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Fluids and Barriers of the CNS

RESEARCH

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Aging alters the expression of trophic factors and tight junction proteins in the mouse choroid plexus

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Abstract

Background The choroid plexus (CP) is an understudied tissue in the central nervous system and is primarily implicated in cerebrospinal fluid (CSF) production. CP also produces numerous neurotrophic factors (NTF) which circulate to different brain regions. Regulation of NTFs in the CP during natural aging is largely unknown. Here, we investigated the age and gender-specific transcription of NTFs along with the changes in the tight junctional proteins (TJPs) and the water channel protein Aquaporin (AQP1).

Methods Male and female mice were used for our study. Age-related transcriptional changes were analyzed using quantitative PCR at three different time points: mature adult, middle-aged, and aged. Transcriptional changes during aging were further confirmed with digital droplet PCR. Additionally, we used immunohistochemical analysis (IHC) for the evaluation of in vivo protein expression. We further investigated the cellular phenotype of these NTFS, TJP, and water channel proteins in the mouse CP by co-labeling them with the classical vascular marker, Isolectin B4, and epithelial cell marker, Plectin.

Results Aging significantly altered NTF gene expression in the CP. Brain-derived neurotrophic factor (*BDNF*), Midkine (*MDK*), *VGF*, Insulin-like growth factor (*IGF1*), *IGF2*, Klotho (*KL*), Erythropoietin (*EPO*), and its receptor (*EPOR*) were reduced in the aged CP of males and females. Vascular endothelial growth factor (*VEGF*) transcription was gender-specific; in males, gene expression was unchanged in the aged CP, while females showed an age-dependent reduction. Age-dependent changes in VEGF localization were evident, from vasculature to epithelial cells. IGF2 and klotho localized in the basolateral membrane of the CP and showed an age-dependent reduction in epithelial cells. Water channel protein AQP1 localized in the tip of epithelial cells and showed an age-related reduction in mRNA and protein levels. TJP's *JAM*, *CLAUDIN1*, *CLAUDIN2* and *CLAUDIN5* were reduced in aged mice.

Conclusions Our study highlights transcriptional level changes in the CP during aging. The age-related transcriptional changes exhibit similarities as well as gene-specific differences in the CP of males and females. Altered transcription of the water channel protein AQP1 and TJPs could be involved in reduced CSF production during aging. Importantly, reduction in the neurotrophic factors and longevity factor Klotho can play a role in regulating brain aging.

Keywords Choroid plexus, Blood–cerebrospinal fluid barrier, Tight junctional proteins, Neurotrophic factors, Aging, Epithelial cells, Klotho, Cerebrospinal fluid, Vascular endothelial growth factor

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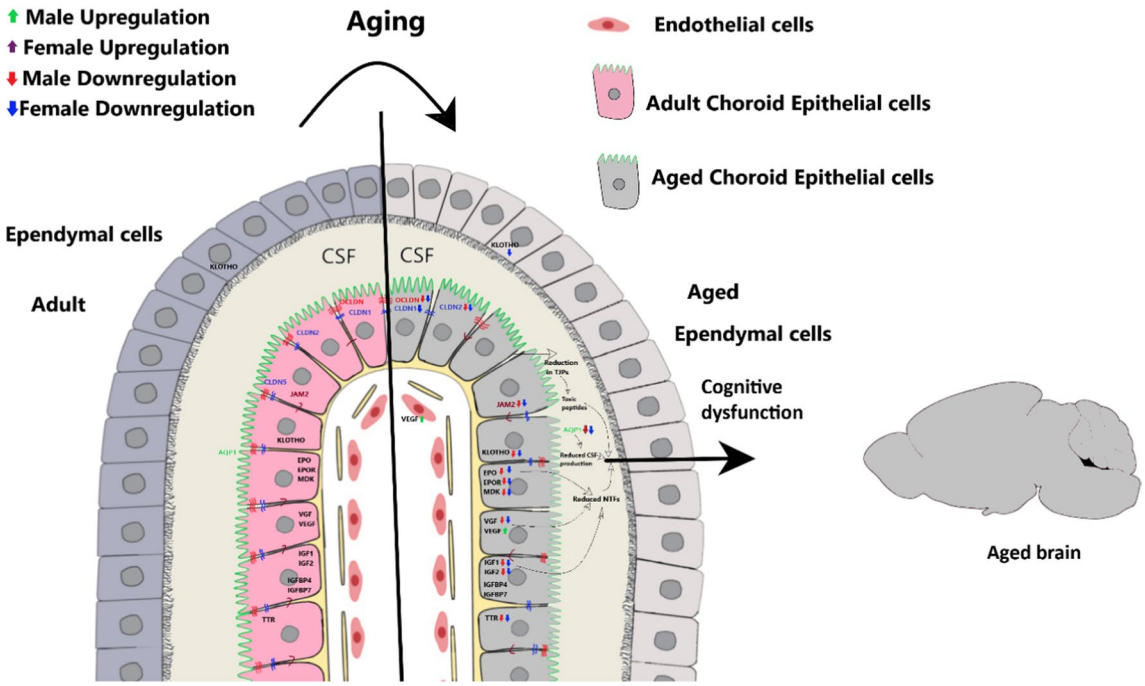
Graphical Abstract



Background

The blood-cerebrospinal fluid barrier (BCSFB) is a physicochemical barrier established by choroid plexus (CP) epithelial cells in the lateral, third, and fourth cerebral ventricles. The CP is composed of highly specialized cuboidal epithelium that is continuous with ependymal cells lining the ventricles of the brain. These cuboidal epithelial cells are interconnected by tight junctions on their apical surface along with a core of fenestrated capillaries, allowing the filtration of plasma [1]. Besides their barrier function, choroid plexus epithelial cells have a secretory function by producing 70–80% of cerebrospinal fluid (CSF) [2]. With the support of fenestrated capillaries and elevated blood flow, the cuboidal epithelial cells provide the brain with a high turnover rate of fluid containing hormones, peptides, and micronutrients to the neuronal network [3–5].

The structure of the CP lends itself to involvement in an extensive spectrum of physiological actions on the brain. In addition to secretory functions, the CP also performs excretory functions, such as the removal of toxic peptides from the brain through various transporters [6]. Maintenance of epithelial-tight junctional integrity by tight junctional proteins is necessary to protect the central nervous system (CNS). These roles are served by junctional proteins such as the claudins, CLDN1, CLDN2, CLDN11, occludin, the zonula occludens protein (ZO-1) and junctional adhesion molecules (JAM), which are present in tight junctions of the choroid plexus epithelium [7–10]. Claudins and occludin are the major transmembrane molecules facilitating epithelial contact [11]. The importance of apical tight junctions in the CP epithelium has largely been neglected, and studies are lacking regarding the expression and regulation of the choroid epithelial tight junction proteins in the later stages of life.

As the brain ages, it undergoes progressive cellular and structural changes, leading to cognitive decline and increased vulnerability to neurobiological diseases. Various trophic factors essential for maintaining neuronal function and survival have been shown to have an altered expression in the aged brain, leading to cognitive decline [12–14]. The choroid plexus plays an essential role in supporting neuronal function by producing a large variety of trophic factors during embryonic and adult stages [15, 16]. In the adult brain, the CP secretes major neurotrophic factors, including fibroblast growth factors, epithelial growth factors, platelet-derived growth factors, insulin-like growth factors, vascular endothelial growth factors, MDK, and BDNF to the CSF, which circulates to

the different parts of the brain, supporting neuronal and vascular function [17–21]. Most of these trophic factors are known to be associated with adult neurogenesis, plasticity, cognition, or angiogenesis [19, 22–24]. Growth factors such as VEGF and TGF-b are known to be involved in the maintenance of the choroid plexus [1, 25]. IGFs are one of the most significant trophic factors produced in the CP, as IGF1 signaling pathways have emerged as essential regulators of the aging process. IGF signaling is regulated by a family of specific IGF-binding proteins (IGFBPs). IGFBPs share substantial sequence homology and can bind IGFs with equal or greater affinity than the IGF1 receptor (IGF1 R). High levels of *IGF2* mRNA expression were reported in the choroid plexus [20]). IGF2 is essential for the maintenance of a subset of adult NSCs, suggesting the importance of IGF2 in aging [26]. Reduced expression of IGF2 in the aged brain can accelerate the risk of neurodegenerative or psychiatric diseases [27, 28].

Natural aging affects the structure and function of the choroid epithelial cells. Aged choroid epithelial cells exhibit increased pathological protein deposits called Biondi ring tangles [29, 30] with depleted glucose metabolism and energy production [31, 32]. During natural aging, cranial and ventricular CSF volume doubles [33–35], and this increase in CSF volume, coupled with the reduction in CSF production and secretion, slows the turnover rate of CSF by three to four-fold [36, 37]. This disruption in CSF turnover can contribute to the etiology of age-related neurocognitive disorders [38–42].

CP shows sex-related functional differences, including differences in the protein and hormone composition of CSF, immune function, BCSFB function, and toxic waste clearance [43]. Various studies have shown that the CP expresses sex hormone receptors such as androgen receptors (ARs), estrogen receptors (ERs), and progestogen receptors (PR) [44–46]. These sex hormonal receptors regulate the transcriptome of the CP expressing secretory proteins and influence the composition of CSF [43, 44]. A previous study by Quintela and co-workers reported that CP exhibits sex-related differences in whole transcriptomes from rat CP [47]. In contrast, a recent study reported that the transcriptomic profiles in rats showed a highly shared expression profile between female and male CP [48]. A focused study on the sex and age-related transcriptional changes in the CP is therefore timely. Age-related alterations in the cerebrospinal fluid (CSF) proteins have been reported [49]. This Dysregulated protein expression could be due to age-related dysfunction of the BCSF barrier. In an aged brain, the integrity of the BCSF barrier is compromised, resulting in protein leakage from blood to CSF [50]. Although the CP is known to produce various trophic factors, age-related changes in trophic factor expression are poorly understood. The present study is focused on age-related and gender-specific trophic factor changes in the choroid plexus of lateral ventricles. We also investigated the cellular-level translational changes in the choroid plexus vasculature and epithelium. Further, we examined age-related transcriptional changes in the epithelial tight junction.

Methods

Animals

We used C57Bl/6J mice at the ages of 5–6 (Matured Adult), 11–12 (Middle-aged), and 18–24 (Aged) months, with five males and five females in each group, except in the 11–12 months group where only four females were available. Mice were bred in our laboratory (breeders from Jackson Laboratories, Bar Harbor, ME). Male and female mice were caged separately after weaning, and females were never exposed to males. Mice were maintained on a standard 12-h light–dark cycle with free access to food and water. All procedures were carried out in strict accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the USD Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used.

RNA extraction and quantitative PCR analyses

The brains of the experimental mice were carefully dissected, and the hemispheres were separated. Ventricles were gently rinsed with RNAlater stabilization solution, and the CP was carefully dissected under a microscope. According to the manufacturer's instructions, total RNA from CP was extracted using an RNAqueous micro kit (Invitrogen). The concentration and purity of RNA at 260/280 nm were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). 200 ng RNA was reverse transcribed into cDNA (Applied Biosystems High-Capacity cDNA Reverse Transcription Kit, USA) using thermal cyclers (Techne Prime). Gene expression analyses were performed by quantitative real-time PCR (applied biosystems QuantStudio 5 using 500 nM of each forward and reverse primers and SYBR green Universal PCR master mix (GenDEPOT, USA and Applied Biosystems, USA). Primers (Additional file 6: Table S1) were designed to amplify specific gene targets using the Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/). The expression levels of each gene target were normalized with the housekeeping gene Cyclophilin A (CypA), and the fold change of transcription was quantified using the relative quantification $2^{-\Delta\Delta Ct}$ method.

QIAcuity dPCR

cDNA preparation was performed as previously described. 200 ng RNA was reverse transcribed into cDNA in a 20 ul reaction volume. After the reaction,

cDNA was diluted in 80 ul of dd water, and 3 ul of diluted cDNA was used for QIAcuity dPCR reactions. QIAcuity dPCR reactions were conducted in the adult and old CP using cDNA generated from common RT reactions. QIAcuity Four 5-plex digital PCR System (Qiagen, Hilden, Germany). Digital PCR reactions consisted of a 12 µL reaction mixture per well containing 4 µL QIAcuity 4× Eva Green Probe PCR master mix (Qiagen), 400 nM of primers (Additional file 6: Table S1), PCR grade water (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA template from the experimental group. Assembled reactions were transferred into QIAcuity 8k 96-well Nanoplates (Qiagen) for partitioning using the Qiagen Standard Priming Profile, and nucleic acids were amplified under the following conditions: enzyme activation for 2 min at 95 °C and 45 cycles of 15 s at 95 °C and 30 s at 60 °C followed by 60 s at 35 °C. Partitions were imaged with 200 ms (Green) exposure time, with gain set to 6 for both target channels. The QIAcuity Software Suite (Qiagen, version 2.0.20) was used to determine the copy number.

Protein isolation from choroid plexus

The brains of the experimental mice were carefully dissected. Two hemispheres were separated using a surgical blade, and the ventricles were carefully rinsed with ice-cold PBS. Removed the floating CP and transferred it to a 1.5 ml Eppendorf tube. 80 ul RIPA buffer with protease and phosphatase inhibitor was added to the Eppendorf tube, followed by 45 min of incubation on ice. Spin the mixture at 14,000×*g* for 20 min in a 4 °C pre-cooled centrifuge. The supernatant was collected in the new centrifuge, and the resulting protein was concentrated using a 10 K MWCO Pierce concentrator (Thermo Scientific). Protein estimation was done using the BSA method.

Automated western blot analysis

Western blots were also performed using the Protein Simple Jess Western instrument (San Jose, CA). Cell and tissue lysates were prepared as described above. 4ul of protein samples were mixed with a 5× fluorescent master mix (Protein Simple) to achieve a final concentration of 1× master mix buffer according to the manufacturer's instructions. Samples were then denatured at 95 °C for 5 min. All materials and solutions added onto the assay plate were purchased from Protein Simple, except the primary antibody. 10 ul of antibody diluent, protein normalizing reagent, primary antibodies, secondary antibodies, chemiluminescent substrates, 3 ul of the sample, and 500 ul of wash buffer were prepared and dispensed into the assay plate. The assay plate was loaded into the instrument, and protein was separated within individual capillaries. Protein detection and digital images were collected and analyzed with Compass software (Protein Simple), and data were reported as an area under the peak, representing the signal's intensity. The data was also normalized using the housekeeping gene beta-actin. For the primary antibody, rabbit polyclonal anti-klotho (Proteintech, Cat No. 28100-1-AP) and rabbit polyclonal anti-beta-actin (4970S, Cell Signaling Technology, Davers, MA) were used at 1:100 dilution; for the secondary antibody, anti-mouse NIR and anti-rabbit HRP secondary antibodies from Protein Simple were used.

Immunohistochemistry

Immunohistochemical analysis was carried out in fresh, frozen mouse brains ($n=3$). Mouse brains were dissected carefully and quickly frozen using dry ice. Immunohistochemical studies were performed in 17 um cryocut coronal brain sections which were fixed using precooled histochoice fixative (Sigma) for 10 min, followed by blocking with Bovine serum albumin (BSA) for 1 h at room temperature. Sections were incubated with different primary antibody (Additional file 7: Table S2) concentrations in antibody solution (2.5% BSA in PBS) at 4 °C overnight. (All the antibody details and concentrations are given in the Additional file 7: Table S2) Following primary antibody incubation, slides were washed in 1× PBS three times for five minutes each at room temperature. Slides were then incubated with appropriate fluorescent secondary antibodies (1:500, Alexa-488, and 640, Invitrogen, Carlsbad, CA, USA) in antibody solution for 1.5 h at room temperature. The slides were then rinsed in 1× PBS three times for five minutes each. Finally, Vascular marker Isolectin B4 (1:1000, Vector Laboratories) was added to the slide and incubated for one hour. Slides were washed in 1× PBS three times, and the cover slipped using VectaMount with DAPI (Vector Laboratories). Sections were viewed, and images were captured using a Nikon Eclipse Ni microscope equipped with DS-Qi1 monochrome, a cooled digital camera, and NIS-AR 4.20 Elements imaging software.

Immunohistochemistry image analysis

Immunohistochemical image analysis was performed using ImageJ software, version 1.54 g, and Java 1.6.0_20 (32-bit) engine. Briefly, the multi-channel images were split into separate channels. The desired image channel was then selected and converted into an 8-bit grayscale. After converting the image into an 8-bit grayscale, we used an inverted feature in the ImageJ. After inverting the image, we adjusted the threshold plugin and kept the area constant. We used three mice each for adult and aged mice, and a total of 12 sections were analysed to obtain the mean gray value.

Blood-CSF barrier permeability analysis

Male mice were anesthetized, and fluorescein isothiocyanate dextran (FITC-dextran—40,000 da, 100 µl, 50 mg/ml, in saline, Sigma-Aldrich, St. Louis, MO) was injected intravenously in the inferior vena cava vein. After 1 min, the brains were removed and frozen quickly on dry ice. Coronal sections (20 µm thickness) were taken using the cryostat (Leica Microsystems, CM1860, Buffalo Grove, USA). Fluorescent images of the sections were captured using a Nikon Eclipse Ni microscope and NIS Elements software.

Data analysis

Statistical analysis was performed using GraphPad Prism 8.4 software. Outliers greater than 2 standard deviations from the mean were identified using the Grubbs test ($\alpha = 0.05$) using GraphPad Prism 8.4 software and removed from further analysis. Normality was assessed using the Shapiro–Wilk test (GraphPad Prism 8.4). All statistical tests use biological replicates and are indicated by group size (n) in the figure legend. Error bars indicate the standard error of the mean (SEM). The following statistical tests were applied to determine statistical significance: an unpaired two-tailed t-test (GraphPad Prism 8.4.3) was used for QIAcuity dPCR, IHC mean gray intensity (Image J version 1.54g) and western blot, and One-way ANOVA was used for the analysis of real-time PCR. The unequal variance was corrected using Welch's correction (GraphPad Prism 8.4).

Results

See Fig. 1.

Neurotrophic factor expression is significantly altered in the aged choroid plexus

Neurotrophic factors are highly expressed and secreted in the CP and other brain regions. CP secretes several trophic factors into CSF and circulates it to different brain regions [2]. Here, we studied the gender-specific expression patterns of major neurotrophic factors in the murine CP. To address the age-dependent transcriptional level changes in murine CP, we first analyzed neurotrophic factor gene expression patterns at three different ages: 5–6 m.o. (from now on referred to as mature adult), 11–12 m.o. (middle-aged) and 18–24 m.o. (aged). Further, we confirmed transcript levels using digital droplet PCR. Most of the trophic factors examined showed transcriptional dysregulation in the aged CP; some were gender specific. First, we analyzed the transcription pattern of the *VEGF* gene, which is actively involved in the maintenance of the CP vasculature and has a role in the permeability of blood vessels. *VEGF* showed a differential transcriptional pattern in males and females. In male CP, the relative gene expression showed a consistent *VEGF* gene expression throughout the lifespan (Additional file 1: Fig. S1a), and the ddPCR gene expression analysis further confirmed that in males, *VEGF* mRNA transcription is not affected by aging (Fig. 1a). However, in females, *VEGF* mRNA expression is significantly ($P < 0.0001$) reduced with age (Fig. 1b), and the reduction is evident from middle age ($P < 0.01$) onwards (Additional file 1: Fig. S1b). We used immunohistochemical analysis to determine whether these gene expression changes are mediated at the protein level. Interestingly, unlike gene expression changes, VEGF protein is more highly expressed in the aged CP than in adults in both males ($P < 0.01$) and females ($P < 0.05$) (Fig. 1c). We also found that VEGF localization was not limited to the choroid vasculature. The expression was also observed in the basal membrane of epithelial cells or stroma (Fig. 1d, e).

Next, we investigated the expression pattern of *MDK*, another trophic factor highly expressed in the CP. *MDK* is known to provide neurotrophic support and neurite outgrowth [51]. *MDK* mRNA transcription showed a significant ($P < 0.0001$) reduction in the aged male and female mice (Additional file 1: Fig. S1a, b). The decline in *MDK* gene expression starts in middle age and continues throughout the later stages of life. ddPCR gene expression (Fig. 1a, b) also confirmed the reduction of *MDK* transcripts in CP of older males ($P < 0.05$) and female mice ($P < 0.001$). Then, we turned our attention to BDNF, which is widely expressed in the CNS and is involved in neuronal survival, development, and synaptic plasticity [52–54] QIAcuity ddPCR analysis showed a significantly ($P < 0.001$) lower copy number of BDNF (Fig. 1a) in CP than other trophic factors, indicating reduced

(See figure on next page.)

Fig. 1 Differential regulation of trophic factors in the aged choroid plexus: The QIAcuity dPCR analysis showed that aging significantly altered the expression of neurotrophic factors in the choroid plexus (CP) of male (**a**) and female (**b**) mice ($n = 5$). The copy number of neurotrophic factors *VGF*, *MDK*, *BDNF*, *EPO*, and *EPOR* was significantly reduced in the CP of aged males and females. *VEGF* showed a gender-specific expression pattern. In males, concentration was unchanged while females showed an age-related decline in the copy number. VEGF (Green)-Isolectin B4 (Red) co-labeling indicates that the aged CP of males (**d**) and females (**e**) showed an increased VEGF localization in and out of the CP vasculature. The mean gray value (**c**) of the immunohistochemical analysis showed an increased VEGF staining in the aged CP of both genders. Scalebar-100 µm
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

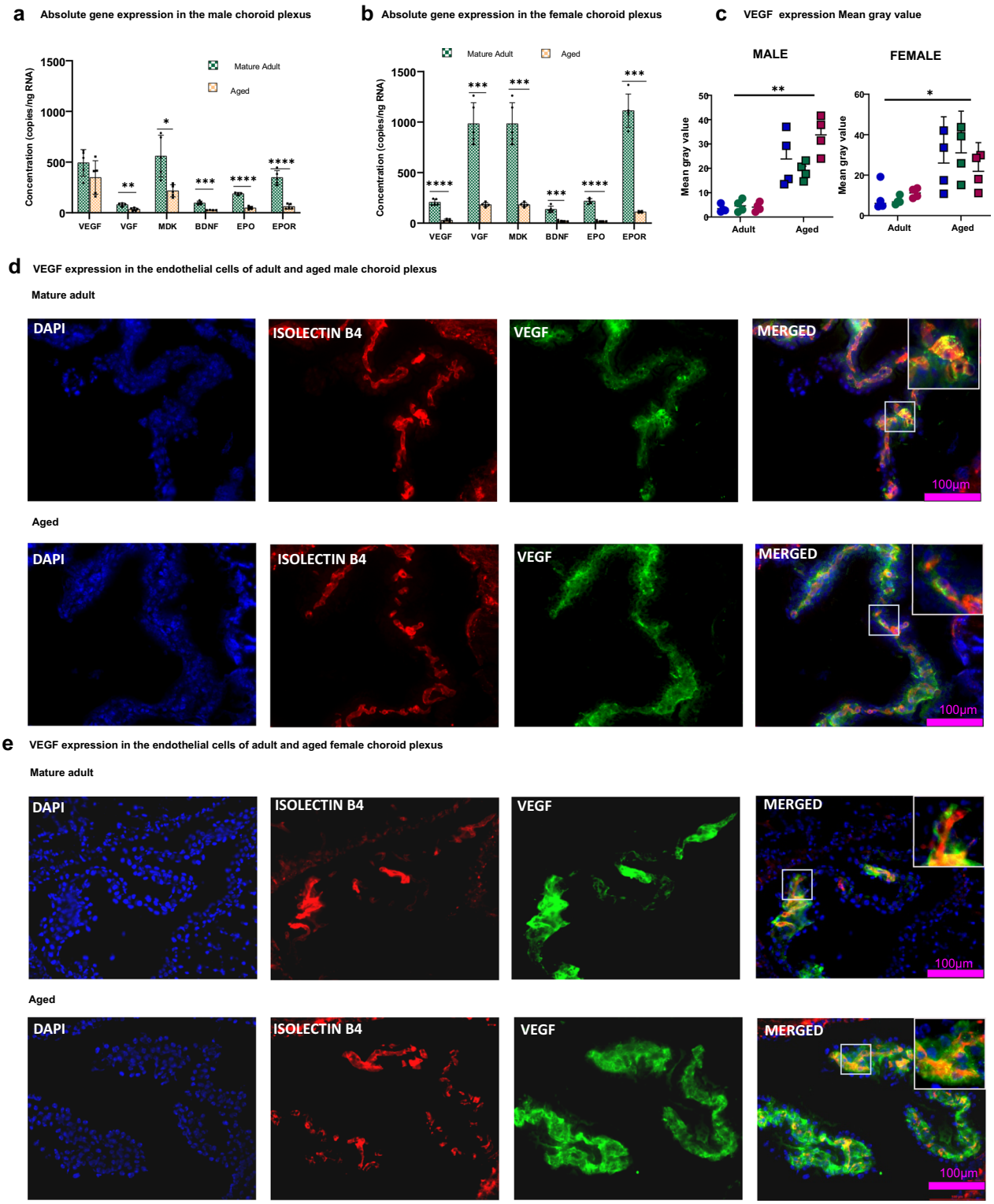


Fig. 1 (See legend on previous page.)

transcription of *BDNF* mRNA in the CP. However, an age-dependent reduction is evident in *BDNF* mRNA levels (Additional file 1: Fig. S1a, b) in the CP of both males ($P<0.0001$) and females ($P<0.001$). Even though another important trophic factor, VGF, did not show any difference in the real-time PCR (Additional file 1: Fig. S1a, b), QIAcuity ddPCR analysis showed an age-dependent reduction in *VGF* mRNA transcription in males ($P<0.01$) and females ($P<0.001$). Interestingly, females exhibit a higher copy number than males. EPO, a neurotrophic and neuroprotective cytokine, exhibits a significantly ($P<0.0001$) lower copy number (Fig. 1a, b) in the CP of aged males and female mice, indicating reduced transcription of the *EPO* gene during aging. EPO acts through its classical receptor EPOR. *EPOR* gene copy number is significantly decreased (Fig. 1a, b) in the aged CP of both males ($P<0.001$) and females ($P<0.0001$), indicating reduced EPO signaling in the later stages of life.

IGF expression decreased in the choroid plexus of aged mice

Insulin-like growth factors (IGFs) are essential growth-promoting peptides that act as endocrine, paracrine, and autocrine factors. IGF signaling plays a crucial role in controlling aging and life span in invertebrates [55, 56]. To analyze IGF signaling in aged mice, we first examined the mRNA of the *IGF1* gene. The relative expression of *IGF1* mRNA showed differential expression patterns in the aged males and females. We found a significant down-regulation ($P<0.0001$) in *IGF1* transcription in aged females (Additional file 1: Fig. S1d). Age-related changes were significant ($P<0.0001$) in middle-aged female mice. ddPCR (Fig. 2b) ($P<0.001$) gene expression confirmed the altered transcription of *IGF1* mRNA in aged females. Even though the relative expression (Additional file 1: Fig. S1c) appeared unchanged in male CP, absolute gene expression showed (Fig. 2a) a significant reduction ($P<0.001$) in *IGF1* transcript levels.

IGF2 is a significant growth factor in the brain and is involved in memory consolidation. *IGF2* transcription in the brain is mainly localized at the CP and leptomeninges [57]. Quantitative PCR analysis showed an age-dependent reduction in *IGF2* gene transcription in the CP of both males ($P<0.001$) and females ($P<0.0001$) (Additional file 1: Fig. S1c, d). The reduction was evident from middle age onwards. QIAcuity ddPCR further confirmed the reduced concentration of *IGF2* transcripts in aged CP of males ($P<0.05$) and females ($P<0.01$) (Fig. 2a, b). Further, we focused on age-related protein-level changes in IGF- II expression in the CP epithelium and vasculature. The co-labeling study showed that in adult CP, IGF2 protein is expressed in the epithelial cells, particularly in the basal membrane of epithelial cells (Fig. 2e; Additional file 3: Fig. S3b). The vasculature of the CP is free from IGF2 expression (Fig. 2d; Additional file 3: Fig. S3a). Natural aging dramatically reduced IGF2 protein expression (Fig. 2c; Additional file 2: Fig. S2c) in the choroid epithelium of both males and females ($P<0.05$).

IGF1 and IGF2 act through the IGF receptor, and the *IGF1R* gene expression showed a differential expression in both males and females (Fig. 2a, b). Aging did not influence *IGF1R* gene expression in the male CP (Fig. 2a; Additional file 1: Fig. S1c), whereas, in females, both the absolute ($P<0.01$) and relative ($P<0.0001$) expression indicate a significant reduction in *IGF1R* gene transcription (Fig. 2b; Additional file 1: Fig. S1d). The IGF peptides have a short lifespan unless they are bound by specific binding proteins that transport them in circulation and deliver them to specific tissues. IGFBPs are found throughout the body in various fluids and tissues [58, 59]. IGFBP has binding affinities for IGF-I and IGF2 comparable to the ligands for IGF-IR. Most IGFBPs inhibit IGF-induced cell growth by binding to IGFs and acting as a time-release mechanism. Here, we analyzed the transcription pattern of *IGFBP4* and *IGFBP7* genes. Quantitative PCR gene expression analysis showed that *IGFBP4* and *IGFBP7* gene expression in CP is gender-specific; in males, gene expression remains unchanged (Additional file 1: Fig. S1c), and in females (Additional file 1: Fig. S1d), both *IGFBP4* ($P<0.001$) and *IGFBP7* ($P<0.0001$) expression significantly reduces as age progresses. Gene expression analysis using QIAcuity dPCR (Fig. 2b) confirms the age-induced reduction in the *IGFBP4* ($P<0.01$) and *IGFBP7* ($P<0.001$) transcription.

(See figure on next page.)

Fig. 2 Insulin-like growth factor expression is reduced in aged choroid plexus. IGF transcription was significantly altered in the aged CP of males (**a**) and females (**b**) ($n=5$). *IGF1* and *IGFII* mRNA showed a reduced copy number in both genders. IGF1R expression showed a gender-specific expression, where *it* was unchanged in the males (**a**) and reduced in the females (**b**). *IGFBP4* and *IGFBP7* in the females showed a reduction (**b**) in copy number, indicating a downregulation of IGFBP gene expression. IGF2 (red)-IsolectinB4 (Green)co-labeling showed that IGF2 is absent in the choroidal vasculature of males (**d**). The co-labeling study using plectin (Green) showed that IGF2 (red) is expressed in the basolateral side of choroidal epithelial cells of male mice (Fig. 1e). **c** The reduction of IGF2 Protein expression in male mice is evident from the mean gray value of IHC images (Fig. 1c). Scalebar-100 µm. *$P<0.05$, **$P<0.01$, ***$P<0.001$,

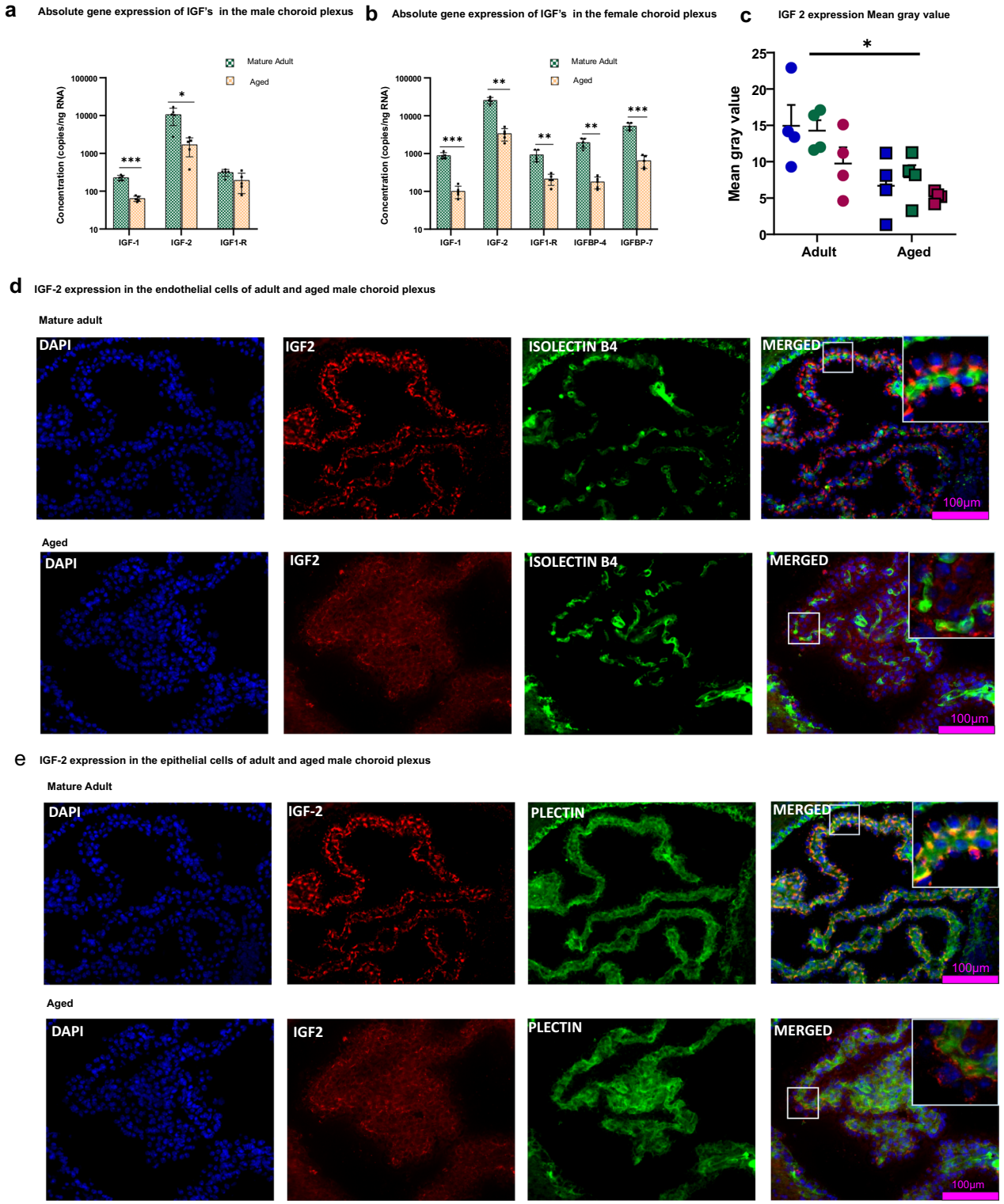


Fig. 2 (See legend on previous page.)

Longevity factor Klotho is reduced in the aged choroid plexus

α-Klotho is a glycosylated transmembrane protein that has been extensively studied as an anti-aging protein. Klotho is expressed highly in the choroid plexus, and its precise functions are largely unknown. We examined the gender and age-specific expression pattern of klotho in the CP. *KL* gene showed a reduced copy number (Fig. 3a) in the aged CPs of both males ($P<0.05$) and females (Fig. 3b) ($P<0.01$), indicating an age-related reduction in *KL* gene expression. Realtime PCR analysis showed that in females, the downregulation (Additional file 1: Fig. S1b) of *the KL* gene is evident from middle age onwards, and male mice show a trend towards reduction (Additional file 1: Fig. S1a) in the later stages of life.

We extended our investigation to examine whether the changes in the mRNA level are reflected at the protein level. First, we used Western blot analysis to examine membrane-bound Klotho protein expression in the aged CP of both sexes (Fig. 3c, e). Klotho protein expression revealed an age-related reduction (Fig. 3d, f) in the CP of males ($P<0.01$) and females ($P<0.01$). Klotho bands were visible in the 130 kDa, suggesting the presence of membrane-bound klotho. Also, we did not observe any bands at 62 kDa, indicating the absence of soluble klotho in the CP of both adults and aged CP. Next, we used IHC analysis to identify age-related changes in klotho protein expression patterns in the choroid epithelium and vasculature. Co-localization of Klotho with plectin indicates that Klotho protein expression is mainly concentrated in the choroid epithelial cells (Fig. 3h; Additional file 4: Fig. S4b). The apical part of epithelium lacks klotho expression, whereas the basolateral side is rich in klotho protein. Klotho is not co-labeled with isolectin B4, indicating the absence of Klotho protein in the choroid vasculature (Fig. 3g; Additional file 4: Fig. S4a). IHC analyses clearly indicate an age-dependent reduction in klotho protein expression in CP of males and females ($P<0.05$) (Fig. 3g; Additional file 4: Fig. S4c). Reduced klotho protein expression was observed in the ventricular lining of aged mice.

Water channel protein AQP1 expression is altered in the aged choroid plexus

A primary function of choroid epithelium is the production of CSF. Aquaporins (AQPs) are a family of small transmembrane proteins that facilitate water transport across plasma membranes during CSF production. AQP1 is the primary water channel expressed in the choroid plexus epithelium. We examined the age-dependent transcriptional changes in the male and female CP. *AQP1* gene expression (Additional file 2: Fig. S2a, b) is significantly lowered in the aged CP of both males ($P<0.001$) and females ($P<0.0001$). The reduction is evident from middle age onwards. The ddPCR analysis further confirmed the low copy number in the aged CP of males ($P<0.05$) and females ($P<0.01$), indicating a significantly reduced concentration of the *AQP1* gene (Fig. 4a, b). Protein expression analysis using IHC indicates that, like *AQP1* mRNA transcription, AQP1 Protein expression also showed an age-dependent reduction ($P<0.01$) in the CP epithelium of aged males and females (Fig. 4e; Additional file 5: Fig. S5c). IHC analysis indicates that the AQP1 protein is absent in the vasculature (Fig. 4d; Additional file 5: Fig. S5a) and robustly expressed in the ventricular-facing surface of the choroid plexus epithelium. We noted low-level expression of AQP-1 in the basolateral membrane of adult epithelial cells, which diminished further as age progressed and was almost absent in the aged CP.

Aging-altered tight junction protein expression compromises tight junction integrity

The distribution of tight junctions in the choroid plexus differs from other brain regions. Choroid vasculature is fenestrated and lacks tight junctions [60]. The epithelial cells are connected by tight junctions in the apical region and maintain BCSF integrity [61]. Claudins are one of the most crucial tight junction proteins in the choroid epithelium. *CLDN-1* gene expression did not show any significant changes in the old male choroid plexus (Additional file 2: Fig. S2a, d). In females, CP, *CLDN1* m RNA transcription showed a trend toward decrease but was not statistically significant. QIAcuity ddPCR analysis

(See figure on next page.)

Fig. 3 Longevity factor klotho expression was significantly altered in the aged choroid plexus of both genders: Age-related transcriptional level reduction was observed in the CP of males and females (**a**). The QIAcuity ddPCR analysis showed reduced KL mRNA copy numbers in the aged mice of both genders ($n=5$). A quantitative analysis of Klotho protein was conducted using Jess capillary separation in the CP of (**b**) males and females (**d**). Bands were observed at 130 kDa, indicating that the Klotho protein in the CP is membrane-bound, and an age-related reduction was evident in the CP of males (**c**) and females (**e**). We used β actin as a reference gene for Klotho with a molecular weight of 45 kDa. Klotho (Red)-IsolectinB4 (Green) co-labeling confirmed the absence of Klotho from the choroid vasculature of male mice (**g**). (←) **g** indicate the expression of klotho in the ependymal ventricular lining, and the expression was significantly reduced in the aged mice. Pectin (green) was used to mark the epithelial cells, and the basolateral sides of the choroid epithelial cells are rich in Klotho protein (**h**). The mean gray value (**f**). of IHC images confirmed the protein level reduction of Klotho in aged male CP. Scalebar-100 µm. *$P<0.05$, **$P<0.01$

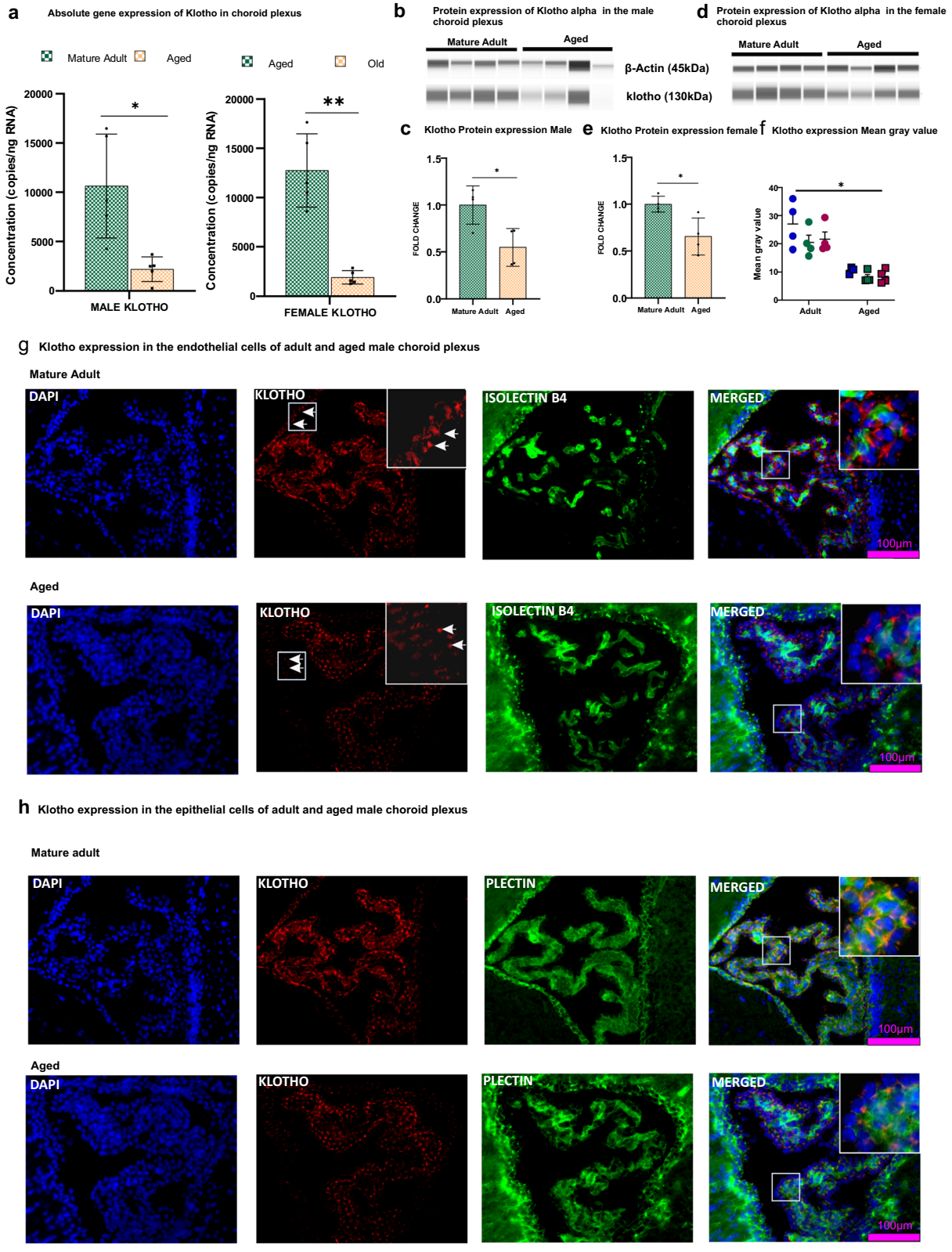


Fig. 3 (See legend on previous page.)

showed a consistent copy number of *CLDN1* transcripts in the adult and aged male CP (Fig. 5a). Copy number in females was significantly ($P<0.01$) reduced in aged mice (Fig. 5b). *CLDN2* and *CLDN5* were significantly downregulated in the aged CP of both males and females, and reduction is evident from 11–12 months of age (Additional file 2: Fig. S2c, d). QIAcuity ddPCR further confirmed the same pattern of reduction in *CLDN2* ($P<0.01$) and *CLDN5* ($P<0.001$) gene expression (Fig. 5a, b).

The mRNA expression of *JAM2* is significantly downregulated in the aged CP of both males ($P<0.001$) and females ($P<0.0001$) (Fig. 5a, b; Additional file 2: Fig. S2c, d). DdPCR (Fig. 5a, b) confirms the JAM2 mRNA gene expression reduction in males ($P<0.0001$) and females ($P<0.01$). To examine whether the altered tight junction transcription affects the BCSF barrier integrity, we injected FITC-Dextran (MW 40000) intravenously. Fluorescent imaging showed that barrier permeability was intact in adult CP, and fluorescence was restricted within the basolateral side (Fig. 5c). However, dextran leakage can be observed from the apical membrane in the aged CP, indicating that BCSF integrity is compromised.

Aging altered transport protein transthyretin gene expression

Transthyretin (TTR) is the major protein synthesized by the choroid epithelial cells and is expressed at remarkably high levels. Functionally, TTR binds and distributes thyroid hormones (THs) in the blood and cerebrospinal fluid [62]. Here, we examined the age and gender-specific changes in *TTR* gene transcription. Relative gene expression showed that aging significantly accelerated the downregulation of *TTR* mRNA (Additional file 1: Fig. S1e, f) in the CP of males ($P<0.001$) and females ($P<0.001$). The ddPCR further confirmed the reduced TTR expression in males ($P<0.001$) and females ($P<0.0001$) (Fig. 6a, b) in aged mice.

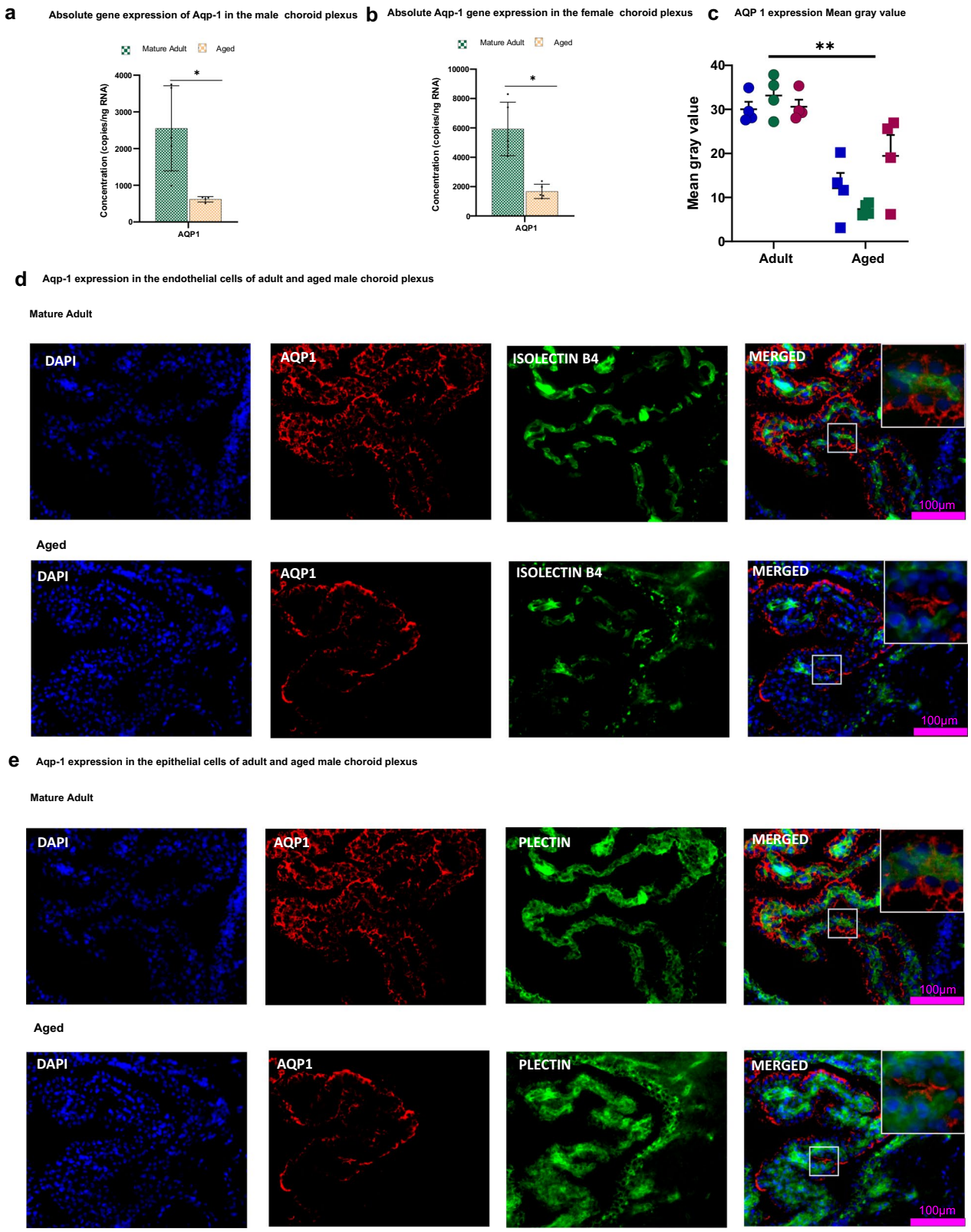
Discussion

The CP is known for producing CSF but it is comparatively understudied beyond that role. CSF contains key proteins and growth factors involved in CNS development that could be brain-derived or produced by the CP. In the present study, we analyzed the age and sex-dependent transcriptional changes in trophic factors, tight junctional proteins, water channel protein AQP1, and the antiaging protein klotho. The choroid plexus plays an indispensable role in supporting neuronal function by producing CSF and is also involved in the regulation of various neurotrophic factors. CP secretes trophic factors directly to the CSF, which circulate to different parts of the brain [63]. Here, we analyzed the impact of aging on neurotrophic factor transcription in the CP of both males and females. VEGF showed a gender-specific gene transcription in the CP. *VEGF* gene expression was unchanged in the aged male CP, while in female CP, gene expression was significantly reduced. The reduction in the female CP was evident from middle age onwards. Interestingly, unlike the pattern of gene transcription, VEGF protein levels increased in the aged CP of males and females. Typically, VEGF is in the vasculature, but in the CP, expression is observed in endothelial and epithelial cells. Aging increases the translocation of VEGF protein from the vasculature to the surrounding tissues, possibly stroma or the basal membrane of epithelial cells. VEGF is involved in the maintenance of fenestrated vasculature of the choroid plexus and has known roles in vascular permeability [25]. It would, therefore, be worthwhile to investigate the role of VEGF in epithelial permeability. BDNF, an important trophic factor involved in the neuroplastic changes related to learning and memory, exhibited reduced transcription in the aged CP. The reduction is evident from middle age onwards in both males and females. The hippocampal BDNF protein expression is not known to decline with age, while the age-related reduction is evident in the cerebral cortex and CSF [64, 65]. Reduced CSF BDNF has been suggested as a potential mechanism in the cognitive decline observed in older individuals [55]. This reduction in CSF BDNF could be due to reduced transcription of *BDNF* mRNA in older CP.

In addition to the above trophic factors, both IGF-I and IGF2 are produced in the choroid plexus. The production of IGF1 reaches its peak in the postnatal brain to support oligodendrocyte differentiation and myelination, and it decreases thereafter [66]. An age-related reduction in hepatic IGF1 production leads to a decline in circulating IGF1, resulting in impaired neurovascular coupling responses in older adults [67]. IGF1 and IGF2 can cross the BBB, so the reduction in the circulating

(See figure on next page.)

Fig. 4 Altered water channel protein expression in the aged choroid plexus: Aqp-1 is the primary water channel present in the CP. Aging significantly reduces the water channel protein *AQP-1* gene expression in the CP of males ($n=5$) (**a**) and females (**b**). Aqp-1(Red) -Isolectin B4 (Green) co-labeling confirmed the absence of Aqp-1 protein expression in the adult and old male CP vasculature (**d**). Aqp1 (Red)- Plectin (Green) co-labeling study indicates Aqp1 localization in the apical part of the choroid epithelial cells (**e**). **c** The mean gray value (**c**) of IHC images showed a reduction in Aqp-1 Protein expression in the aged CP of male mice. Scalebar-100 µm. *$P<0.05$, **$P<0.01$



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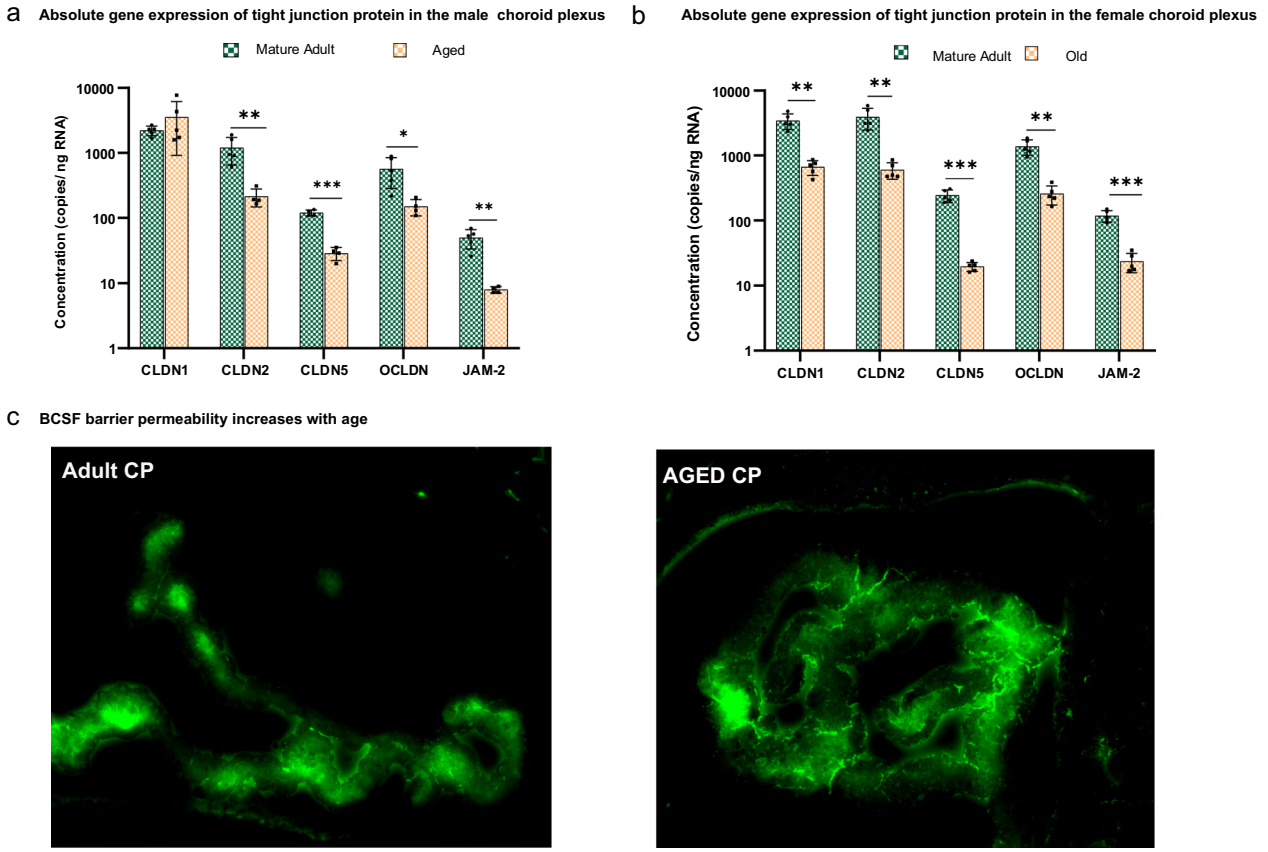

Fig. 5 Tight junctional protein gene expression showed a differential expression in the aged male CP (**a**) *CLDN-1* mRNA transcription was unchanged along with reduced CLDN-2, CLDN-5, OCLDN, and JAM2 expression. In females (**b**), *CLDN-1, CLDN-2, CLDN-5, OCLDN*, and *JAM2* transcription was significantly reduced. BCSF barrier permeability was assessed by injecting dextran average mol wt 40,000 intracardially. In adult CP, fluorescence is mainly observed in the choroid vasculature and basolateral side of the epithelium, indicating that the tight junction prevents the diffusion of FITC dextran to the ventricles (**c**). In aged mice, fluorescence can be observed in and out of the apical side of endothelial cells, indicating that dextran 40,000 was able to cross the tight junction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

levels can influence the concentration of IGFs in the CSF and CNS. Females showed an age-dependent reduction in *IGF1* transcription from middle age onwards, but in males, expression was unchanged in the middle-aged CP and significantly reduced thereafter. Even though IGF2 is abundantly expressed in the CNS, only CP, leptomeninges, and parenchymal microvasculature produce IGF2 [68]. In a healthy brain, CP secretes IGF2 directly to CSF, and CSF distributes this IGF2 to different brain regions. IGF2 is critical for preserving neural stem cells (NSCs) in the adult hippocampus [69] and also plays a vital role in adult neurogenesis, memory formation, neuronal growth, and neuroprotection [70]. A previous study conducted in sheep by Chen [71] and co-workers reported that *IGF2* mRNA expression in CP did not change with age. Our results in mice are at variance with these findings. We observed a significant reduction in *IGF2* mRNA transcription in the CP of aged males and females. The protein level analysis using IHC also confirmed the age-related reduction in the choroid epithelial cells. CP vasculature lacks IGF2 localization, indicating IGF2 bioactivity is observed only in epithelial cells. Numerous reports suggest that reduced IGF2 protein in aged mice can lead to memory deficit and cognitive impairment [72–74]. One possibility is that reduced protein expression in the hippocampus could stem from age-related transcriptional changes in the CP. IGF1 and IGF2 act through their corresponding receptors, IGF1 R and IGF2R [75]. The reduced IGF1R transcription in aged CP could be due to the age-related reduction in IGF1 protein. IGF signaling is regulated by a family of specific IGF-binding proteins (IGFBPs). We observed a gender-specific expression of *IGFBP 4 and IGFBP 7 (IGFBP-rp1)* genes in aged CP; *IGFBP4* and *IGFBP 7* mRNA transcription remained unchanged in male CP, whereas in female CP, reduction in the *IGFBP4 and IGFBP 7* gene transcription was observed. This reduction in IGFBP could be due to the reduced availability of IGF proteins in the aged CP.

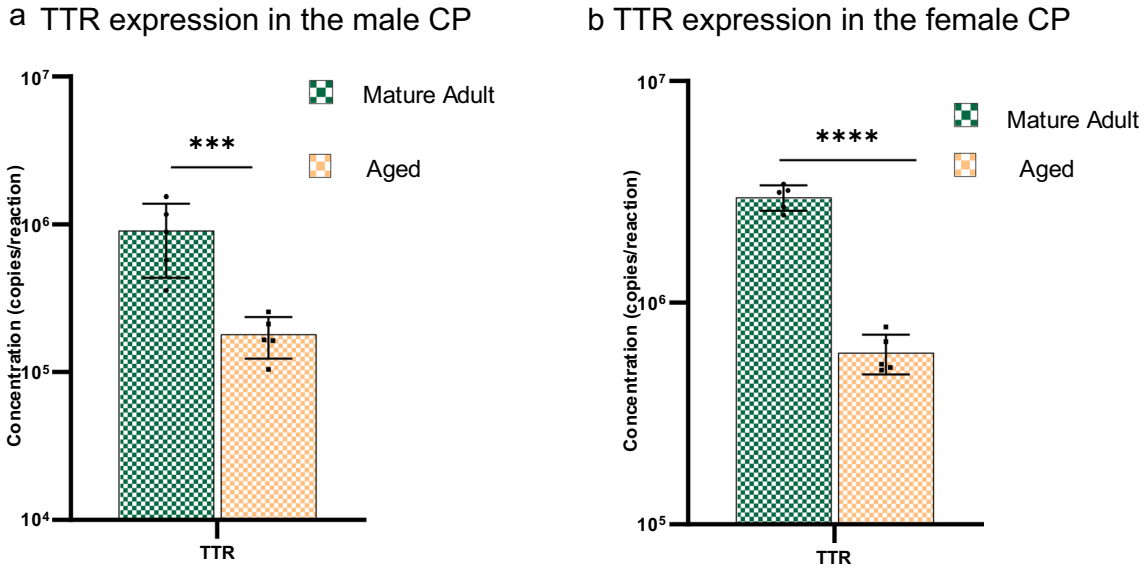

Fig. 6 Transthyretin gene expression was significantly downregulated in the aged CP of males and females. Thyroid hormone distributor protein TTR gene expression in the aged CP of males (**a**) and (**b**) females. TTR transcription was significantly downregulated in the aged CP of both genders. ***$P < 0.001$, ****$P < 0.001$

Klotho is known to regulate several pathways involved in aging, including Wnt signaling, insulin signaling, and intracellular pathways, including p53/p21, cAMP, Protein kinase C, and TGF β [76–79]. In mice, the overexpression of the Klotho gene extends life span, whereas mutations reduce life span [80–82]. In humans, serum levels of Klotho, produced from kidneys, decrease after 40 years of age [83–85]. Previous studies from our lab, using in situ hybridization, showed that *KL* mRNA and protein are abundantly localized in the CP [86]. We observed a reduction in the transcription of *KL* mRNA in the aged CP of males and females. The reduced transcription is reflected at the translation level as well. WB and IHC analyses point towards reduced transmembrane klotho protein secretion and localization in the epithelial cell's basal membrane. We did not observe a band at 68 kDa, indicating the absence of soluble klotho in CP epithelium. A previous study reported that selective knockout of klotho in the CP using a Flox/Cre mouse model revealed a novel regulatory role of klotho in the expression of inflammation-related genes [87]. Klotho knockout in the CP resulted in the increased expression of cytokine response factors, intracellular adhesion molecule 1 (ICAM1), and interferon regulatory factor 7 (IRF7) [87]. The same study also points out that the deceased production of klotho in the choroid plexus led to enhanced macrophage infiltration into the CNS, which further promoted the activation of microglia. The imbalance between different immune signals can perpetuate brain inflammation and cognitive decline in aging and neurodegeneration [88]. Neuroinflammation is considered a universal characteristic of brain aging and neurological disorders, and age-dependent Klotho reduction could potentiate this neuroinflammation. Abraham and co-workers [89] in 2018 reported that normal brain aging in rhesus monkeys was associated with a downregulation of Klotho expression, and the reduction in Klotho could accelerate age-related cognitive deficits. As klotho does not cross the BBB [90], the CP is likely the primary source of klotho for the brain. The reduction in CSF and CNS klotho levels with advanced age is likely due to the altered klotho mRNA transcription and translation in the CP.

Next, we turned our attention to the expression of water channel protein AQP1. CSF is composed of 99% water, and the remaining 1% is accounted for by proteins, ions, neurotransmitters, and glucose [91]. A high-water permeability of the BCSF barrier is essential for the optimal production of the CSF [92, 93], and this is met by the abundant expression of water channels AQP4 and AQP1. CSF enters from the perivascular spaces surrounding arteries into the brain parenchyma through the AQP4 water channels in the astrocytic end-feet [94]. AQP1 is a cGMP-gated cation channel [95, 96] that serves as a water channel and a gated ion channel in the choroid plexus, contributing to the regulation of CSF production [97–99]. We observed an age-related reduction in the transcription of *AQP1* mRNA in the CP of both males and females. Transcriptional level alterations in the *AQP1* gene were evident from middle-aged CP and continued

till later stages of life. Both males and females showed a similar pattern of gene expression. The reduction in *AQP1* gene transcription was reflected at the protein level, showing reduced AQP1 protein expression in the aged CP. The majority of AQP1 localization was observed in the apical region of cuboid epithelial cells, which is in accordance with previous studies [100–103]. A previous study in humans reported that a few adult choroid epithelial cells showed low basolateral expression of AQP1. We also found low-level expression of AQP-1 in the basolateral membrane of adult epithelial cells, and the expression diminished as age progressed and was almost completely absent in the aged CP. In situ hybridization in rats also reported a reduced expression of AQP1 mRNA levels in CP of old rats [104]. AQP1 allows water to follow the osmotic gradient and is also involved in maintaining the osmotic permeability of the apical membrane. The secretory role of AQP1 is well established, and AQP1-null mice possess 56% lower CSF pressure and a 20% reduction in CSF production [90]. The age-related reduction in water channel *AQP1* in the CP could be responsible for the reduced production of CSF in natural aging. Another highly expressed protein in the CP is TTR, a carrier protein for thyroxine and retinol in plasma and CSF [105]. CP synthesizes 90% of TTR in CSF [106], and its level in CP and CSF reflects the health of BCSFB. Our results show a reduction in TTR mRNA transcription in both sexes. TTR has been reported to bind with Aβ peptide, preventing its deposition in the brain and related toxicity [107, 108]. These age-related decreases in transport protein and water channels in CP can cause the aged brain to be more vulnerable to Alzheimer's disease.

he regulation of BCSF barrier permeability and integrity is a key role of choroid epithelial tight junctions. These apical tight junctions regulate the paracellular diffusion of water-soluble molecules through this barrier. Unlike the BBB, CP vasculature is fenestrated and lacks tight junctions to connect the endothelial cells [109–112]. Hence, the expression of the tight junctional proteins in CP is primarily from the apical tight junctions of the choroid epithelium. Like BBB, the blood–CSF barrier is also highly restricted to soluble tracers and has higher electrical resistance [113]. The tight junction proteins, claudins occludin and zonula occludens are integral to maintaining epithelial integrity, as they greatly limit paracellular permeability and preserve electrical resistance of the epithelial layer in the choroid plexus. Zonula occludens are sub-membrane proteins attaching occludin and claudins to actin filaments [114, 115], while occludin and claudins are transmembrane proteins facilitating contact between epithelial cells [11]. We found that aging impacts the transcription of the epithelial tight junction proteins, with most of the tight junctional proteins showing a reduction in transcription, potentially resulting in increased permeability in both males and females. Interestingly, this tight junctional protein expression reduction was evident from middle age onwards. The reduced transcription of the tight junction protein in the aged CP can compromise epithelial tight junction integrity and alter the electrical resistance of the BCSF membrane. The changes in the BCSFB permeability potentially result in the entry of inflammatory and toxic molecules into the brain. We cannot rule out the possibility of age-related inflammation in the choroid plexus as a cause behind the reduced expression of TJPs. The altered water channel and reduced CSF can in turn lead to the accumulation of toxic molecules in the aged brain. Furthermore, it can lead to age-related cognitive impairment.

Our results highlight gene and protein level changes in the CP during aging. The age-related gene expression changes are comparable in the CP of males and females. Altered transcription of the water channel protein AQP1 and TJ proteins could be contributing factors that lower CSF production in natural aging. Importantly, aging decreases the expression of neurotrophic factors, and the longevity factor Klotho can play a role in regulating the aging of the brain. In the later stages of life, the CP-CSF axis shows a decline in all aspects of its function, including CSF secretion [38, 116, 117], barrier, and secretory functions. This decline in function could increase the risk of developing late-life diseases and cognitive deficits.

Abbreviations

AQP1	Aquaporin1
AQP4	Aquaporin4
BBB	Blood–brain barrier
BCSFB	Blood cerebrospinal fluid barrier
BDNF	Brain-derived neurotrophic factor
CLDN-1	Claudin1
CLDN-2	Claudin2
CLDN5	Claudin5
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
DDPCR	Digital droplet polymerase chain reactor
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ICAM1	Intracellular adhesion molecule 1
IGF1	Insulin-like growth factor1
IGF1R	Insulin-like growth factor1 receptor
IGF2	Insulin-like growth factor2
IGFBP	Insulin-like growth factor binding protein
IGFBPrp	Insulin-like growth factor binding protein-related protein
IRF7	Interferon regulatory factor 7
IHC	Immunohistochemistry
JAM	Junctional adhesion molecule
KL	Klotho
NSCs	Neural stem cells
NTF	Neurotrophic factors
TGF β	Transforming growth factor-β
THs	Thyroid hormones
TJP	Tight junctional protein
TTR	Transthyretin
VEGF	Vascular endothelial growth factor
VGF	Nerve growth factor inducible

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12987-024-00574-0.

Additional file 1. Figure S1. Real-time PCR analysis showed an altered neurotrophic factors expression in the CP of male mice. Gene expression of MDK BDNF and FGF17. VEGF A, EPO, and EPOR significantly reduced aged CP along with unchanged Klotho and VGF expression. Real-time PCR analysis showed a differential expression in neurotrophic factor n in the CP of female mice. Neurotrophic factors MDK, BDNF VEGF, and FGF17 transcription were downregulated in the female mice, while VGF and EPO remained unchanged. Longevity factor Klotho gene expression was also reduced in the females.IGF transcription in male mice showed an unchanged expression of IGF1, IGF1R, IGFBP4, and IGFBP7, while IGF2 expression was significantly downregulated.IGF transcription was significantly downregulated in the female CP along with the IGF-binding protein and receptor. Thyroid hormone transporter TTR gene expression was downregulated in the CP of € males andFemales. *P<0.05, **P<0.01, *** P<0.001, *** P<0.0001.

Additional file 2. Figure S2. The expression of the water channel protein AQP1 gene was significantly downregulated in the maleand femaleCP. This decrease is evident from middle age onwards. The Tight junctional protein showed a differential transcriptional pattern in the male CP. OCLDN and CLDN 1 gene expression was unchanged, while JAM2, CLDN2, and CLDN5 gene expression was significantly downregulated. Tight junctional protein expression was significantly altered in female CP. Tight junctional proteins OCLDN JAM2, CLDN2, and CLDN5 showed a reduced expression, while CLDN1 expression was unchanged. *P<0.05, **P<0.01, *** P<0.001, **** P<0.0001.

Additional file 3. Figure S3.Immunohistochemical analysis showed an age-related decrease in the CP of female mice. IGF2- IB4co-labelingindicates IGF2 localization outside the choroid vasculature. The co-labeling study using plectinshowed that IGF2is expressed in female mice's basolateral side of choroidal epithelial cells. The reduction of IGF2 Protein expression is obvious from the mean gray valueof IHC images. *P<0.05.

Additional file 4. Figure S4.Klotho—IB4co-labelingshows that the female choroid vasculature did not express the Klotho protein. Most of the Klotho localization was observed outside the vasculature. Klotho expression in epithelial cells was examined using klotho- Plectinco-labeling. Klotho was highly expressed in the basolateral membrane of epithelial cells. The reduction in the mean gray value of aged CP points towards the reduced klotho protein localization in the epithelial cells of aged CP. * P<0.05.

Additional file 5. Figure S5.AQP1-IB4co-labelingstudy indicates that AQP-1 expression is almost absent in the choroid epithelium. AQP1-Plectinco-labelingindicates that most AQP1 was localized on the apical part of the epithelial cells. Very few cells showed basolateral expression of AQP1 in adult choroid plexus. Mean gray valueanalysis of IHC images showed that AQP1 protein localization is reduced in aged CP ** P<0.01.

Additional file 6. Table S1. Primer details.

Additional file 7. Table S2. Antibody details

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Not applicable.

Author contributions
JS—Performed experiments, collected and analyzed data, and prepared the manuscript MS—Collected samples, prepared protocols and intellectual input; SSN—study design, intellectual input, and manuscript preparation. All authors reviewed the manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All procedures were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the USD Institutional animal care and use committee (01-01-23-26D, 12-02-2023).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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