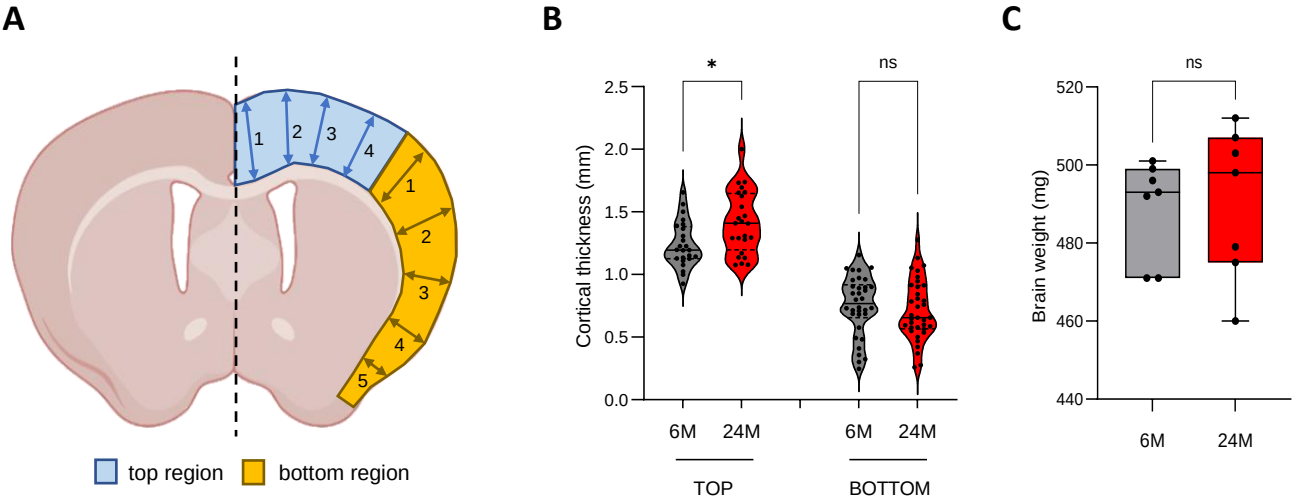
Figure 1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1
2
3
4
1
2
3
4
5
top region
bottom region
B
*
ns
Cortical thickness (mm)
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2.0
1.5
1.0
0.5
0.0
6M
24M
6M
24M
TOP
BOTTOM
C
ns
Brain weight (mg)
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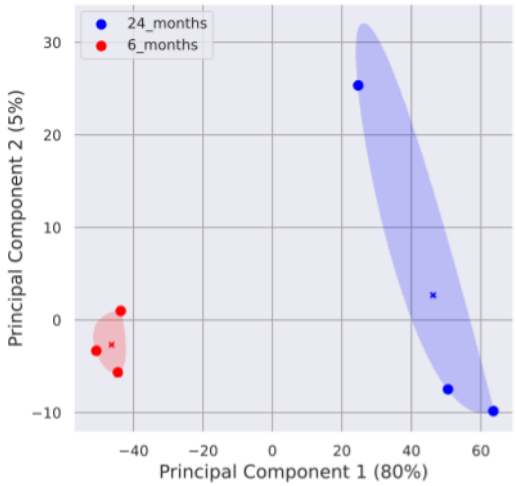
6M
24M

Figure 2

A



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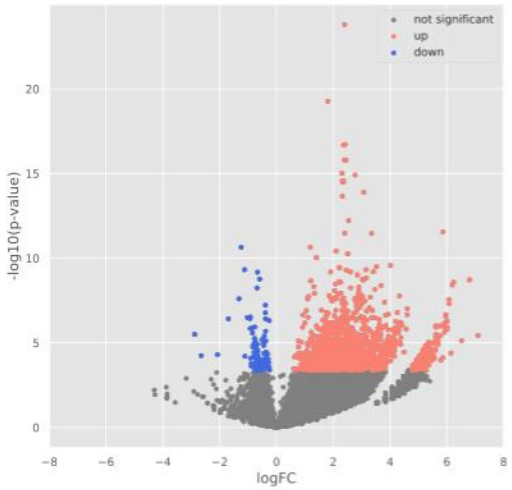

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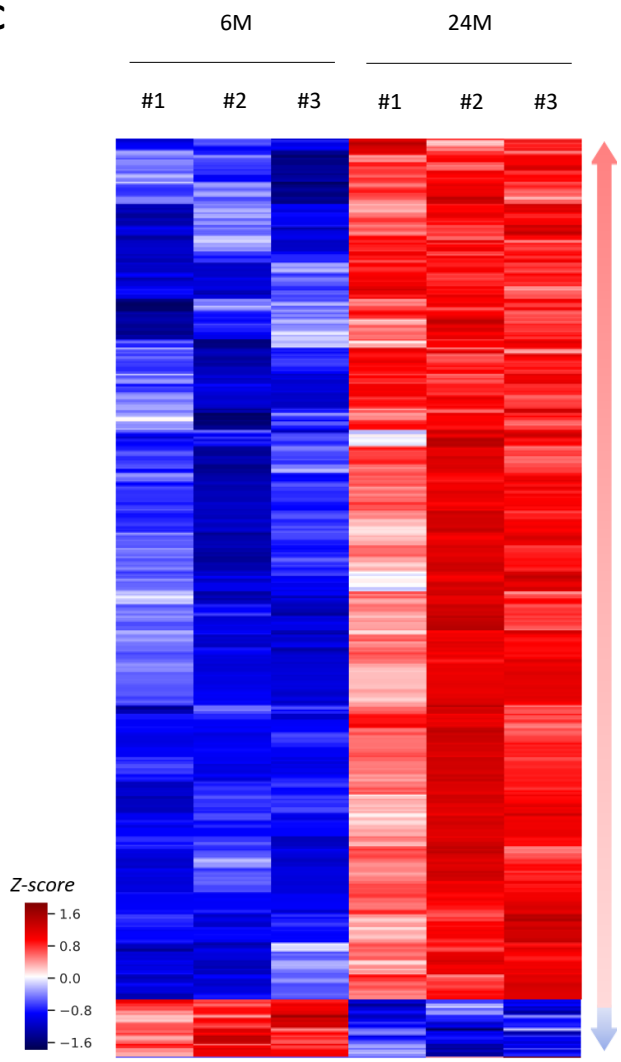

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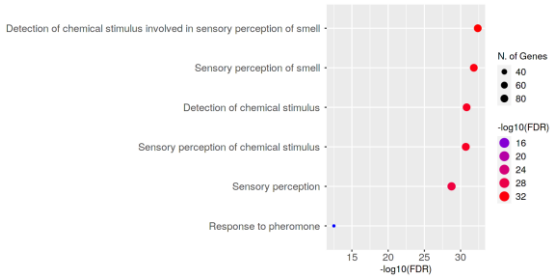

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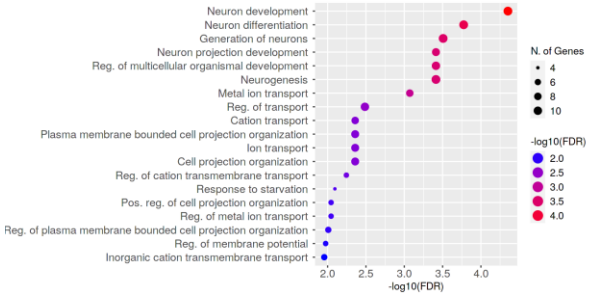


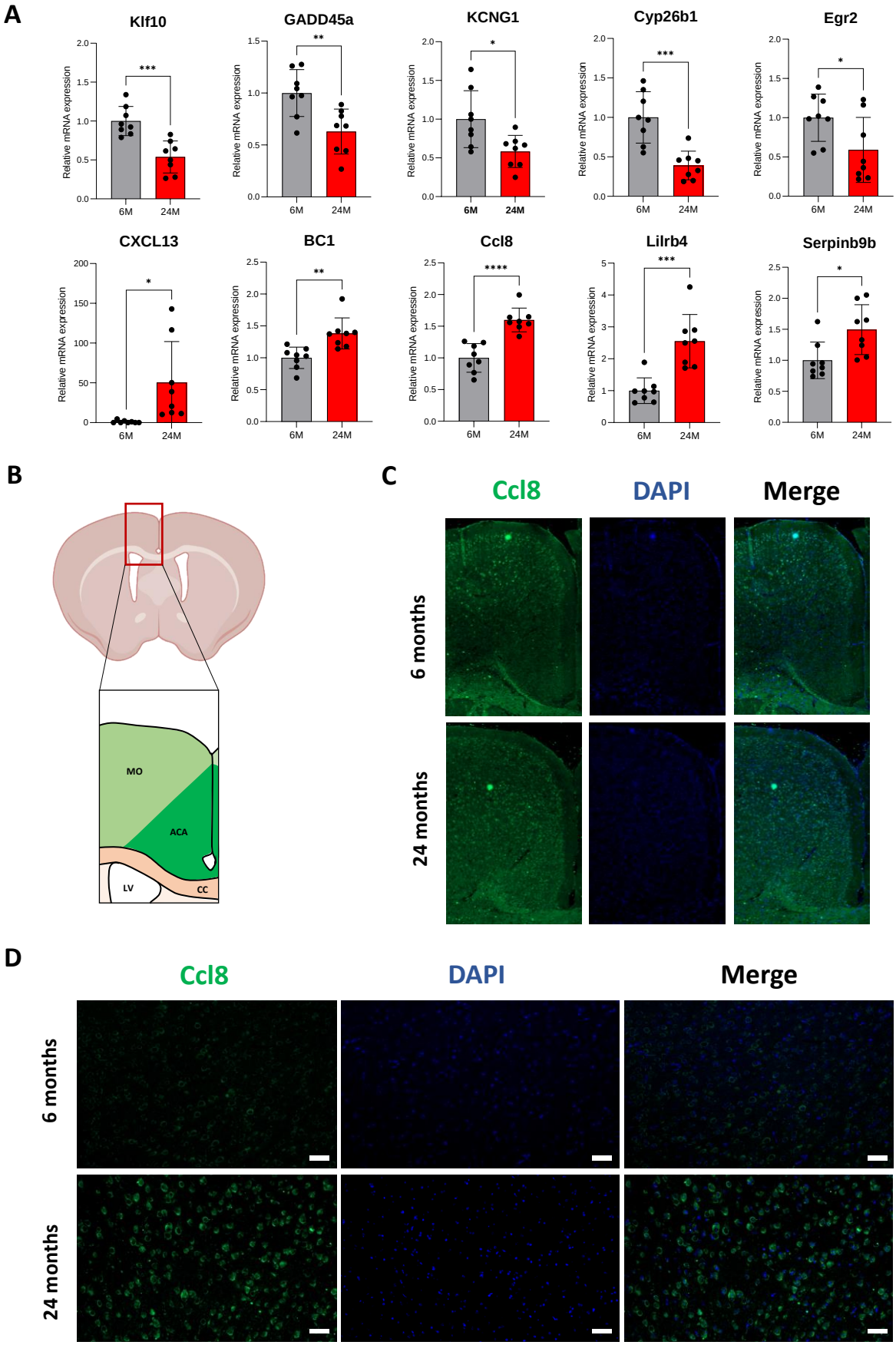
F

Description Pathways	P-value
Apoptotic cleavage of cell adhesion proteins	0.01948
Monocyte and its surface molecules	0.01948
Adhesion and diapedesis of granulocytes	0.03528
Cell surface interactions at the vascular wall	0.04039
Cells and molecules involved in inflammatory response	0.04452

G

Description Pathways	P-value
BDNF signaling pathway	0.00002325
Neuronal system	0.00003424
Myometrial relaxation and contraction pathways	0.00005300
Transmission across chemical synapses	0.0001167
GluR2-containing AMPA receptor trafficking	0.0002082

Figure 3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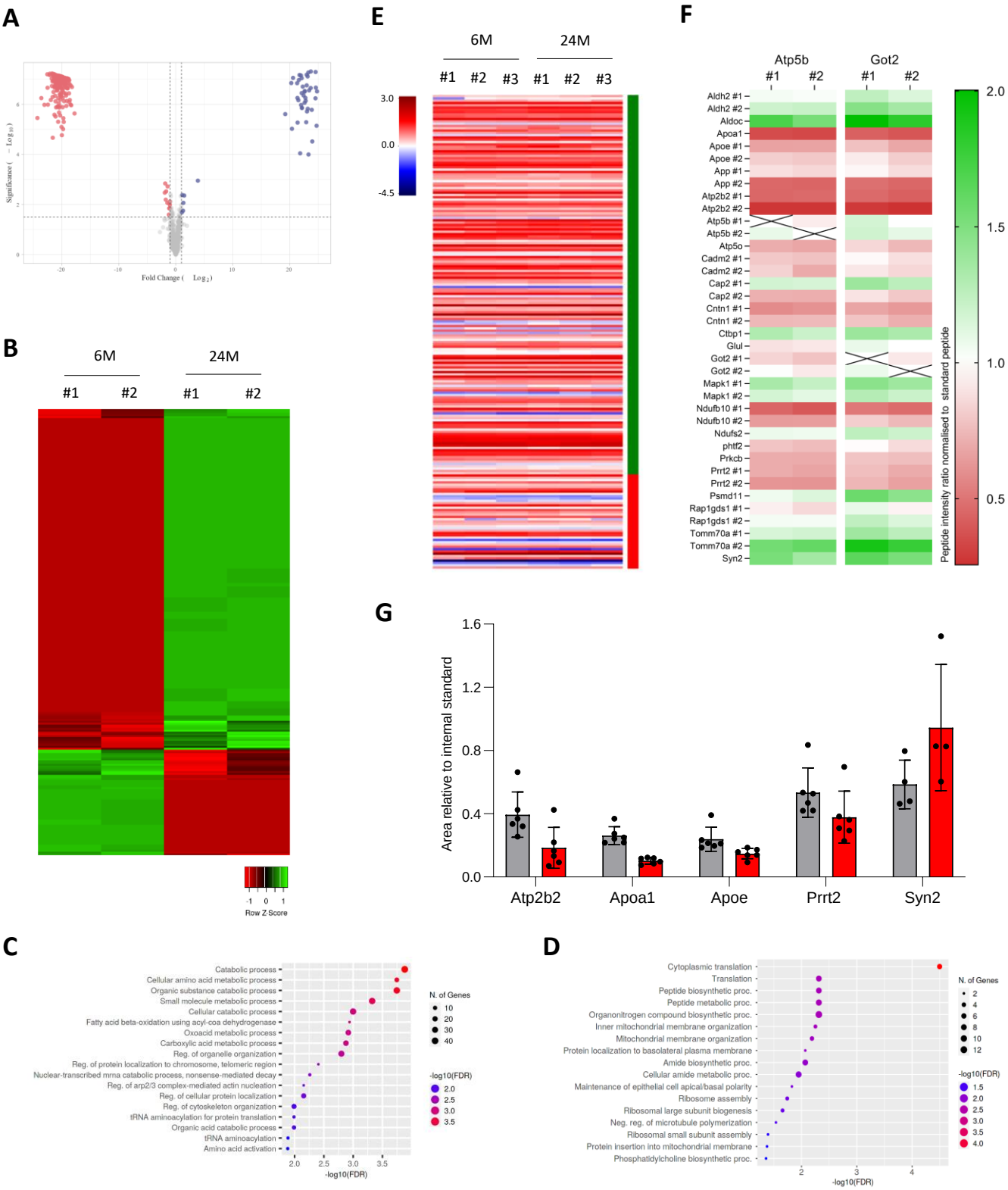
6M
24M
B
MO
ACA
LV
CC
C
Ccl8
DAPI
Merge
6 months
24 months
D
Ccl8
DAPI
Merge
6 months
24 months

Figure 4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Fold Change (Log 2)
Significance (− Log 10)
B
6M
24M
#1
#2
#1
#2
Row Z-Score
C
Catabolic process
Cellular amino acid metabolic process
Organic substance catabolic process
Small molecule metabolic process
Cellular catabolic process
Fatty acid beta-oxidation using acyl-coa dehydrogenase
Oxoacid metabolic process
Carboxylic acid metabolic process
Reg. of organelle organization
Reg. of protein localization to chromosome, telomeric region
Nuclear-transcribed mrna catabolic process, nonsense-mediated decay
Reg. of arp2/3 complex-mediated actin nucleation
Reg. of cellular protein localization
Reg. of cytoskeleton organization
tRNA aminoacylation for protein translation
Organic acid catabolic process
tRNA aminoacylation
Amino acid activation
-log10(FDR)
N. of Genes
-log10(FDR)
D
Cytoplasmic translation
Translation
Peptide biosynthetic proc.
Peptide metabolic proc.
Organonitrogen compound biosynthetic proc.
Inner mitochondrial membrane organization
Mitochondrial membrane organization
Protein localization to basolateral plasma membrane
Amide biosynthetic proc.
Cellular amide metabolic proc.
Maintenance of epithelial cell apical/basal polarity
Ribosome assembly
Ribosomal large subunit biogenesis
Neg. reg. of microtubule polymerization
Ribosomal small subunit assembly
Protein insertion into mitochondrial membrane
Phosphatidylcholine biosynthetic proc.
N. of Genes
-log10(FDR)
-log10(FDR)
E
6M
24M
#1
#2
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#3
F
Atp5b
Got2
#1
#2
#1
#2
Aldh2 #1
Aldh2 #2
Aldoc
Apoa1
Apoe #1
Apoe #2
App #1
App #2
Atp2b2 #1
Atp2b2 #2
Atp5b #1
Atp5b #2
Atp5o
Cadm2 #1
Cadm2 #2
Cap2 #1
Cap2 #2
Cntn1 #1
Cntn1 #2
Ctbp1
Glul
Got2 #1
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Mapk1 #2
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phtf2
Prkcb
Prrt2 #1
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Rap1gds1 #1
Rap1gds1 #2
Tomm70a #1
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Area relative to internal standard
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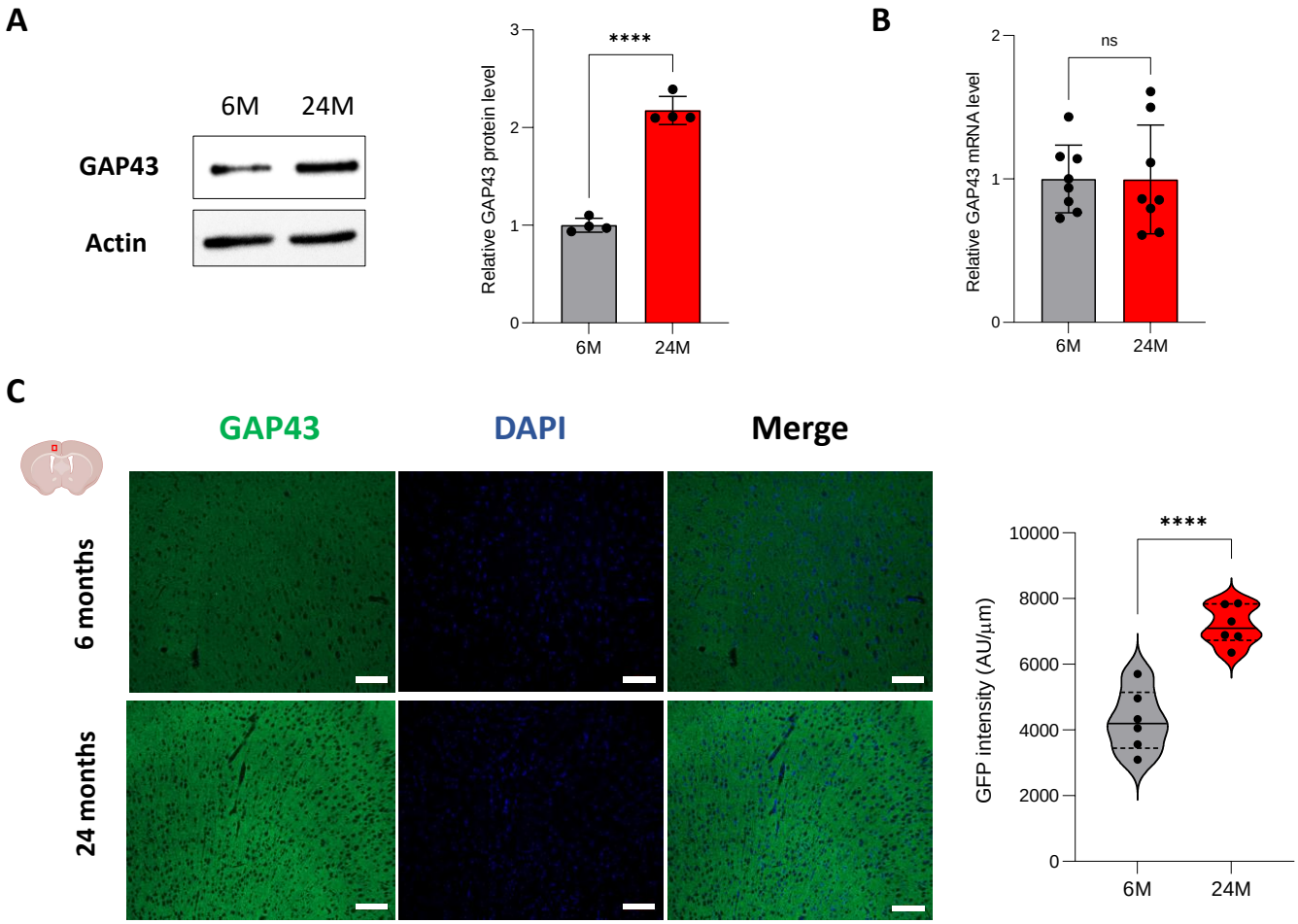
Prrt2
Syn2

Figure 5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Relative GAP43 protein level
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0
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2
6M
24M
C
GAP43
DAPI
Merge
6 months
24 months
GFP intensity (AU/µm)
****
0
2000
4000
6000
8000
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