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Aging lowers PEX5 levels in cortical neurons in male and female mouse brains

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Abstract

Peroxisomes exist in nearly every cell, oxidizing fats, synthesizing lipids and maintaining redox balance. As the brain ages, multiple pathways are negatively affected, but it is currently unknown if peroxisomal proteins are affected by aging in the brain. While recent studies have investigated a PEX5 homolog in aging *C. elegans* models and found that it is reduced in aging, it is unclear if PEX5, a mammalian peroxisomal protein that plays a role in peroxisomal homeostasis and degradation, is affected in the aging brain. To answer this question, we first determined the amount of PEX5, in brain homogenates from young (3 months) and aged (26 through 32+ months of age) wild-type mice of both sexes. PEX5 protein was decreased in aged male brains, but this reduction was not significant in female brains. RNAScope and real-time qPCR analyses showed that *Pex5*

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mRNA was also reduced in aged male brain cortices, but not in females. Immunohistochemistry assays of cortical neurons in young and aged male brains showed that the amount of neuronal PEX5 was reduced in aged male brains. Cortical neurons in aged female mice also had reduced PEX5 levels in comparison to younger female mice. In conclusion, total PEX5 levels and *Pex5* gene expression both decrease with age in male brains, and neuronal PEX5 levels lower in an age-dependent manner in the cortices of animals of both sexes.

Keywords

PEX5; aging brain; peroxisomal protein

Introduction

Peroxisomes are organelles present in most cells, excluding mature red blood cells (Gronowicz, Swift, and Steck 1984; Amaral et al. 2013). Their functions include beta-oxidation of fatty acids, lipid synthesis, and simultaneously generating and degrading reactive oxygen species (ROS) (Duve and Baudhuin 1966; Antonenkov et al. 2009). In the central nervous system, peroxisomes generate myelin sheath lipids, an important part of axonal communication and conduction (Trompier et al. 2014; Baes and Aubourg 2009). While peroxisomes are crucial in maintaining cellular health, their turnover seems to be equally important, as they have an average lifespan of around two to three days (Huybrechts et al. 2009). Peroxisomes are degraded via a selective recycling process known as pexophagy, the autophagy of peroxisomes (Iwata et al. 2006; Nordgren et al. 2013; Abdrakhmanov, Gogvadze, and Zhivotovsky 2020; Germain and Kim 2020). In pexophagy, a nascent autophagic membrane identifies peroxisomes for degradation, and engulfs them. The autophagosome then fuses with an acidic lysosome, forming an autophagolysosome that breaks down the peroxisomes, freeing up resources to be used by the cell (Mizushima and Komatsu 2011; Uzor, McCullough, and Tsvetkov 2020).

Peroxisomes accumulate in fibroblast models of senescence, indicating a possible defect in clearance with age (Legakis et al. 2002). In *C. elegans* models, the gene expression of *Prx-5* (the nematode homolog of *Pex5*, a peroxisomal import protein gene) and the levels of PRX-5 protein, are lowered in aging animals (Narayan et al. 2016). Another aging study investigating the expression of peroxisomal proliferation genes in mouse livers showed that knocking down peroxisomal biogenesis genes reduced cellular peroxide markers and increased cellular tolerance to oxidative stress, increasing lifespan (Zhou et al. 2012). Interestingly, PEX5 plays a major role in modulating peroxisomal biogenesis by importing peroxisomal matrix proteins into the peroxisomal membrane (Stanley et al. 2006). Taking this evidence together, PEX5 protein and *Pex5* expression may be affected in other aging cell types, which may hint at an age-associated change in peroxisomal health. While some evidence indicates that peroxisome clearance is inhibited in the senescent brain (Stroikin et al. 2005), it is unknown how peroxisomal proteins in general are affected as the brain ages, particularly in neurons (Uzor, McCullough, and Tsvetkov 2020).

Here, we hypothesized that aging lowers the amount of PEX5 protein and *Pex5* expression in the mouse brain. To test this, we ran a Western blot probing for PEX5 protein levels using young (3-month-old) and aged (32-months+) mouse brains of both sexes. Next, to determine if *Pex5* expression was affected in the cortex, we ran RNAScope and RT-qPCR assays on young and aged male and female brain cortices. RNAScope analysis of the cingulate cortices of young and aged male and female mice revealed a reduction in *Pex5* mRNA in aged male cortices in comparison to young male cortices, while *Pex5* mRNA did not significantly change in with age in female brains. RT-qPCR confirmed this trend: *Pex5* gene expression was lower in aged male cortices in comparison to young male cortices, while there was no significant difference in *Pex5* expression between female samples. Finally, immunohistochemistry was used to determine the relative amount of PEX5 in cortical neurons in young and aged male and female brains, where PEX5 levels were reduced in both aged male and female cortical neurons. We conclude that as the brain ages, PEX5 levels go down in cortical neurons of both male and female mice, indicating a potential age-related change to the peroxisomal pathways that PEX5 is involved in, such as peroxisomal protein import and pexophagy.

Methods

Antibodies

Antibodies against PEX5 were from Proteintech (12545–1-AP; 1:100). Antibodies against HRP-beta actin were from Sigma-Aldrich (#A3854, 1:50,000). Antibodies against ACAA1 were from Abcam (#ab110289, anti-mouse, 1:1000). Antibodies against NeuN were from Abcam (#702022, anti-mouse, 1:1000) and MilliporeSigma (#ABN78, anti-chicken, 1:500). Secondary antibodies against rabbit (#PI-1000, 1:3000) and mouse (#PI-2000, 1:3000) were from Vector Laboratories.

Histology and immunohistochemistry (IHC)

Mice were anesthetized with avertin (2,2,2-Tribromoethanol, #T48402, Sigma-Aldrich), and the animals were perfused through the heart with 1% heparin in ice-cold PBS, followed by 4% ice-cold PFA. Brains were then post-fixed in 4% PFA overnight. Next, mouse brains were placed in 30% sucrose until the brains no longer floated in the solution, sectioned using a microtome (Thermo Scientific Microm HM 450) at 20 μm, and stored in an antifreeze solution until IHC was run. The personnel in charge of sectioning and slicing was blinded to the age of the animal each brain was taken from. Blinding was also achieved by assigned randomized numbers to each sample, while another person kept track of the identities of the samples.

For IHC, brain slices were mounted on slides overnight, then hydrated in 0.1% PBS-Tween (PBS-T) briefly. After washing, slides were subjected to antigen retrieval in citrate buffer, pH 6 in a rice steamer for 35 minutes, followed by 20 minutes of cooling. Slides were then briefly rinsed in cold PBS-T, then blocked and permeabilized in 5% BSA in PBS (0.1% Triton, 1% Tween) for 4 h at room temperature. To mask lipofuscin fluorescence and autofluorescence in aged brain slices, slides were washed in PBS, and stained with TrueBlack Autofluorescence Quencher (Biotium). Next, samples were incubated in primary

antibody dilutions in 5% BSA in PBS (*Pex5* 1:100, NeuN 1:1000) overnight, then washed in PBS-T and incubated in secondary antibody dilutions (Alexa Fluor 594 goat anti-rabbit 1:500, Alexa Fluor 488 goat anti-mouse 1:500, Alexa Fluor 694 goat anti-chicken 1:500) for 1 h at room temperature in the dark. 1.5H coverslips were mounted on slides using ProLong Gold Antifade Mountant from Thermo Fisher Scientific (#P36930), sealed with nail polish, and stored in 4°C overnight before imaging under a confocal microscope (Leica DMi8, LAS X software).

Western blotting

Brain tissue was prepared by first anesthetizing mice with avertin, then perfusing through the hearts with 1% heparin in ice-cold PBS. Next, brains were collected, placed on ice, then manually homogenized in RIPA buffer, analyzed for protein content using BCA assays; resulting lysates were then diluted to 5 μg/μL concentrations, and stored in aliquots.

Lysates were prepared for Western blotting in 2x Laemmli buffer, then heated at 95°C for 10 mins, before being loaded on 12% Mini-PROTEAN TGX Protein Gels (#456–1043, Bio-Rad). Gels were run for 30 minutes at 75 V, then 35 – 55 minutes at 120 V. Gels were then transferred to PVDF/nitrocellulose membranes using the wet electroblotting method, after which the membranes were blocked in 5% milk in TBS-Tween (TBS-T) overnight. The next day, membranes were incubated in primary antibody (in 2% milk in TBS-T) for 1 h, washed, then incubated for 1 h in secondary (in 2% milk in TBS-T). After washing, blots were developed using Pierce ECL Western Blotting Substrate (#32106, Thermo Scientific), and the Image Lab software for the ChemiDoc imager (Bio-Rad).

Fluorescence microscopy

Coronal sections were imaged using the Leica DMi8 confocal system at 40x magnification, with laser settings, brightness, contrast, gain and offset kept the same between images of the same type. Z-stacks were taken of cortical regions and combined using the max projection setting. Three random images per region were taken for each region. To measure PEX5 fluorescence intensity in cortical neurons, the raw images were split into three fluorescent channels in ImageJ, after which the green channel underwent thresholding for NeuN signal. The resulting image was then processed by the “Watershed” and “Analyze Particles” functions to create regions of interest (ROI). The ROIs were then used to measure the PEX5 signal intensity in the red channel; three ROIs for each image were also collected to calculate background signal (which was subtracted from all the intensity data). If the red channel of an image had high background intensity, the background was corrected by the “Adjust > Window/Levels” function before ROIs were generated. For each section of each sample, around one hundred neurons were analyzed for PEX5 fluorescence intensity. The analysis was performed blinded to age and sex of the samples.

PCR and primers

The mouse *Pex5* primers used for PCR were adapted from Origene (#MP210755). The forward sequence is: GCTGAGGAGTATCTGGAGCAGT and the reverse sequence is: CCTTGGACACAAAGTCACTGGC. Mouse *Acaa1a* primers were from Origene (#MP200195), and mouse *Cat* primers were from Sino Biological (#MP20177). Mouse

Gapdh primers were synthesized by IDT DNA. Brain tissue was prepared by dissecting fresh cortical tissue on ice, then using the Qiagen RNeasy Lipid Tissue Mini Kit (#74804); the resulting RNA was reverse-transcribed using synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, #1708890). After reverse transcription, the cDNA samples were diluted 1:5 in RNase-free water, and were run at a volume of 2 μL in a 96-well-plate, in duplicate. The total volume of each well was 20 μL, made up of 10 μL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, #1725274), 0.5 uL forward primer, 0.5 uL reverse primer (or 1 μL primer mix), and 7 μL RNAse-free water. The PCR was performed using the Bio-Rad CFX96 Real-Time System, and programmed for 30 seconds at 95 °C (for initial denaturation), followed by 40 cycles of 10 seconds at 95 °C (denaturation) and 30 seconds at 65 °C (annealing), and an extension step for 5 seconds at 65 °C, and 5 minutes at 95 °C. qPCR data was analyzed by ΔΔCt analysis of the *Gapdh* housekeeping gene and the *Pex5*, *Acaa1a* and *Cat* genes.

RNAScope assay and analysis

To measure the relative amount of *Pex5* mRNA in young and aged cortical samples, the RNAScope Multiplex Fluorescent v2 assay was run (ACDBio). A target probe against mouse *Pex5* was generated by ACDBio, and the protocol for the RNAScope Intro Pack for Multiplex Fluorescent Reagent Kit – Fixed Frozen was used (#323132, ACDBio). Fixed-frozen mouse coronal brain sections, 20 μm-thick, were mounted on slides and allowed to dry overnight. The next day, the sections were baked for 1h at 60°C. Sections were then post-fixed with ice-cold 10% normal buffer formalin for 15 minutes at 4°C, air-dried for 5 minutes and then dehydrated in 50%, 70% and two changes of 100% ethanol. Next, RNAScope Hydrogen Peroxide was added to sections for 10 minutes, after which they underwent target retrieval for 5 minutes in RNAScope Target Retrieval Reagent, in a vegetable steamer. After dehydration for 5 minutes in 100% ethanol and slides were air-dried, a hydrophobic barrier was applied to each slide. The slides were then air-dried overnight, treated with RNAScope Protease III for 30 min and washed.

Next, the *Pex5* probe (assigned to the C1 channel) was warmed to room-temperature, and 4 drops were used to cover each slide. The slides were placed in the oven for 2 hours at 40°C to hybridize the probe. While the probe was hybridizing, a 1:1500 solution of Opal 690 fluorophore was made in Pex5 probe solution, and stored in the dark. Slides were washed, then AMP 1, 2 and 3 added to cover each slide, in two 30-minute and one 15-minute incubation in the oven at 40°C. After washing, signal on the slides was developed as follows: slides were incubated in RNAScope horse radish peroxidase (HRP)-C1 for 15 minutes at 40°C, washed, incubated in diluted Opal solution (690 or 570) for 30 minutes at 40°C, washed, incubated in RNAScope HRP Blocker for 15 minutes, then washed. Finally, slides were incubated with RNAScope DAPI, coverslipped with ProLong Gold Antifade Mountant, and stored in the dark overnight.

The next day, slides were imaged under the confocal microscope at 20x magnification (Leica DMi8). Due to the semi-quantitative nature of the assay, an n of 2 was used for each group. The number of puncta in the region of interest (ROI) (i.e. the cingulate cortex) were calculated in ImageJ by a blinded investigator, by first splitting the images into red channels

(Pex5 puncta) and blue channels (DAPI). The puncta in the red channel underwent thresholding, and the "Analyze Particles" function was used to count the thresholded particles in each region. Next, the number of DAPI-positive nuclei was calculated by thresholding the nuclei in the blue channel, and using "Analyze Particles" to count the nuclei in the same region as above. For each sample, the resulting number of puncta was divided by the resulting number of DAPI-positive nuclei to give the mean number of puncta per DAPI-positive nucleus.

Statistical analysis

Unpaired t test was used for two-group comparison. For data with two factors such as age and sex, two-way ANOVA was used followed by post-hoc group comparisons, adjusted by the Sidak method for multiple testing. Values in the aged group were normalized by the values in the corresponding young group for males and females, respectively. Statistical significance was defined by a p value less than 0.05. All data analyses were performed in Prism 8 (Graphpad).

Animals and ethics

Mice were maintained in accordance with the guidelines and regulations of The University of Texas Health Science Center at Houston McGovern Medical School (protocol #350687). All experimental protocols were approved by the McGovern Medical School, and the experiments were carried out in accordance with approved guidelines compliant with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Wild-type C57BL/6 mice were obtained from JAX Lab, and were euthanized via avertin during the histology process, followed by cervical dislocation. Mice were sacrificed at the following ages: 3 months (young) and 26 to 32+ months (aged).

Results

PEX5 levels differ in young and aged male mouse brains

To examine age-dependent changes in PEX5 levels, we analyzed brains from 3-month-old and 26 to 33-month-old male and female mice. We first evaluated whole brain lysates for levels of PEX5, as well as ACAA1, (a peroxisomal matrix protein that is transported by PEX5) in males (Fig. 1A) and females (Fig. 1B). We also examined PEX5 and ACAA1 protein levels in males and females of the same age, to determine if there was a sex difference due to age (Fig. 1C, D). PEX5 was reduced in aged male samples in comparison to young male samples, while there was no significant difference in PEX5 protein levels between young and aged female brain samples (Fig. 1E). On the other hand, there was no significant difference in ACAA1 levels between young and aged male and female samples (Fig. 1F). PEX5 expression levels were not significant when comparing young or aged male and female samples, but there was still a trend of reduced PEX5 levels in aged males, when compared to aged females (Fig. 1G). There was also no significant difference between ACAA1 levels in young and aged male and female samples (Fig. 1H).

We next evaluated if *Pex5* gene expression was affected by age in the cortex. A possible age-dependent reduction in *Pex5* fascinated us, due to a recent proteomic study of aging *C. elegans* models that concluded aged animals had lower amounts of *Pex5* mRNA than their younger counterparts (Narayan et al. 2016). Cortical sections from young and aged male and female mouse brains were probed for *Pex5* expression using RNAScope, with focus on the cingulate cortex (Fig. 1I). In our RNAScope experiments, *Pex5* expression was reduced in aged male cortices in comparison to young male cortices, while there was no significant difference in *Pex5* expression in the female samples (Fig. 1J). Rt-qPCR assays on young and aged male and female cortices also revealed a similar trend: *Pex5* expression was lower in aged male cortices in comparison to young male cortices, and there was no significant difference in *Pex5* expression between female samples (Fig. 1K). Taking this evidence together with our previous Western blotting data, it can be assumed that the age-related changes to PEX5 protein levels in the male mouse cortex may be mRNA-driven. Further RT-qPCR analysis of genes for two peroxisomal proteins transported by PEX5, *Acaa1a* (ACAA1) and *Cat* (catalase) showed no significant change in expression levels with age in both male and female cortices (Fig 1L, IM).

PEX5 levels are lower in cortical neurons of aged male mouse brains, in comparison to young male brains

While the male brain samples we examined via Western blot may have showed an age-dependent difference in PEX5 protein, the lysates were whole brain, masking a cell- and/or region-dependent difference in PEX5 levels. We selected cortical areas due to their being affected in aging: the cingulate cortex and the cerebral cortex (Heumann and Leuba 1983; Peters 2002; Fjell et al. 2014). Then, we ran an IHC assay to determine the age-dependent change in PEX5 in these cortical structures, using NeuN as a neuronal marker, and a PEX5 antibody.

We first tested our PEX5 antibody on sagittal sections of a 12-week old wild-type (WT) mouse brain, and that of an age-matched *Nestin-Cre Pex5*$^{-/-}$ (*Pex5* KO) knockout mouse (Bottelbergs et al. 2010). Only the wild-type brain exhibited positive PEX5 fluorescence, indicating that our antibody was specific (Fig. 2A, B). We then stained for PEX5 in cortical neurons in young and aged brains in 4 major areas: Bregma 1.32 mm, or the septo-striatal region (SSR), Bregma 0.38 mm, or the septo-diencephalic region (SDR), and the caudal and rostral diencephalon (CD and RD respectively, Bregma −1.28 mm and −2.12 mm, respectively). In the most two anterior slices, we analyzed neurons in the cingulate cortex, and the frontal cerebral cortex. In the last two anterior slices, we analyzed neurons in the retrospenial, somatosensory, and motor cortices (Fig. 2C). To understand if aging had an effect on PEX5 peroxisomal levels, we subsequently examined the levels of PEX5 in neurons of aged mice (32–33 months), using younger 3-month-old mice as a control. PEX5 levels were reduced in the aged cohort in comparison to the younger male cohort, indicating that PEX5 levels reduce with age (Fig 2D, E). We conclude that in the male mouse brain, PEX5 levels diminish in neurons in an age-dependent manner.

PEX5 levels decrease in aged female neurons in the mouse cortex compared to young female mice

IHC experiments were also run on cortices from young (3-month-old) and aged (32-month-old) female cohorts. PEX5 levels in the aged female cortical neurons were reduced in comparison to PEX5 in young female cortical neurons (Fig. 3A, B). We conclude that in the female mouse cortex, neuronal PEX5 levels also drop in an age-dependent manner.

Discussion

In this study, we found an age-dependent and sex-dependent difference in PEX5 levels and *Pex5* expression, as indicated by Western blot, RNAScope, RT-qPCR, and fluorescent IHC analyses of young and aged mouse cortices. Our Western blot data indicates that PEX5 levels are lower in aged male brains. From our rt-qPCR analysis, *Pex5* expression levels drop with age in aged male brain cortices, but there is no significant difference in *Pex5* expression between young and aged female cortices. Our IHC data suggests that in aging, PEX5 levels decrease in male mouse cortical neurons, and the same occurs in aging female cortical neurons, indicating that neuronal PEX5, in particular, is reduced by age.

PEX5 is a peroxisomal receptor involved in peroxisomal protein import, maintaining peroxisomal homeostasis after peroxisomal biogenesis by importing peroxisomal proteins into the peroxisomal matrix (Wang et al. 2017; Carvalho et al. 2007; Wang and Subramani 2017; El Magraoui et al. 2019). It also plays a major role in peroxisomal degradation via pexophagy, a peroxisome-specific form of autophagic clearance (Sargent et al. 2016; Tripathi et al. 2016; Uzor, McCullough, and Tsvetkov 2020; Deosaran et al. 2013; Subramani 2015; Nazarko 2017; Pascual-Ahuir, Manzanares-Estreder, and Proft 2017; Sedlackova, Kelly, and Korolchuk 2020). Recently, its role has been further elucidated as a sensor for oxidative stress, by being monoubiquitinated in situations of high oxidative stress, inducing pexophagy, or by moving catalase into the cytosol to respond to oxidative stress (Apanasets et al. 2014; Walton et al. 2017). Lower PEX5 levels in the aging mouse brain, as our findings suggest, might leave neurons more vulnerable to oxidative stress, as peroxisomes will not be able to function properly due to insufficient amounts of PEX5 transporting the enzymes necessary for their antioxidant activity. As a result, this oxidative stress could further stress the aging neuron, possibly contributing to neurodegeneration. As an alternative, the age-related reduction in neuronal PEX5 levels in our study could also be a sign of decreased peroxisomal metabolism due to aging, as seen with other metabolic pathways in aging, such as autophagy and mitochondrial respiration. On the other hand, reduced PEX5 levels in the aging mouse brain could be a possible neuroprotective mechanism that leaves more peroxisomal antioxidant enzymes in the cytosol to fight oxidative stress, rather than the opposite, where high levels of PEX5 are importing the antioxidant enzymes into peroxisomal matrices and membranes.

Our data showed that PEX5 was reduced in aged male and female neurons in comparison to younger male and female neurons, respectively, and that *Pex5* expression was reduced in the brains of aging male animals. This trend is similar to that seen in previous *C. elegans* proteomic research, which points to an age-related role of PEX5, as the expression of *Prx-5*, the nematode homolog of *Pex5*, was strongly reduced in aging animals (Narayan et al.

2016). Also, recent proteomic studies on the expression of genes involved in mitochondrial activities have observed reduced expression of these genes in the aging brain, independent of neurodegenerative disease (Wingo et al. 2019). Our immunoassay findings in our aged samples are also consistent with studies that have found an age-dependent reduction in the levels of autophagy proteins in the brain, such as ATG7, p62, NBR-1, LC3-II, ULK-1 and Beclin-1; PEX5 plays a major role in the autophagy of peroxisomes in particular (Cuervo 2008; Kaushik et al. 2012; Yu et al. 2017; Moruno-Manchon et al. 2020; Zhang et al. 2015; Nordgren et al. 2015).

Sex differences in gene expression levels have been widely reported in the brain, particularly in proteins involved in autophagy; therefore, we decided to investigate if PEX5, a peroxisomal protein with a role in peroxisomal autophagy, may be influenced by sex (Du et al. 2009; Olivan et al. 2014; Weis et al. 2014; Koenig et al. 2014; Campesi et al. 2013; Chen et al. 2013; Demarest et al. 2016; Camuzard et al. 2016). Unexpectedly, our investigation of the expression levels of *Pex5* in male and female samples revealed a sex difference: there was a reduction of *Pex5* mRNA in our aged male cortices in comparison to younger male samples, but there was no statistically significant reduction in *Pex5* expression in aged female cortices (Fig. 1J, K). This is unlikely due to cyclical estrogen effects, as our aged female (32 months of age) mice can be considered to be “post-menopausal” in comparison to the young female cohort, due to the cessation of the estrous cycle, which occurs between the ages of 11 and 16 months of age (Yan et al. 2017). Another unexpected finding was that the *Pex5* mRNA levels and neuronal PEX5 protein levels in aged female brains did not necessarily correlate i.e. aged female brains had no significant change in *Pex5* mRNA in comparison to young females, but aged cortical neurons still possessed lower PEX5 protein levels than their younger female counterparts. This could be due to post-translational modification of PEX5, particularly polyubiquitination, which induces the degradation of PEX5 via the proteasome (Wang et al. 2017). However, more likely than not, this reduction in PEX5 levels could be the result of protein synthesis declining with age, which occurs in the rodent brain with time (Ori et al. 2015). Sexual dimorphic differences have been reported in at least two peroxisomal proteins outside the brain. The livers of male and female mice showed a difference in the protein levels and expression of sterol carrier protein-x (SCP-X) and sterol carrier protein-2 (SCP-2) at basal levels, and when the mice were fed with phytol, a dietary stressor that liver peroxisomes convert into phytanic acid (Atshaves et al. 2004). Taking this evidence together, the relatively unchanged *Pex5* expression in aged female cortices may be due to upstream regulators that remain unaffected in the female mouse brain, while the reduction of PEX5 protein levels may be related to age-related protein synthesis decline. If *Pex5* expression levels in the brain are indeed modulated by sex, then further research will need to be done to investigate sex-associated expression levels for other peroxisomal genes, as well as regulators of *Pex* genes (like *Pex5*) in the aging brain.

Previous studies have shown aging cell models have an abundance of peroxisomes, suggesting that PEX5’s interaction with peroxisomes for degradation may be disrupted by aging (Legakis et al. 2002). A biomedical consequence of this age-related disruption would be increased oxidative stress in the aging brain, leading to insults to different cellular compartments due to increased ROS; high peroxisomal ROS is known to damage mitochondria in human and mouse fibroblasts, which may further contribute to cellular

aging (Wang et al. 2013; Homma, Tsunoda, and Kasai 1994; Mecocci et al. 2018). As peroxisomes are potentially affected in Alzheimer disease, leading to reduced plasmalogen levels in the brains of Alzheimer patients, it is possible that the levels of PEX5 are negatively affected, impeding transport of the peroxisomal proteins necessary for plasmalogen synthesis (Kou et al. 2011). Therefore, PEX5 could be a possible marker of peroxisomal health in brain aging and age-related neurodegenerative disorders.

Experimentally, our next step is to determine whether PEX5 can be restored in the brains of aging mice models, as our results suggest that PEX5 levels may be associated with neuronal health. Another future direction is to investigate whether the changes in PEX5 levels are due to sex-associated changes in post-translational modification; PEX5 activity and amount is modulated by ubiquitination and phosphorylation (Wang and Subramani 2017; El Magraoui et al. 2019; Carvalho et al. 2007; Zhang et al. 2015; Sargent et al. 2016; Costello et al. 2019). To understand the overall change to peroxisomal pathways in neuronal aging, we plan to look into the change to levels of other peroxisomal import proteins, such as ABCD3/PMP70 and PEX14, which also transport peroxisomal proteins into the peroxisomal membranes and matrices respectively, as PMP70 is also ubiquitinated in the pexophagy process, and markers of peroxisomal mass, such as ACAA1 and catalase, to further understand how peroxisomes are directly affected by the changes to peroxisomal transport proteins (van Roermund et al. 2014; Barros-Barbosa et al. 2019; Osumi et al. 1991; Yamamoto et al. 1988; Sargent et al. 2016). It would also be fascinating to examine whether PEX5 in aging is affected by USP30, a deubiquitinating enzyme recently shown to counter pexophagy by preventing peroxisomal loss or HSPA9, a novel pexophagy regulator (Jo et al. 2020; Marcassa et al. 2019; Riccio et al. 2019). In sum, future studies will investigate the upstream and downstream mechanisms of neuronal PEX5 alteration and of other peroxisomal import proteins, so that the mechanism of age-related changes to PEX5 and related peroxisomal pathways in neurons may be fully elucidated.

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Highlights

- PEX5 protein is lower in aged mouse neurons than in young mouse neurons
- *Pex5* gene expression is lower in aged mouse brains than young mouse brains
- Aging's effect on PEX5 may highlight peroxisome changes that happen in aging brain

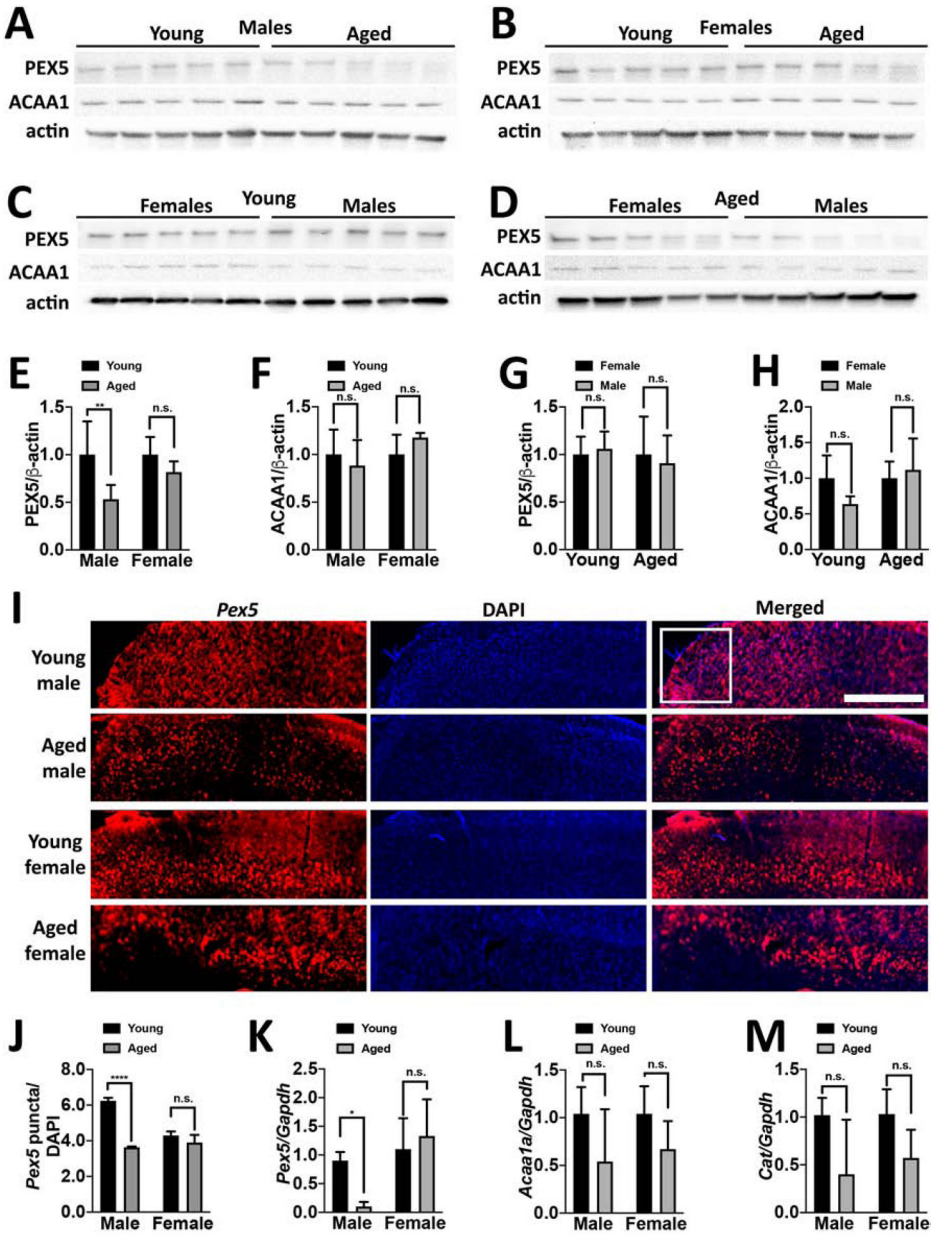

Figure 1: PEX5 levels differ in young and aged male mouse brains

(A, B): PEX5 and ACAA1 immunoblots of young and aged male (A) and female brain lysates (B) are shown. **(C, D)**: PEX5 and ACAA1 immunoblots comparing aged male and female (C) and young male and female brain lysates (D) are shown. **(E)**: The quantification of the PEX5 Western blots in 1A and 1B. Bars are mean ± the standard deviation (SD). Young male: n=5, young female: n=5, aged male: n=5, and aged female n=5 (total n=20). **P<0.001, n.s. = not significant. **(F)**: The quantification of the ACAA1 Western blots in 1A and 1B. Bars are mean ± the standard deviation (SD). Young male: n=5, young female: n=5, aged male: n=5, and aged female n=5 (total n=20). n.s. = not significant. **(G)**: The quantification of the PEX5 Western blots in 1C and 1D. Bars are mean ± the standard deviation (SD). Young male: n=5, young female: n=5, aged male: n=5, and aged female n=5

(total n=20). n.s. = not significant. **(H)**: The quantification of the ACAA1 Western blots in 1C and 1D. Bars are mean ± the standard deviation (SD). Young male: n=5, young female: n=5, aged male: n=5, and aged female n=5 (total n=20). **P<0.001, n.s. = not significant. **(I)**: Representative cortices of young and aged male and female brains from the rostral diencephalon (Bregma −1.28mm). White rectangle outlines cingulate cerebral cortex, which was analyzed. Scale bar = 4 mm. Four slices for each group were analyzed. **(J)**: Quantification of the average number of *Pex5* probe puncta divided by the number of DAPI-positive nuclei per region of interest (ROI) in each group. ****P<0.0001, n.s. = not significant. **(K)**: Quantification of relative RT-qPCR *Pex5* expression. Young male: n=4, young female: n=4, aged male: n=4, and aged female n=4 (total n=16). *P<0.01, n.s. = not significant. **(L, M)**: Quantification of relative RT-qPCR *Acaa1a* and *Cat* expression. Young male: n=4, young female: n=4, aged male: n=4, and aged female n=4 (total n=16). n.s. = not significant. Two-way ANOVA was run, adjusting for multiple comparisons with Sidak's multiple comparisons test.

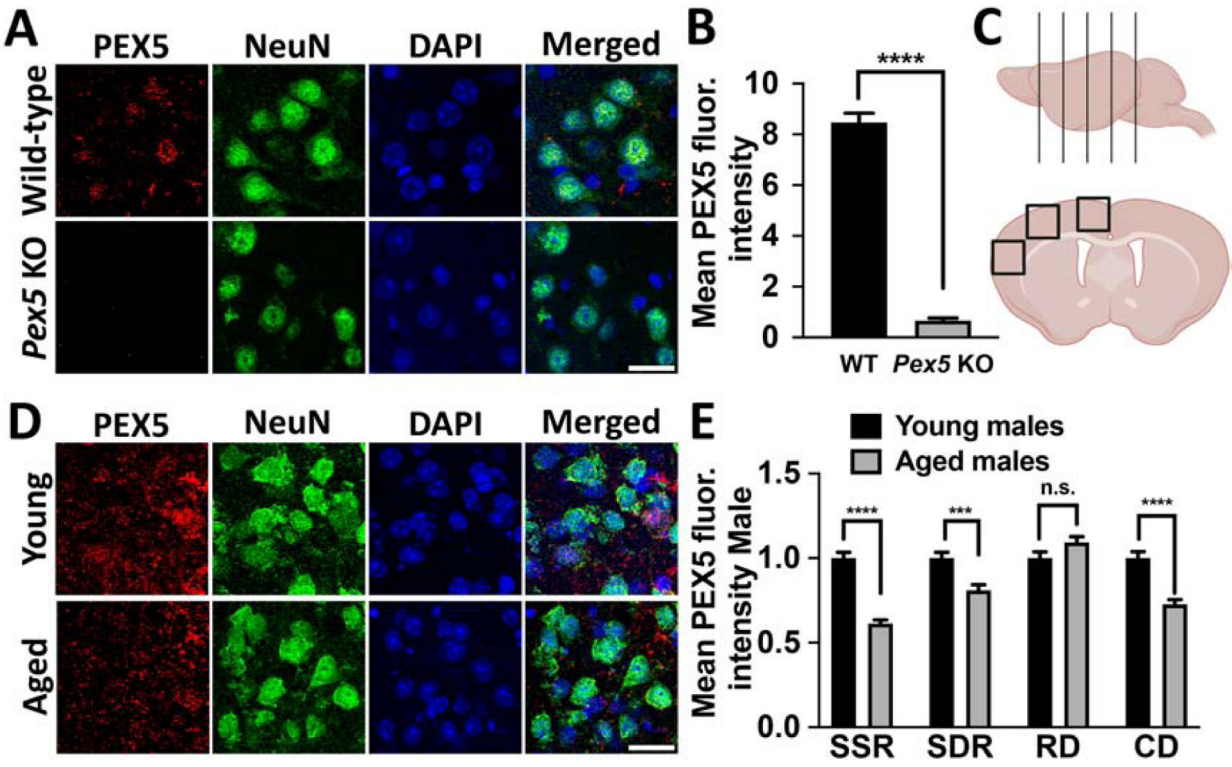

Figure 2: PEX5 protein levels are lower in cortical neurons of aged male mouse brains, in comparison to young male brains

(A, B): Representative images of wild-type and *Pex5* KO mouse 12 week-old sagittal brain sections stained for PEX5 (red), neuronal marker NeuN (green), and DAPI (blue), and the quantification of PEX5 fluorescence intensity. Bars are mean ± SEM, n=150. ****P<0.0001. Scale bar=15 μm. Unpaired t-test was run to determine statistical significance. **(C)**: Representative scheme of brain sections (top) analyzed for PEX5 immunohistochemistry in young and aged groups; sections of cortex (bottom) analyzed in PEX5 immunohistochemistry experiments. **(D, E)**: Representative images of young (3 months) and aged (32+ months) male cortical neurons, and PEX5 fluorescence intensity quantification. SSR = septo-striatal region, SDR = septo-diencephalic region, RD = rostral diencephalon, CD = caudal diencephalon. Bars are mean ± SEM, n = 4; about 400 cells per section were analyzed. ****P<0.0001, n.s. = not significant. Scale bar = 15 μm. Two-way ANOVA was run for each region, adjusting for multiple comparisons with Sidak's multiple comparisons test.

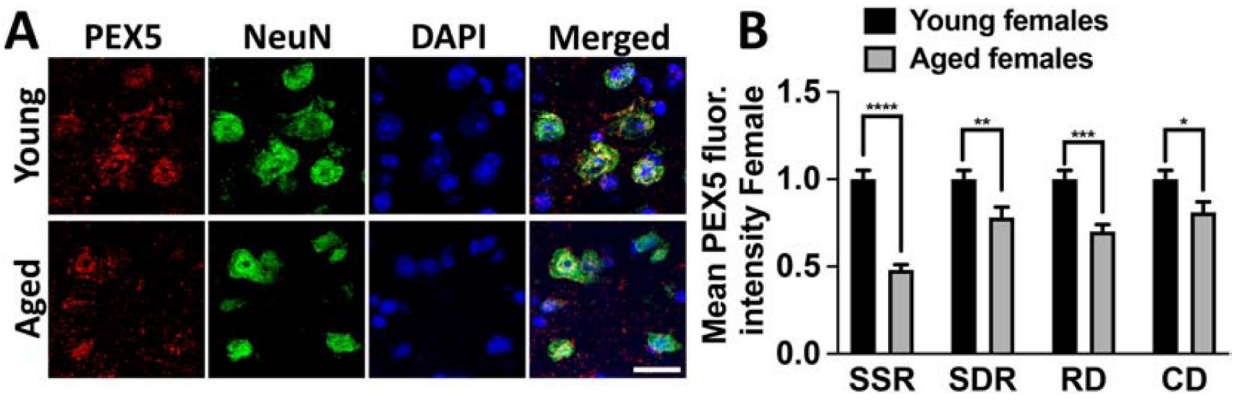

Figure 3: PEX5 levels increase in aged female cortical neurons in comparison to young female cortical neurons, while *Pex5* levels drop in aged female cortical neurons

(A, B): Representative images of young (3 months) and aged (32+ months) female cortical neurons, and PEX5 fluorescence intensity quantification. SSR = septo-striatal region, SDR = septo-diencephalic region, RD = rostral diencephalon, CD = caudal diencephalon. Bars are mean ± SEM, n = 3; 300 cells per section were analyzed for the aged group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Scale bar = 15 μm. Two-way ANOVA was run for each region, adjusting for multiple comparisons with Sidak's multiple comparisons test.