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## **Aging Lowers PEX5 Levels in Cortical Neurons in Male and Female Mouse Brains**

Ndidi-Ese Uzor

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AGING LOWERS PEX5 LEVELS IN CORTICAL NEURONS IN MALE AND FEMALE  
MOUSE BRAINS

by

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## Dedication

This dissertation is dedicated to my late grandfather, Chief C. Harrison Jefia.

It is also dedicated to my late uncle, Dr. Abraham Jefia, who passed when I was writing this dissertation, and whose medical career inspired me to do this thing in the first place, and to finish it.

Finally, this dissertation is dedicated my late uncle, Azubuike Uzor, whom I never met. I am sure you will agree with the following:

One could not be a calm, cool, and detached scientist while Negroes were lynched, murdered and starved. – W.E.B. Du Bois.

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To my late hedgehog, Cornelius, who started this journey with me.

AGING LOWERS PEX5 LEVELS IN CORTICAL NEURONS IN MALE AND FEMALE  
MOUSE BRAINS

Ndidi-Ese Uzor, B.S.

Advisory Professors: Andrey S. Tsvetkov, Ph.D., and Louise D. McCullough, M.D., Ph.D.

Peroxisomes are small organelles with critical functions: lipid synthesis, breakdown of reactive oxygen species by antioxidant enzymes, and amino acid degradation. In the brain, peroxisomal lipids make up the myelin sheath. Brain peroxisomal dysfunction leads to lipid disruption or neurological consequences if key peroxisomal proteins are absent. Still, it is unclear how peroxisomes are affected in neurodegenerative diseases and in normal brain aging. This work examines peroxisomal markers in three settings: 1) in a neuronal and 2) animal model of Huntington disease (HD), where mutant huntingtin (mHtt), the causative protein in Huntington disease pathogenesis is expressed, and 3) in the cortices of aged mouse brains.

First, we found that the rate of peroxisomes being moved to acidic lysosomes increased in a neuronal HD model compared to control neurons, indicating that mHtt expression amplified the peroxisomal degradation process. We also found that in the cortices of a presymptomatic HD mouse model, neuronal levels of PEX5, (a peroxisomal protein involved in clearance and homeostasis of peroxisomes) and ACAA1 (a peroxisomal marker), were lower than in control groups, suggesting that before HD symptoms begin, mHtt expression may reduce peroxisomal number in neurons, and affect peroxisomal homeostasis by reducing

PEX5 levels. These changes, while unexpected, had us wondering if brain aging affected PEX5 levels, since metabolic pathways become impaired as the brain ages.

Secondly, we investigated how age and sex affect cortical PEX5 levels of aged male and female mice. We discovered that PEX5 is lower in aged male brains than in young male brains, lower *Pex5* cortical expression in aged males compared to younger males, and lower neuronal PEX5 levels in aged male and female cortices, compared to young male and female cortices. In conclusion, aging has a negative effect on neuronal PEX5 levels in aging mouse brains of both sexes.

This novel work investigates how PEX5 levels are affected in models of a neurodegenerative disease and in the typical aged mouse brain, setting a foundation for further investigation of the role of peroxisomal proteins in the progression of normal neuronal aging and neurodegenerative disease.

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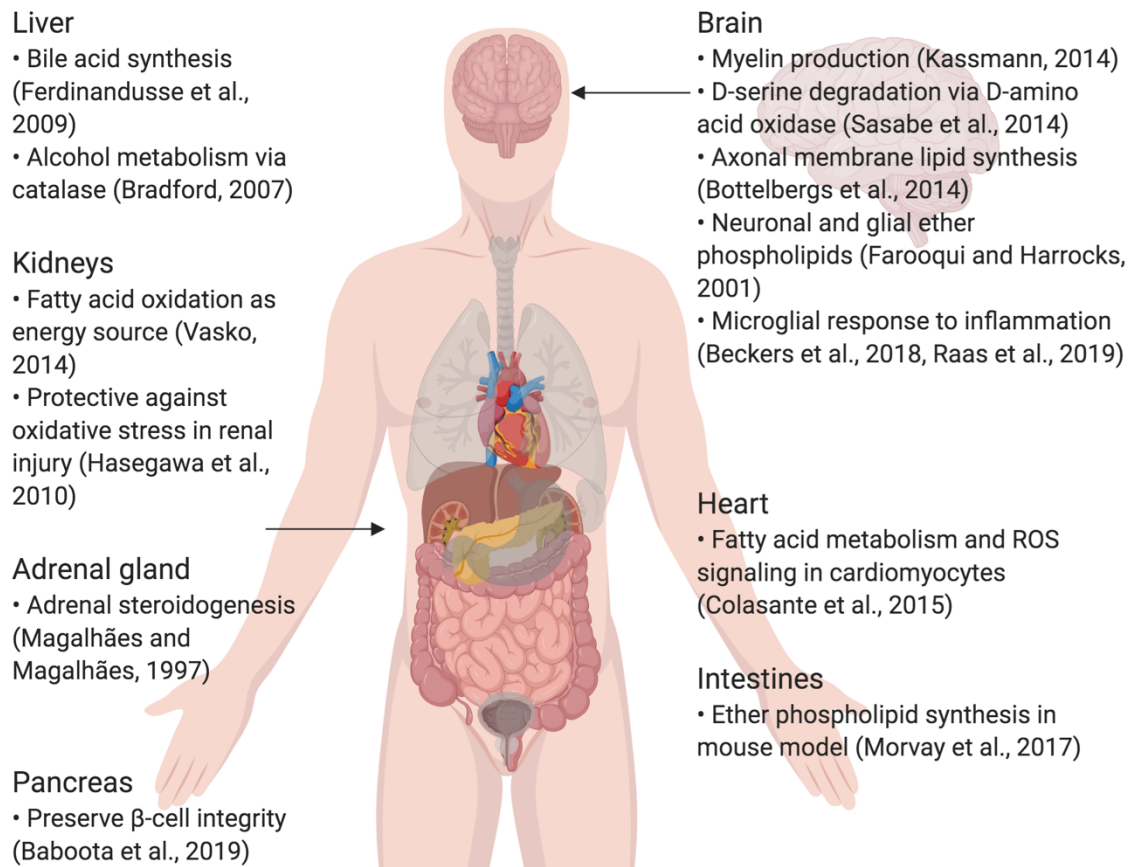
## **Chapter 1: Introduction**

This chapter is based upon “Peroxisomal dysfunction in neurological diseases and brain aging”, courtesy of *Frontiers in Cellular Neuroscience*, © Uzor, McCullough, and Tsvetkov. 2020.

### **Introduction**

Peroxisomes are small, nearly ubiquitous organelles found in almost all cell types, except maturing red blood cells (Gronowicz, Swift et al. 1984). Their major functions include the beta-oxidation of very long chain fatty acids and lipid peroxidation; as a result of this metabolism, they secrete reactive oxygen species (ROS) as by-products (Reddy and Hashimoto 2001, Poirier, Antonenkov et al. 2006, Lodhi, Wei et al. 2015, Park, He et al. 2019). Peroxisomes also possess enzymes that break down ROS, such as catalase and glutathione peroxidase, which breaks down hydrogen peroxide, and superoxide dismutase, which breaks down superoxide (Nordgren and Fransen 2014). They degrade prostaglandins, amino acids, polyamines, and purines, and are commonly enriched in the kidneys, liver, pancreas and adrenal glands, which are involved in fat metabolism and detoxification (Magalhães and Magalhães 1997, Bradford 2007, Ferdinandusse, Denis et al. 2009, Hasegawa, Wakino et al. 2010, Smith and Aitchison 2013, Vasko 2016, Baboota, Shinde et al. 2019). Furthermore, they are implicated in lipogenic and ROS signaling roles in the heart and intestines (Colasante, Chen et al. 2015, Morvay, Baes et al. 2017). In the central nervous system (CNS) in particular, peroxisomes synthesize lipids that make up the myelin sheath and cellular membranes, as well as ether phospholipids in neurons and glia; peroxisome dysfunction is also known to impair neuronal migration and membranes (Farooqui and

Horrocks 2001, Powers 2001, Bottelbergs, Verheijden et al. 2010, Kassmann 2014). They also play a critical role in breaking down D-serine via D-amino acid oxidase, important in glutamatergic signaling (Sasabe, Suzuki et al. 2014) (**Figure 1.1**).



**Figure 1.1: Summary of specialized roles of peroxisomes in some organs, including the brain.**



Certain diseases, such as peroxisomal biogenesis disorders, underscore the importance of functional peroxisomes in the central nervous system. Peroxisomal biogenesis disorders are a subset of diseases where 1) peroxisomes are either not present, leading to severe neurological phenotypes (as seen in neonatal adrenoleukodystrophy, where seizures, hypotonia and loss of vision and hearing occur) and a short lifespan, or 2) genes coding for a single peroxisomal protein are defective, where the symptoms are not as severe (Fujiki, Yagita et al. 2012, Aubourg, Wanders et al. 2013). To conclude, peroxisomes are small, but important organelles that play supportive, yet critical roles in maintaining cellular health, especially in the central nervous system.

This review summarizes peroxisomal biogenesis, and yeast and mammalian pexophagy, with an extended focus on peroxisomes in cellular senescence models, and the peroxisomal dysfunction shared by both age-related neurodegenerative diseases and peroxisomal biogenesis disorders. In all of these conditions, functional peroxisomes move from understudied, secondary organelles to critical sustainers of cellular homeostasis that are disrupted by disease. Future studies will elucidate the role of peroxisomes in aging and CNS function in other diseases and models.

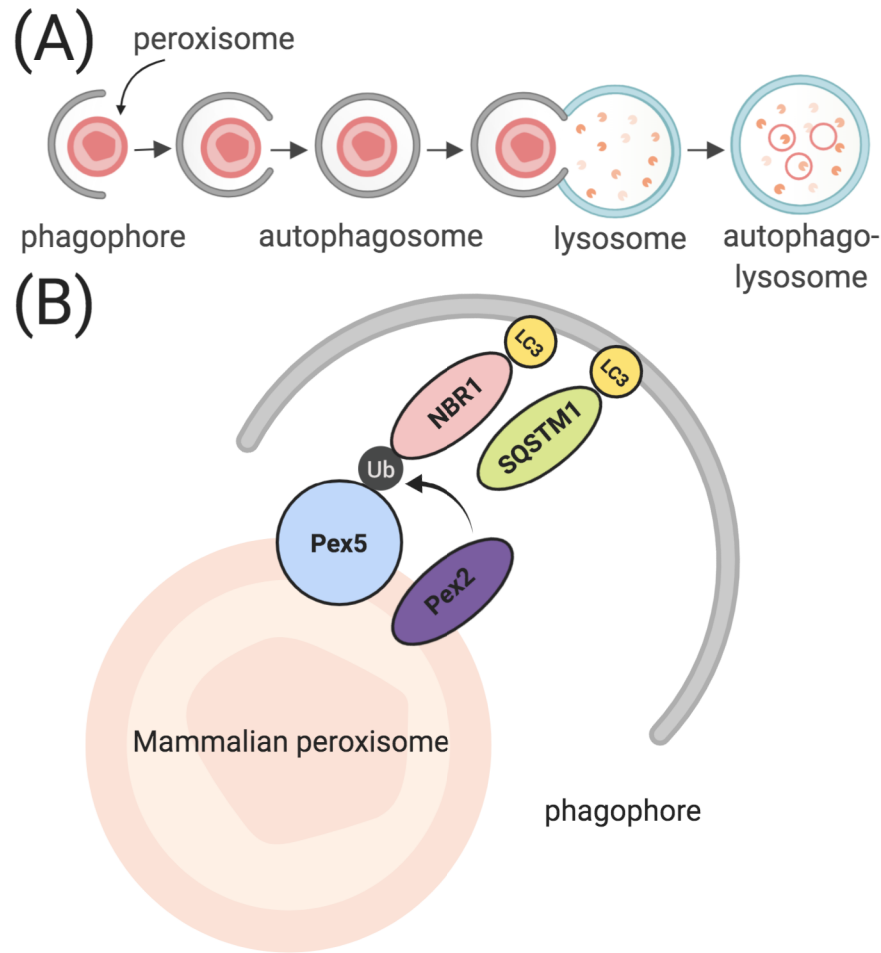
### **Peroxisomal biogenesis**

Peroxisomes begin their lifecycle by budding off the endoplasmic reticulum in response to peroxisome proliferator-activated receptor (PPAR) activation due to signaling of the PPAR gamma coactivator- 1 $\alpha$  (PGC-1 $\alpha$ ) protein (Bagattin, Hugendubler et al. 2010). Unlike mitochondrial proteins, peroxisomal proteins are synthesized on free ribosomes in the cytosol (Koehler 2000, Jan, Williams et al. 2014). After this, peroxisomal proteins are inserted into

peroxisomal membranes and matrices by the peroxisomal protein Pex5 (Smith and Aitchison 2013). Pex5 recognizes the peroxisomal targeting sequence (PTS1) serine-lysine-leucine (SKL), which is found on the C-terminal of many peroxisomal proteins (Brocard and Hartig 2006). After proteins are inserted, peroxisomes are considered mature and functional. For peroxisomal maintenance, division and maturation, peroxisomes are known to make contact with the endoplasmic reticulum (Hua, Cheng et al. 2017). To conclude, peroxisomal division and maintenance are modulated by the endoplasmic reticulum, and peroxisomes mature due to peroxisomal protein import into their matrices and membranes.

### **Autophagy and pexophagy**

The peroxisomal lifespan in mammalian cells lasts about two to three days (Poole, Leighton et al. 1969, Huybrechts, Van Veldhoven et al. 2009, Moruno-Manchon, Uzor et al. 2018). Peroxisomes are then degraded by a selective form of macroautophagy: macropexophagy, which specifically targets peroxisomes (Yang and Klionsky 2010, Bartoszewska, Williams et al. 2012, Cho, Kim et al. 2018). A lesser-known form of pexophagy micropexophagy, exists, but has only been, so far, observed in yeast models (Strømhaug, Bevan et al. 2001, Mukaiyama, Baba et al. 2004). In macroautophagy, targets for degradation are recognized by a phagophore, which matures to form an autophagosome (Moruno Manchon, Uzor et al. , Reggiori and Tooze 2009, Mizushima, Yoshimori et al. 2011, Feng, He et al. 2014, Biazik, Ylä-Anttila et al. 2015, Moruno Manchon, Uzor et al. 2016). The autophagosome envelops the targets and then fuses with an acidic structure known as the lysosome. Together, they form the autophagolysosome, which degrades the target (**Figure 1.2A**) (Nakamura and Yoshimori 2017, Sasaki, Lian et al. 2017).



**Figure 1.2: Summary of pexophagy**

(A): In macropexophagy, a form of macroautophagy selective for peroxisomes, a single membrane known as a phagophore engulfs a peroxisome for degradation. The phagophore matures into an autophagosome, which then fuses with a lysosome. Their fusion creates an autophagolysosome that degrades the target.

(B): In mammalian systems, pexophagy occurs when Pex2 ubiquitinates Pex5. As a result, autophagy adaptor proteins NBR1 or SQSTM1 (p62) bind to ubiquitinated Pex5, and then eventually bind to LC3 on the phagophore. Due to this process, pexophagy occurs.

Pexophagy itself uses the same process; however, peroxisomes are targeted via particular proteins on their membrane (Jin, Liu et al. 2013, Cho, Kim et al. 2018). Once recognized, peroxisomes are enveloped by the phagophore, and eventually degraded by the autophagolysosome. Recently, a study in HeLa SH-SY5Y cells and mutant *Drosophila* flies unearthed a novel pexophagy inducer: HSPA9, a heat shock protein which responds to cellular changes such as glucose deprivation (Jo, Park et al. 2020). In summary, peroxisomes that have reached the end of their life cycle are degraded through a selective autophagic process known as pexophagy, due to the enzymatic action of the autophagolysosome.

### **Pexophagy in mammals**

The foundation of the pexophagy mechanism (and peroxisome biology) began in studies using yeast and plants as models (Lefevre, van Roermund et al. 2013, Williams and van der Klei 2013, Kao and Bartel 2015, Young and Bartel 2016). The *Saccharomyces cerevisiae* yeast homolog of PEX5, Pex5, recognizes cytosolic peroxisomal matrix proteins and delivers them to the peroxisomal membrane (Carvalho, Pinto et al. 2007). In this model, pexophagy involves Pex3, which is recognized and bound by phosphorylated Atg36, which is itself recognized by Atg8 or Atg11, which are bound to the phagophore (Motley, Nuttall et al. 2012, Motley, Nuttall et al. 2012, Farré, Burkenroad et al. 2013, Yamashita, Abe et al. 2014). In some cases, such as when mitochondria and peroxisomes interact, peroxisomal fission occurs before pexophagy, modulated by Dnm1 and Vps1 (Mao, Liu et al. 2014).

Further studies in mammalian models revealed that in order for mammalian pexophagy to begin, PEX5 has to be monoubiquitinated by PEX2, an E3 ubiquitin ligase (Nordgren, Francisco et al. 2015, Sargent, van Zutphen et al. 2016, Germain and Kim 2020). Previously,

it was not clear what directly induces pexophagy; however, recent evidence has shown that increased ROS in the cytosol can stimulate this monoubiquitination, allowing PEX5 to act as a ROS sensor, leading to an increase in peroxisome degradation (Kim, Hailey et al. 2008, Zhang, Tripathi et al. 2015, Walton, Brees et al. 2017). After PEX5 is monoubiquitinated, it is recognized by one of two LC3 adaptor proteins: NBR1, or p62 (SQSTM1); these proteins are then bound to LC3, which is bound to the autophagosome (**Figure 1.2B**) (Kabeya, Mizushima et al. 2000, Kirkin, Lamark et al. 2009, Deosaran, Larsen et al. 2013). Together, the interaction of these proteins induces pexophagy in the mammalian cell.

### **The known: dysfunctional peroxisomes and pexophagy in neurodegenerative disease, peroxisomal disorders, and neuropathies**

In the central nervous system, neurons rely on different forms of autophagy (general and selective) to clear organelles and proteins that are no longer of use; this use of autophagy is due to neurons being post-mitotic and unable to divide, making them more vulnerable than cells that can divide and dilute toxic protein build-up (Moore and Holzbaur 2016, Evans and Holzbaur 2019, Stavoe and Holzbaur 2019, Stavoe and Holzbaur 2019). Neuronal autophagy is compartment-specific: it begins at the distal axon, after which axonal autophagosomes then move into the cell soma; the soma also contains its own autophagosomes (Maday and Holzbaur 2016, Kulkarni, Chen et al. 2018, Moruno-Manchon, Uzor et al. 2018). Neurons also respond to autophagy inducers differently than other neural cells, underscoring the uniqueness of neuronal autophagy among other forms of autophagy (Ferguson, Lenk et al. 2009, Pamerter, Perkins et al. 2012, Bordi, Berg et al. 2016, Moruno Manchon, Uzor et al. 2016, Sung and Jimenez-Sanchez 2020).

Interestingly, a common trait of neurodegenerative diseases is the impairment of protein and organelle turnover. Alzheimer disease (AD) is the most common form of dementia in elderly people, with patients exhibiting symptoms such as memory loss and mood changes; the disease eventually destroys neurons in the hippocampus and the cortex (Liang, Dunckley et al. 2008, Mortality and Causes of Death 2015). In AD, beta-amyloid and tau accumulate, and senescent mitochondria are also present (Zilka, Filipcik et al. 2006, Mitchell 2009, Nilsson, Loganathan et al. 2013, Shi, Zhu et al. 2016, Harada, Ishiki et al. 2018). While Parkinson disease (PD) has a lower prevalence than AD, the number of people with PD has increased over time, as the number of aged people has increased (Dorsey, Elbaz et al. 2018). In PD, neurodegeneration occurs in the substantia nigra, leading to tremors, bradykinesia, postural instability, and rigidity (Jagadeesan, Murugesan et al. 2017). Huntington disease (HD) occurs due to the mutated huntingtin gene and affects the medium spiny neurons in the striatum as well as neurons in the cortex, leading to symptoms such as chorea (jerky movements), rigidity and progressive motor failure (Ehrlich 2012, Wyant, Ridder et al. 2017). In Parkinson disease and Huntington disease, damaged mitochondria and causative proteins (alpha-synuclein and to a much smaller extent, tau in PD, and mutant huntingtin in HD) accumulate in affected neurons, indicating a problem with autophagy or the ubiquitin/proteasome system (Bloom 2014, Atik, Stewart et al. 2016, Zhao, Hong et al. 2016, Chiasseu, Alarcon-Martinez et al. 2017, Zhang, Gao et al. 2018, Finkbeiner 2019, Harrison, La Joie et al. 2019). Amyotrophic lateral sclerosis (ALS) can be familial or sporadic, leading to neurodegeneration of motor neurons in the CNS; a wide range of genetic mutations can induce this neurodegeneration, including the *SOD1* gene, which codes for superoxide dismutase (Peters, Ghasemi et al. 2015). Inducing autophagy improves survival in neuronal

ALS models (Barmada, Serio et al. 2014). In aging neurons, mitochondrial senescence is observed (Gilmer, Ansari et al. 2010, Menzies, Fleming et al. 2017). However, not much is known about how pexophagy, or how peroxisomal proteins are affected by these diseases. First, we will summarize the present data on peroxisomes and pexophagy in neurodegenerative disease studies, then review cases where global peroxisomal disturbances lead to neurodegenerative phenotypes.

In some neurodegenerative diseases, the amount and/or function of peroxisomes may be compromised. In Alzheimer's disease, in which beta-amyloid and tau accumulate in neurons, peroxisomes may be affected. In one study, rat hippocampal cultures with beta-amyloid overexpression were treated with Wy-14.463, a peroxisomal proliferator. This treatment increased peroxisomal number and catalase activity, reduced ROS production, and overall, reduced the degenerative effects of beta-amyloid such as the instability of beta-catenin and the increase of calcium (Santos, Quintanilla et al. 2005). In a clinical study, plasmalogens (which peroxisomes synthesize) were negatively affected in post-mortem samples of Alzheimer patients' brains, suggesting a reduction in peroxisomal activity, or a shorter half-life of plasmalogens (Goodenowe and Senanayake 2019). Amyotrophic lateral sclerosis (ALS), a disease in which motor neurons degenerate, is linked to peroxisome dysfunction through a genetic mutation that codes for D-amino acid oxidase (DAO), a peroxisomal enzyme that specifically breaks down D-serine (Kondori, Paul et al. 2017, Kondori, Paul et al. 2018).

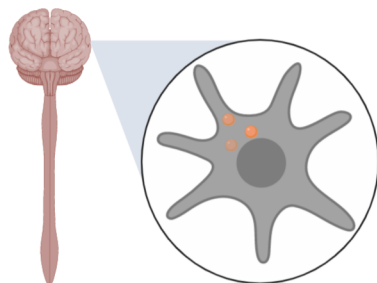
In other cases, peroxisome dysfunction, as seen in peroxisome biogenesis disorders, may lead to degenerative neurological symptoms. Peroxisome biogenesis disorders occur due to peroxisome genetic defects, either resulting in single peroxisomal enzyme dysfunction, or in

rare cases, the absence of peroxisomes themselves (Braverman, Raymond et al. 2016). Two groupings of peroxisome biogenesis disorders exist: those under the Zellweger spectrum (neonatal adrenoleukodystrophy, Zellweger syndrome and infantile Refsum disease), and those outside of it. In Zellweger syndrome, which is inherited in an autosomal recessive manner, one of 13 peroxin (*PEX*) genes is mutated (*PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX11 $\beta$* , *PEX12*, *PEX13*, *PEX14*, *PEX16*, *PEX19*, *PEX26*), leading to issues with neuronal migration, myelination and brain development (Waterham and Ebberink 2012, Klouwer, Berendse et al. 2015, Wang, Yik et al. 2015). A cellular model of Zellweger syndrome, particularly of a *Pex5* mutation, has shown an increase in alpha-synuclein Lewy bodies; alpha-synuclein is thought to be a causative agent in Parkinson disease, particularly in familial cases (Yakunin, Moser et al. 2010, Riederer, Berg et al. 2019). *In vivo*, *Pex5*<sup>-/-</sup> mouse brain samples exhibited an increase in alpha-synuclein oligomers in comparison to control, suggesting a correlation between peroxisome dysfunction and Parkinson disease (Yakunin, Moser et al. 2010). Neonatal adrenoleukodystrophy is also an autosomal recessive PBD, but with multiple peroxisomal enzymes affected; infant patients exhibit neurological symptoms such as hearing loss, neuropathy and demyelination (Aubourg, Scotto et al. 1986). The last PBD under the Zellweger spectrum is infantile Refsum disease, where a build-up of phytanic acid and other very long-chain fatty acids in the body (a result of mutated *PEX* genes) leads to neurological symptoms such as mixed neuropathy and hearing loss (Warren, Mierau et al. 2018). Outside the Zellweger spectrum, adult Refsum disease has similar symptoms to infantile Refsum disease, but the adult-onset disease is due to a mutation in the *PHYH* gene that codes for the peroxisomal enzyme phytanoyl-CoA dioxygenase, which



peroxisomes use to break down phytanic acid into pristanic acid (Wanders, Komen et al. 2011, Wanders and Poll-The 2017, Gettelfinger and Dahl 2018).

Rhizomelic chondrodysplasia punctata (RCDP) is a set of peroxisome biogenesis disorders where peroxisomal genes coding for proteins involved in plasmalogen synthesis are mutated (Barøy, Koster et al. 2015). Of note is RCDP type 1, which is due to the mutation of *PEX7*, which codes for PEX7, a peroxisomal receptor that inserts proteins into the peroxisomal membrane that carry a peroxisome targeting signal 2 (PTS2) (Purdue, Skoneczny et al. 1999). This mutation results in severe neurological symptoms such as epilepsy and age-related conditions such as cataracts (Purdue, Skoneczny et al. 1999, Malheiro, da Silva et al. 2015, Landino, Jnah et al. 2017). In conclusion, peroxisomal dysfunction in the central nervous system is shared by both neurodegenerative and peroxisomal disorders, leading to disrupted cellular homeostasis that contributes to the pathogenesis of those diseases (**Figure 1.3** and **Table 1**).



### Peroxisomal dysfunction in the CNS

Age-related CNS diseases and models	Other CNS diseases and models
<ul style="list-style-type: none"> <li>• Lower level of plasmalogens in Alzheimer's patients' brains (Santos et al., 2005; Kou et al., 2011)</li> </ul>	<ul style="list-style-type: none"> <li>• Various peroxisomal genes negatively affected in peroxisomal biogenesis disorders (Braverman et al., 2016)</li> </ul>
<ul style="list-style-type: none"> <li>• D-amino acid oxidase mutation in amyotrophic lateral sclerosis (Kondori et al., 2017; Kondori et al., 2018)</li> </ul>	<ul style="list-style-type: none"> <li>• Catalase gene expression and protein amount impaired in oxaliplatin neurotoxicity models (Zanardelli et al., 2014)</li> </ul>
<ul style="list-style-type: none"> <li>• Increased number of peroxisomes and catalase expression in ischemic stroke models (Young et al., 2015)</li> </ul>	<ul style="list-style-type: none"> <li>• Increased peroxisomal oxidative stress in doxorubicin chemotherapy neuronal models (Moruno-Manchon et al., 2018b)</li> </ul>
<ul style="list-style-type: none"> <li>• Higher amounts of D-amino acid oxidase in post-stroke dementia patients (Chen et al., 2019)</li> </ul>	

**Fig 1.3: Summary of peroxisomal dysfunction in age-related diseases in the CNS, and other diseases that affect the CNS. Created using BioRender.**

**Table 1: Summary of neurological symptoms in neurological and peroxisomal disorders that arise as a result of peroxisomal dysfunction.**

<b>Neurological disorder</b>	<b>Peroxisomal protein/function affected</b>	<b>Neurological result</b>
Alzheimer disease	Plasmalogen production	Lowered plasmalogens in brain, increase in peroxisomal density and VLCAS in gyrus frontalis; peroxisome loss correlated with tau (Santos, Quintanilla et al. 2005, Kou, Kovacs et al. 2011)
Amyotrophic lateral sclerosis (ALS)	D-amino acid oxidase enzyme	DAO inactivity; increase in D-serine (Kondori, Paul et al. 2017, Kondori, Paul et al. 2018)
Oxaliplatin	Catalase expression	Lipid peroxidation;

neuropathy: astrocytic and amount neuropathic  
 culture, human phenotype in animal  
 colorectal cancer line model (Zanardelli,  
 and rat model Micheli et al. 2014)

<b>Peroxisomal disorder</b>	<b>Peroxisomal affected</b>	<b>gene</b>	<b>Neurological result</b>
Adult Refsum disease	<i>PHYH</i>		Phytanic acid buildup, anosmia, polyneuropathy, hearing and vision loss (Wanders, Komen et al. 2011, Wanders and Poll-The 2017, Gettelfinger and Dahl 2018)
Infantile Refsum disease		<i>PEX1, PEX3, PEX6, PEX12, PEX26</i>	Phytanic acid buildup, hypomyelination, hearing and vision loss, polyneuropathy (Warren, Mierau et al. 2018)
Neonatal		<i>PEX1, PEX2, PEX3,</i>	Buildup of VLCFAs,

adrenoleukodystrophy	<i>PEX5, PEX6, PEX10, PEX11<math>\beta</math>, PEX12, PEX13, PEX14, PEX16, PEX19, PEX26</i>	seizures, hearing loss, neuropathy (Aubourg, Scotto et al. 1986)
Rhizomelic chondrodysplasia punctata	<i>PEX7; PEX5</i> (short isoform)	Epilepsy, seizures, cataracts, neuroregression (Purdue, Skoneczny et al. 1999, Malheiro, da Silva et al. 2015, Landino, Jnah et al. 2017)
Zellweger syndrome	<i>PEX1, PEX2, PEX3, PEX5, PEX6, PEX10, PEX11<math>\beta</math>, PEX12, PEX13, PEX14, PEX16, PEX19, PEX26</i>	Limited neuronal migration, issues with myelination and brain development (Waterham and Ebberink 2012, Klouwer, Berendse et al. 2015)

Outside of the central nervous system, peroxisomal dysfunction plays a role in neuropathies. For instance, oxaliplatin, a chemotherapy drug for colorectal, has been known to cause peripheral neuropathies in patients (Grothey 2003, Banach, Zygulska et al. 2018). A study uncovered the role of peroxisomes in this mechanism using primary rat astrocyte cultures, a human colon cancer cell line and *ex vivo* analysis of an oxaliplatin neuropathy rat model: peroxisomal catalase expression and levels were impaired with oxaliplatin treatment of cell cultures, and in the dorsal root ganglia and spinal cords of treated animals; this change was also linked with lipid peroxidation in the spinal cord of treated animals (Zanardelli, Micheli et al. 2014). More recent research has strengthened the role of peroxisome function in neuropathies: the peripheral nerves in peroxisomal mutation mouse models exhibited various abnormalities, such as impaired lysosomal function, accumulation of ganglioside, and a changed redistribution of Kv1 channels and their anchoring proteins that may lead to impaired signaling (Kleinecke, Richert et al. 2017). These studies, in conclusion, highlight the important, but previously hidden role that peroxisomal function plays, not only in the central nervous system, but in peripheral nerves as well.

### **The somewhat known and the unknown: cellular biology of peroxisomes in neural cell types and in the normal aging brain**

As previously mentioned, peroxisomes are negatively affected in disorders that affect the central nervous system, leading to undesirable consequences. Some characterization of basal peroxisomal pathways has been made in oligodendrocytes and astrocytes in the central nervous system (Chistyakov, Aleshin et al. 2014, Di Cesare Mannelli, Zanardelli et al. 2014, Aguirre-Rueda, Guerra-Ojeda et al. 2015, Nury, Sghaier et al. 2018). In the case of neurons,

there has also been focus on peroxisomes (Ballister, Ayloo et al. 2015, Olenick, Tokito et al. 2016). In hippocampal neurons, it was discovered that preventing tuberous sclerosis complex 2 (TSC2) (a regulator of mTORC1 activity) from localizing to peroxisomes led to several axons extending from the neuronal body, indicating a change in morphology (Zhang, Kim et al. 2013). In studies of noise-induced hair loss, neurons in mice deficient in pevjakin (a protein associated with neuronal peroxisomes in the auditory pathway), exhibited less peroxisomal proliferation in response to loud sounds in comparison to their wild-type counterparts; peroxisomal proliferation is protective against oxidative stress produced by loud sounds (Defourny, Aghaie et al. 2019). We recently discovered that in neuronal models of doxorubicin treatment (a chemotherapy drug that leads to chemobrain), peroxisomes exhibited increased oxidative stress, which eventually damaged neurons (Kesler 2014, Wefel, Kesler et al. 2015, Kesler and Blayney 2016, Manchon, Dabaghian et al. 2016, Moruno-Manchon, Uzor et al. 2016, Moruno-Manchon, Uzor et al. 2018). A more positive link has been found between peroxisomes and ischemic stroke; peroxisomal volume in *in vitro* and *in vivo* models of ischemia increased after injury, leading to increased number of peroxisomes, as well as increased expression of peroxisomal catalase (Young, Nelson et al. 2015). Inhibiting catalase or dynamin-related protein 1 (Drp1), a protein needed for peroxisomal fission, led to increased neuronal susceptibility to death from oxygen-glucose deprivation (OGD), a cellular model of ischemic stroke (Young, Nelson et al. 2015). These findings inspired a clinical study, which investigated the link between post-stroke dementia (PSD) and peroxisomal D-amino acid oxidase (DAO), an enzyme that oxidizes D-serine; plasma levels of PSD patients had higher levels of DAO, indicating its role in stroke and stroke-related dementia (Chen, Chou et al. 2019). In conclusion, these neuronal studies show that

peroxisomal dysfunction can contribute to changes in neuronal morphology, increased oxidative stress, and even death in the CNS.

The link between peroxisomal function and inflammation has been established in non-CNS models; however, a few microglial studies have shed light on potential mechanisms in the CNS (Di Cara, Andreoletti et al. 2019). For one, deleting the MFP2 peroxisomal enzyme (which is responsible for  $\beta$ -oxidation) in mouse microglia, switched their state to a pro-inflammatory one, but this change did not affect neuronal health or the microglial response to injury (Beckers, Geric et al. 2019). Another study looked at a neuron-specific form of MFP2 deletion, and discovered that unlike constitutive *Mfp2*<sup>-/-</sup> knockouts, *Nestin-Mfp2*<sup>-/-</sup> knockout brains possessed microglia that were not primed for an inflammatory response (Beckers, Stroobants et al. 2018). Microglial peroxisomal dysfunction, as seen in a microglial model deficient in acyl-coA oxidase 1 (ACOX1), has also been shown to affect catalase activity, the peroxisome, lipid droplet and mitochondrial number in microglia, as well as the induction of interleukin-1 $\beta$  (IL-1 $\beta$ ), the repression of interleukin-6 (IL-6) and the increased expression of *Trem2*, which codes for a cell surface protein that plays a role in microglial phagocytosis (Raas, Saih et al. 2019). Taking these studies together, it can be assumed that microglial peroxisomal dysfunction does not only affect catalase and organelle amount in response to a possibly altered redox balance, but also the inflammatory response of microglia in the brain, directly and indirectly. The results of these neuronal and microglial studies stress the importance of peroxisomal health in diseases of the central nervous system; the increased oxidative stress or inflammation as a result of peroxisomal dysfunction may lead to irreparable cellular loss. Therefore, it is crucial to keep the negative side effects of treatments on various metabolic pathways, including those that involve peroxisomes, in mind.



Nonetheless, one gap in the literature exists regarding peroxisomes in the aging central nervous system that is unaffected by neurodegenerative disease. Non-neuronal senescence studies have shed some light on peroxisomes in aging cells, such as in senescent human fibroblasts, where there is a reported reduction in import of PTS1-tagged proteins, an increase in hydrogen peroxide and peroxisomal number, and changes in peroxisomal appearance (Legakis, Koepke et al. 2002). Proteomic analysis of *C. elegans* also shows a reduction of peroxisomal protein import, as well as a reduction in about 30 peroxisomal proteins, including PRX-5, the nematode homolog of PEX5; PRX-5 was also found to be mislocalized in the aged animals, suggesting that peroxisomal proteins were not properly localized (Narayan, Ly et al. 2016). Knocking it out reduced brood size, implicating a potential role of PRX-5 in both development and aging (Narayan, Ly et al. 2016). Cell type-specific ribosome profiling of *Drosophila melanogaster* oenocytes (cells involved in liver-like processes) revealed that peroxisomal pathways were downregulated with aging (Huang, Chen et al. 2019). Some related evidence exists in post-mortem Alzheimer's studies, where there is an increase in peroxisomal density and very long-chain fatty acids (but a reduction in plasmalogen levels) in neurons in the gyrus frontalis of AD patients, and a loss of peroxisomes in neuronal processes where phosphorylated tau is present (Kou, Kovacs et al. 2011). However, a search of the literature does not currently reveal evidence of peroxisomal perturbations in the normal aging brain. Another gap in the literature is present when investigating how sex, particularly in age-related neurological disease, affects peroxisomes. For instance, there is evidence that a sex difference exists in response to cerebral ischemia, or ischemic stroke, but it is unknown how these sex-associated differences may affect peroxisomes specifically (Siegel and McCullough 2013, Mirza, Ritzel et al. 2015, Ritzel,

Patel et al. 2017). Future studies on age-related neurological changes should investigate how peroxisomal pathways are affected, given the important roles that peroxisomes play in the brain, and how they are affected in other related diseases.

### **Conclusion**

As small and understudied as they are, there is ample evidence that peroxisomes play a supportive, yet critical role in the maintenance of the central nervous system; future studies should investigate the treatment of neurological diseases, while keeping the peroxisomal role in maintaining cellular homeostasis in mind.

## **Chapter 2: Methods and Materials**

### **Plasmids and chemicals**

Hoechst dye was from Santa Cruz Biotechnology (#sc-394039). pGW1-Keima-per (or Keima-per), a variant of the Keima fluorescent protein targeted to peroxisomes, was cloned from pGW1-Keima-SKL, a variant of Keima tagged with a peroxisome-targeting signal (SKL), which was generated from mt-mKeima/pcDNA3 (Katayama, Kogure et al. 2011). pGW1-mHtt-ex1-46Q was derived from pGW1-ex1-46Q-GFP (tagged with green fluorescent protein).

Antibodies against MAP2c (#sc-20172; 1:1000) were from Santa Cruz Biotechnology. Antibodies against thiolase I (ACAA1) were from Sigma-Aldrich (#HPA007244, 1:1000). 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.

### **Neuronal cultures and transfection**

Cortices and striata from wild-type rat embryos (E17) and wild-type and BACHD mice (P0) were dissected, dissociated, and plated on 24-well tissue-culture plates ( $6.5 \times 10^5$ /well) coated with poly-D-lysine (BD Biosciences, #354210) as described previously (Tsvetkov, Miller et al. 2010). Neurons were grown in Neurobasal Medium supplemented with B-27 supplement, GlutaMAX, and penicillin-streptomycin (each from Thermo Fisher Scientific, #A3582801, #35050079 and #15140122, respectively). Primary cultures were transfected

after 3-4 days with Lipofectamine 2000 (Invitrogen, #12566014) with a total of 1  $\mu\text{g}$  of plasmid DNA per well as described previously (Moruno Manchon, Uzor et al. , Tsvetkov, Miller et al. 2010, Tsvetkov, Ando et al. 2013, Tsvetkov, Arrasate et al. 2013). Transfection efficiency was around 5% on average.

### **Immunocytochemistry and fluorescence microscopy of living neurons**

Neuronal cultures were fixed in warm 4% paraformaldehyde for 15 minutes, permeabilized in phosphate-buffered saline containing 0.1% Triton X-100, and incubated in 1% bovine serum albumin overnight at 4°C. Neurons were washed and incubated with primary antibodies against catalase (1:1000) or thiolase (1:1000) and MAP2c (1:1000) overnight at 4°C, washed with phosphate-buffered saline, and incubated with secondary antibodies for 1 hour at room temperature.

Typical imaging methods rely on snapshots of fixed cells or lysates. These techniques may be misrepresentative, especially when analyzing complex processes such as autophagy. We currently use a live-cell imaging system combined with longitudinal single-cell analysis to overcome this limitation (Tsvetkov, Miller et al. 2010, Tsvetkov, Arrasate et al. 2013, Manchon, Dabaghian et al. 2016, Moruno-Manchon, Uzor et al. 2016); individual neurons transfected with various reporters can be imaged over set intervals (Fig. 2A) to analyze autophagy (Moruno Manchon, Uzor et al. 2015, Moruno Manchon, Uzor et al. 2016, Moruno-Manchon, Uzor et al. 2016). Live neurons were imaged with the EVOS FL Auto Imaging System (Thermo Fisher Scientific, #AMAFD1000). The microscope automatically positions the 20x objective to the center of the first well of the 24-well tissue plate and collects fluorescence images under different filters. Fixed cells were imaged with a Zeiss LSM 510 confocal microscope.

To visualize pexophagy in cells expressing mHtt, we co-transfected neurons with pGW1-Ex1-46Q-untagged and Keima-per, then imaged them using the EVOS microscopy system with the Qdot 625 and Texas Red filters once every 24 hours for 2 days.

### **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  $\mu$ m, 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 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, ACAA1 1:500) 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); the forward sequence was ATGACCTCGGAGAATGTGGCTG and the reverse sequence was AGGACAGTGGTTGTACAGGCA. 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  $\mu$ L in a 96-well-plate, in duplicate. The total volume of each well was 20  $\mu$ L, made up of 10  $\mu$ L SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, #1725274),

0.5 uL forward primer, 0.5 uL reverse primer (or 1  $\mu$ L primer mix), and 7  $\mu$ L 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  $\Delta\Delta$ Ct 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). After incubation with *Pex5* probes, slices 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, and divided by the number of DAPI-positive nuclei in the ROI, also calculated in ImageJ.

### **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 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). We thank Dr. Liang Zhu, Ph.D. for her biostatistics expertise.



**Animals and ethics**

Rats and mice were maintained in accordance with the guidelines and regulations of The University of Texas Health Science Center at Houston McGovern Medical School (protocol number #AWC-16-0081). 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). Pregnant female rats at E19 were purchased from Charles River Laboratories. Wild-type C57BL/6 mice and BACHD 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), 26-27 months, and 32+ months (aged).

## **Chapter 3: Markers of peroxisomes and pexophagy are affected in two models of Huntington disease**

### **Introduction**

Peroxisomal biogenesis disorders, as previously described in Chapter 1, often damage the brain, suggesting that peroxisomes are fundamental to the central nervous system (CNS) (Wanders, Schutgens et al. 1995). Neuronal peroxisomes are essential to brain health: they are found throughout in the brain, being most active during myelination, and produce myelin sheath and axonal lipids (Baes and Aubourg 2009). They also synthesize intermediates for neuronal membrane phospholipids and metabolize both beneficial and toxic compounds such as D-serine and phytanic acid (Wanders, Komen et al. 2011, Schonfeld and Reiser 2016).

Neurodegenerative diseases are driven, in part, by redox imbalance, in which peroxisomes may play a critical role. Oxidative stress and aging occurs when peroxisomes fail to remove ROS (Terlecky, Koepke et al. 2006, Lizard, Rouaud et al. 2012). As peroxisomes age, under-oxidized metabolites accumulate, inducing organelle dyshomeostasis and cellular stress (Baes and Aubourg 2009, Manivannan, Scheckhuber et al. 2012). In the neurons of Alzheimer disease patients, peroxisomes abnormally accumulate (Lizard, Rouaud et al. 2012). In both neuronal and non-neuronal cellular models, peroxisomes proliferate as cells age (Baes and Aubourg 2009, Manivannan, Scheckhuber et al. 2012). In short, if neuronal peroxisomes build up or become dysfunctional, the brain suffers negative consequences.

As previously discussed, dysfunctional peroxisomes are degraded by pexophagy, the selective autophagy of peroxisomes. The main form of pexophagy in mammalian cells is macropexophagy (referred to as pexophagy thereafter): peroxisomes are enclosed by

autophagosomes that fuse with lysosomes, degrading the autophagosomal content (Katarzyna and Suresh 2016). Since neurons do not divide, dysfunctional cellular components need to be removed to maintain homeostasis. Yet, the importance of neuronal pexophagy in maintaining neuronal homeostasis is unknown. This is interesting, as peroxisomal function and overall redox balance are necessary for the CNS to function. Currently, conventional assays study pexophagy using fixed cells, limiting the analysis of a dynamic process. In this work, we examined neuronal pexophagy using fluorescent reporters and time-lapse imaging, which allowed us to see changes in pexophagy with time.

It was concluded in Chapter 1 that the treatment of neurological diseases should keep their effects on peroxisomes in mind, as they are critical to the health of neurons and the CNS at large, and negatively affected in neurological and neurodegenerative disorders. In the spirit of that conclusion, we investigated if pexophagy affected in a mouse model of Huntington disease, a genetically inherited neurodegenerative disease (Finkbeiner 2011).

Huntington disease (HD) is a rare, incurable neurodegenerative disease that is autosomal dominant, with the highest prevalence among people of European descent (Kay, Hayden et al. 2017). The disease is due to CAG repeats in the huntingtin gene, which get translated into a polyglutamine repeat in the huntingtin protein; greater than 35 CAG repeats are known to eventually lead to HD pathogenesis (Ross, Wood et al. 1999, Finkbeiner 2011, Wyant, Ridder et al. 2017). The most common age of patients with symptoms is around the mid-30s, and patients experience symptoms such as chorea (involuntary movement), impaired motor function, weight loss, as well as behavioral and cognitive symptoms (Aziz, van der Burg et al. 2008, Sturrock and Leavitt 2010). The neurons mostly affected in the HD brain are medium spiny neurons, which are found in the striatum. However, other regions such as the

cortex and even the hippocampus can be affected as well (Spargo, Everall et al. 1993, Nana, Kim et al. 2014). This work focused on the cerebral cortex as a site of HD neurodegeneration. In postmortem brains, for instance, all lobes are affected by lower cortical volumes in HD, and this cortical loss is correlated with loss in the striatum (Halliday, McRitchie et al. 1998). Neuroimaging studies have also confirmed that the cerebral cortex is negatively affected in HD pathogenesis (Mangin, Rivière et al. 2020). This work's focus on the effects of HD neurodegeneration on peroxisomal clearance in the cerebral cortex may also have future relevance to other forms of neurodegenerative disease, and normal brain aging, where cortical volume is also lost, such as in Alzheimer disease, frontotemporal dementia, natural brain aging and mild cognitive impairment (Desikan, Thompson et al. 2014{Du, 2007 #966})(Kilimann, Hausner et al. 2016, Armstrong, An et al. 2019). In addition, the effect of normal brain aging on peroxisomal proteins in a mouse model will be discussed in Chapter 4. Also, certain forms of neuronal autophagy, such as bulk autophagy and mitophagy (mitochondrial autophagy) are impaired in HD pathogenesis, it is unclear if this impairment extends to pexophagy, or peroxisomal autophagy (the general mechanism of pexophagy has been previously discussed in the Chapter 1 of this work).

In this work, we tested a few hypotheses; first, that pexophagy is affected by mutant huntingtin (mHtt). We subjected embryonic rat cortical neurons to the overexpression of mHtt, the protein implicated for Huntington disease (HD). By transfecting neuronal cell models of HD with a unique pexophagy marker, and imaging the cells longitudinally, we showed that pexophagy is increased in primary neuronal models of HD. Next, we tested the hypothesis that markers of peroxisomes, as well as pexophagy, will be affected by the overexpression of mutant huntingtin, using immunohistochemistry analysis of cortical

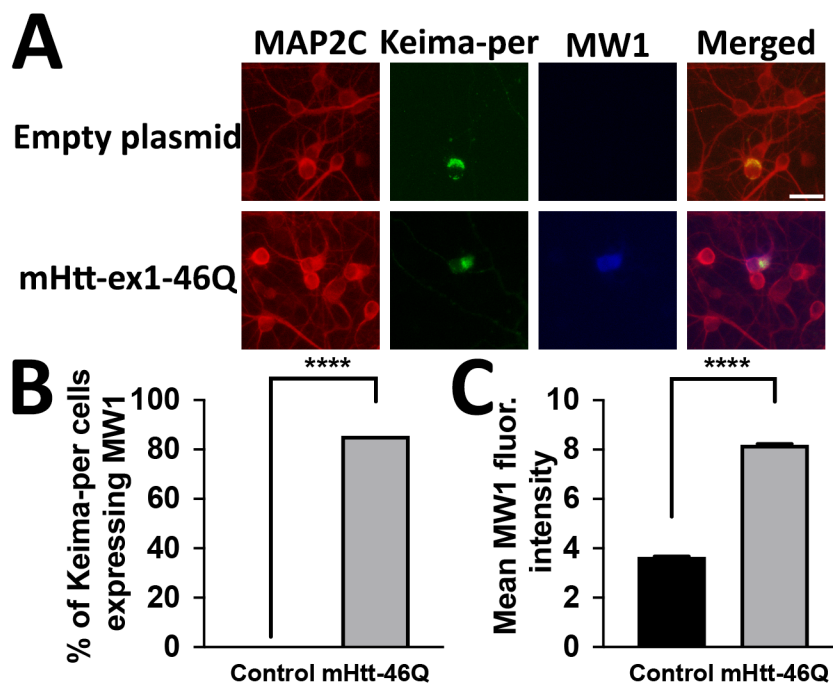
sections of the mouse brain in young wild-type mice and a BACHD mouse model of Huntington disease (Gray, Shirasaki et al. 2008), staining for PEX5, a peroxisomal protein involved in pexophagy, and ACAA1, a peroxisomal marker (Berger, Dorninger et al. 2015, Morvay, Baes et al. 2017, Wang and Subramani 2017, Eun, Lee et al. 2018, Islinger, Voelkl et al. 2018). Our results showed that PEX5 and ACAA1 were both reduced in cortical neurons of the BACHD mouse model. Our findings suggest that peroxisomes and pexophagy are indeed affected in the overexpression of mHtt in the cortex, and the regulation of pexophagy could possibly be a potential therapeutic target in Huntington-related neurodegeneration.

## **Results**

### **Neurons transfected with mHtt-exon1-46Q are positive for MW1, an antibody marker of the mutant huntingtin protein**

To create a neuronal model of Huntington disease, we transfected embryonic rat neurons (P18) with a construct coding for mHtt-exon1-46Q; exon1-46Q stands for the mutant huntingtin gene exon that possesses

46 repeats of the polyQ sequence in the mutant huntingtin protein (mHtt). As a control, a separate cohort of neurons was transfected at the same time with an empty construct (pCAG). After two days, the neurons underwent immunocytochemistry and were probed with anti-MAP2, a neuronal marker, and anti-MW1, an antibody that recognizes the extended polyQ tract in the mutant huntingtin protein (**Figure 3.1A**) (Landles, Sathasivam et al. 2010).



**Figure 3.1: Neurons transfected with mHtt-46Q express MW1, a marker of the huntingtin protein**

3.1A: Two cohorts of neurons were transfected with either Keima-per and an empty plasmid (pCAG), or Keima-per and a construct for the mutant huntingtin exon 1 with 46 polyglutamine repeats (mHtt-ex1-46Q), and were stained for MAP2C (red) and MW1 (blue) two days later.

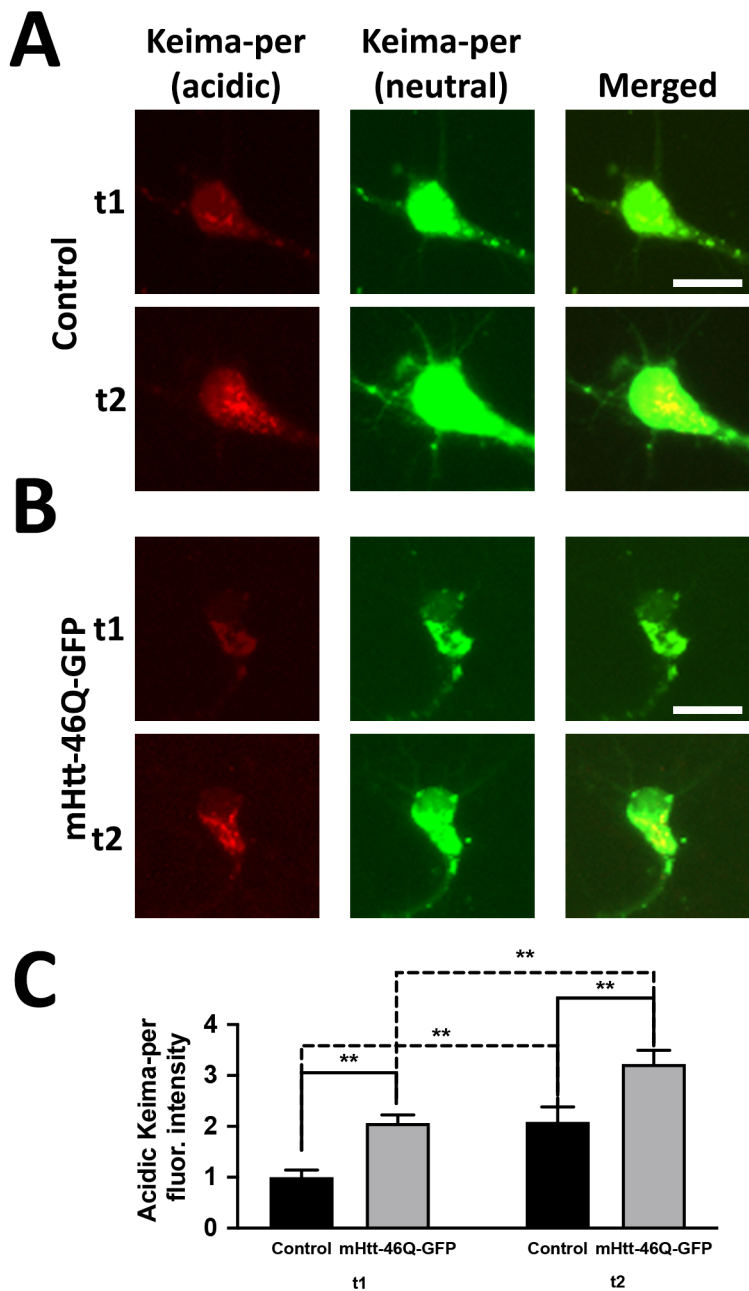
3.1B: Mean percentage of neurons positive for Keima-per and MAP2C that expressed MW1 fluorescence in each group. 100 cells (in random regions) per group were analyzed.

3.1C: Mean MW1 fluorescence intensity per group. 100 cells per group were analyzed. Welch's t-test was used to analyze both groups. Scale bar = 10  $\mu$ M. \*\*\*\*p < 0.0001.

As expected, around 80% of the neurons transfected with mHtt-ex1-46Q and Keima-per expressed positive anti-MW1 signal, while none of the neurons transfected with the empty construct and Keima-per had any signal (**Figure 3.1B**). The mean fluorescence intensity of MW1 was also calculated between groups, and the mHtt-expressing group had higher levels of blue fluorescence intensity compared to the control group, which stayed at baseline (**Figure 3.1C**). Our results suggest that our neuronal culture model of Huntington disease expresses mHtt-46Q as they should, and can be used for our next set of experiments.

### **Neurons expressing mHtt-exon1-46Q exhibit increased peroxisome clearance**

Next, we transfected rat neurons with either an empty plasmid or mHtt-exon1-46Q, along with a pH-sensor construct known as Keima, tagged with a PTS-1 signal; the resulting construct was named Keima-per. In neutral environments such as the cytoplasm, Keima emits fluorescence that can be detected under the Qdot625 channel on a fluorescence microscope; in acidic environments, Keima emits red fluorescence that can be detected under the TexasRed channel; Keima has been successfully used in autophagy assays (Katayama, Kogure et al. 2011, Proikas-Cezanne and Codogno 2011, Sliter, Martinez et al. 2018). Therefore, Keima-per can be used to track peroxisomes that have moved from the neutral cytoplasm into the acidic lysosome compartments, where they are degraded via pexophagy. After transfecting our neurons with a control or mHtt-46Q, as well as Keima-per, we measured the acidic red (TexasRed) fluorescence of Keima-per in both groups, once each day over a two-day period (**Figure 3.2A,B**).



**Figure 3.2: Neurons expressing mHtt-46Q exhibit increased peroxisome clearance**

3.2A,B: Representative images of cortical neurons transfected with Keima-per and GFP, or Keima-per and mHtt-46Q-GFP. 2.2C: Mean fluorescence intensity of red (acidic) Keima-per in both control and mHtt-46Q-GFP groups, over a 2-day period. 100 cells per group were

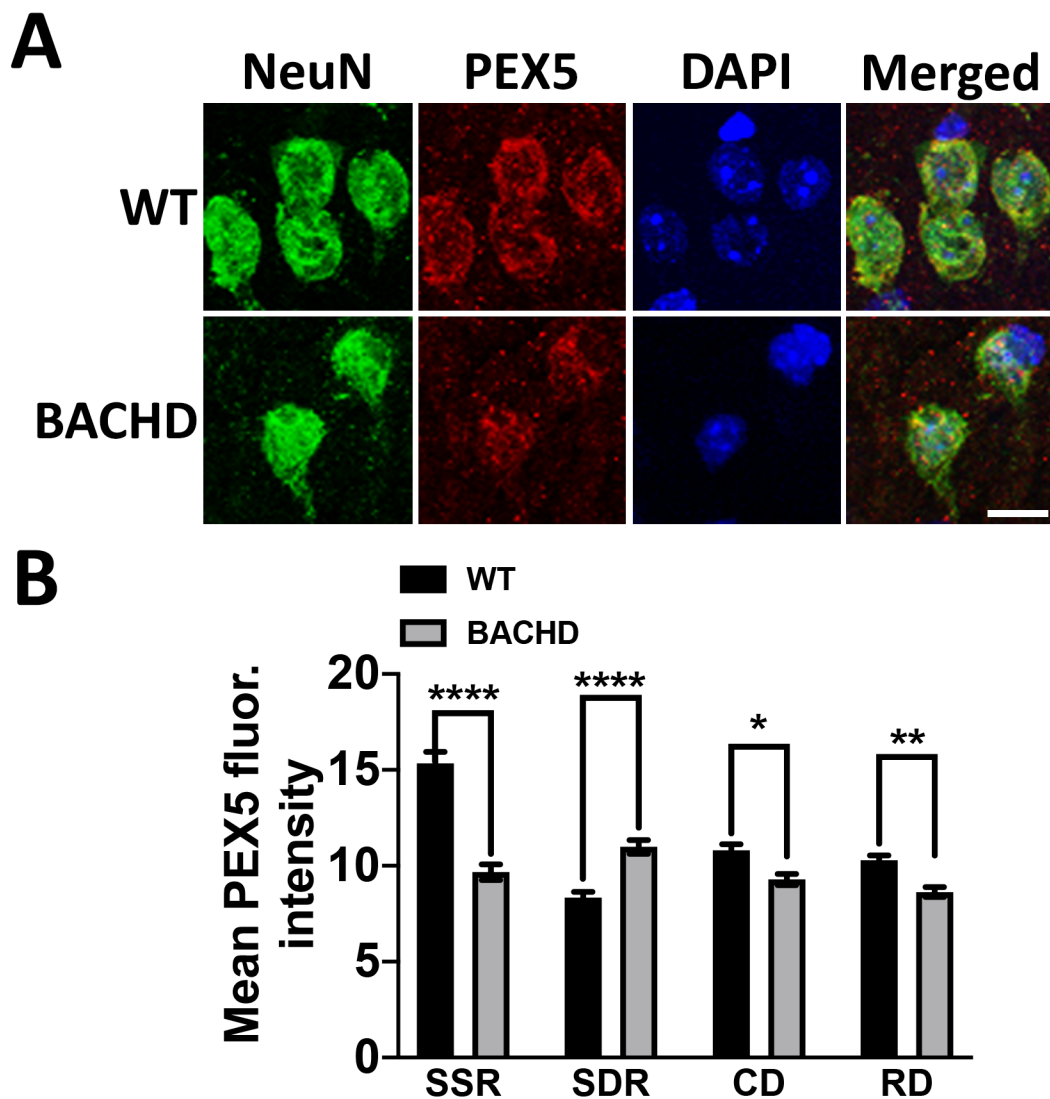


analyzed. Scale bar = 10  $\mu$ M. Two-way ANOVA was used to calculate statistical significance, adjusting for multiple comparisons with Sidak's multiple comparison test.

We discovered that at both time-points, the red fluorescence of Keima-per was significantly higher in the mHtt-46Q-expressing group than in the control group, indicating that more peroxisomes were in the acidic lysosomes (**Figure 2.2B**). Our results suggest that in our models of mutant huntingtin-expressing cells, more peroxisomes are being degraded, compared to neurons that do not express mHtt.

### **PEX5, a peroxisomal protein, is mostly reduced in premanifest BACHD cortical neurons**

To determine if a similar trend was present in an animal model, we analyzed the levels of PEX5, a peroxisomal protein involved in pexophagy, in 12-week-old wild-type and BACHD male mice. BACHD mice do not exhibit symptoms modeling Huntington disease until about a year of age; however, we wondered whether there were changes related to neuronal pexophagy before the onset of symptoms (Gray, Shirasaki et al. 2008, Abada, Schreiber et al. 2013). We stained neurons in the cortical areas of the septo-striatal (SSR), septo-diencephalic (SDR), caudal diencephalic (CD) and rostral diencephalic (RD) regions of wild-type and BACHD mouse brains with antibodies against NeuN (a neuronal marker), and PEX5; their corresponding locations were Bregma 1.32 mm, Bregma 0.38 mm, Bregma -1.28 mm and Bregma -2.12 mm, respectively (**Figure 2.3A**).



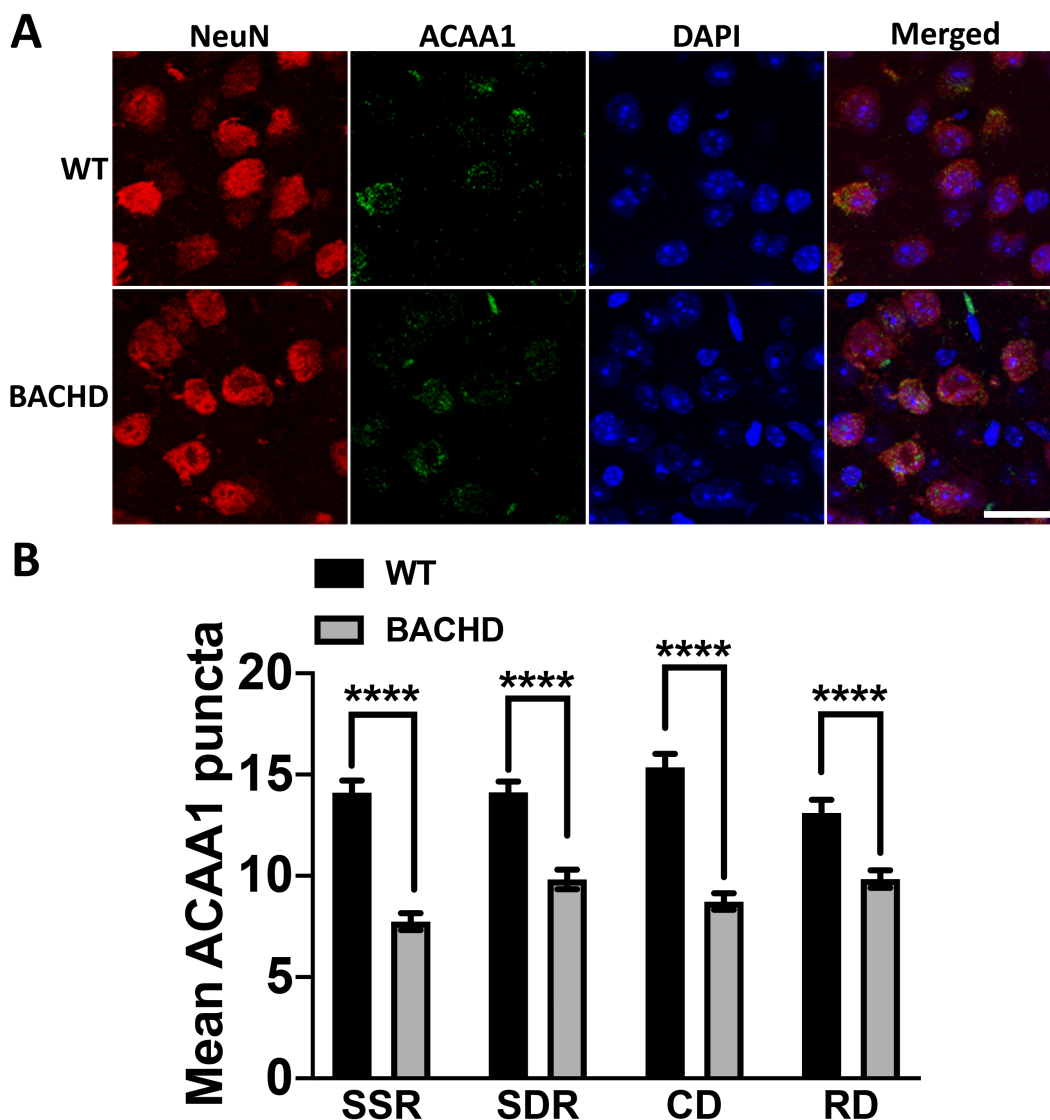
**Figure 3.3: PEX5, a peroxisomal protein, is mostly reduced in premanifest BACHD cortical neurons**

3.3A,B: Representative images of wildtype and BACHD 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  $\pm$  SEM, n = 4; 400 cells per section were analyzed for each group. \*P<0.05, \*\*P<0.01, \*\*\*\*P<0.0001. Scale bar = 15  $\mu$ m. Two-way ANOVA was run for each group, adjusting for multiple comparisons with Sidak's multiple comparisons test.

We discovered that in all regions, except the septo-diencephalic region, cortical neurons of BACHD mice had reduced PEX5 fluorescence in comparison to their wild-type counterparts (**Figure 3.3B**). Our results suggest that in cortical neurons of premanifest BACHD mice, PEX5 levels are lower than in wild-type mice, possibly indicating an aberration in pexophagy dynamics before symptoms begin.

### **Peroxisome number is reduced in premanifest BACHD cortical neurons**

To confirm if peroxisomes are indeed lower in cortical neuronal models of HD, we stained cortical slices of 12-week-old wild-type and BACHD mice for NeuN (a neuronal marker) and ACAA1, a peroxisomal matrix protein and a marker of peroxisomes (**Figure 3.4A**).



**Figure 3.4: Peroxisome number is reduced in premanifest BACHD cortical neurons**

3.4A,B: Representative images of wildtype and BACHD 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  $\pm$  SEM,  $n = 4$ ; 400 cells per section were analyzed for each group. \*\*\*\* $P < 0.0001$ . Scale bar = 15  $\mu\text{m}$ . Two-way ANOVA was run for each group, adjusting for multiple comparisons with Sidak's multiple comparisons test.

Rather than measuring fluorescence intensity, here, we counted the puncta of ACAA1 staining, and we discovered that BACHD cortical neurons had significantly lower numbers of ACAA1 puncta in comparison to wild-type cortical neurons (**Figure 2.4B**). Our results suggest that in premanifest BACHD cortical neurons, the number of ACAA1 puncta is lower than in control, indicating fewer peroxisomes in cortical BACHD neurons before HD symptoms begin.

## **Discussion**

In this study, we used neuronal culture and immunohistochemistry assays to determine if the overexpression of the mHtt protein has an effect on pexophagy, or the autophagic clearance of peroxisomes. We first confirmed that our cellular HD model used in our system for tracking pexophagy was a valid model of HD; the neurons transfected with mHtt-exon-1 did indeed express mHtt, as seen by the MW1-positive staining of neurons transfected with Keima-per and mHtt-46Q (**Figure 3.1**). Next, by transfecting neurons with Keima-per and mHtt-46Q-GFP, and tracking the cell cultures over two days, we discovered that the acidic Keima-per fluorescence was much higher at the end of the two-day period in our mHtt-expressing group than in the control group (**Figure 3.2**). Our IHC analyses also showed that in 12-week-old premanifest BACHD mouse cortices, PEX5 levels and peroxisomal numbers were reduced in comparison to wild-type mouse cortices, suggesting an aberration in peroxisomal pathways in our HD model long before symptoms begin (**Figures 3.3 and 3.4**).

PEX5 is a peroxisomal protein that is implicated in modulating pexophagy and peroxisomal import; see Chapter 1 (Zhang, Tripathi et al. 2015, Nazarko 2017, Walton, Brees et al. 2017). ACAA1, or peroxisomal thiolase I, is a peroxisomal matrix enzyme in the

peroxisomal beta-oxidation pathway; it has also been used as a peroxisomal marker in some studies, as well as in this work (Ezaki, Komatsu et al. 2009, Nordgren and Fransen 2014, Islinger, Voelkl et al. 2018).

Some studies have reported autophagy upregulation in stress conditions, and as a result, we hypothesized that pexophagy may also increase in neurons under stress conditions (Eun, Lee et al. 2018). Furthermore, the mechanism of stress-induced pexophagy in neurons has not been elucidated. Our current study shows that under the stress of expressing mHtt in rat neuronal culture, cortical neurons exhibit increased peroxisomal clearance, or pexophagy. PEX5 levels are decreased in our premanifest BACHD mouse data; however, this change could be due to one of two different mechanisms. The first is that the peroxisomes in mHtt models may not be degraded by PEX5-modulated pexophagy, but by bulk autophagy or another form of pexophagy; organelles can be degraded by bulk autophagy, and PEX2 is also known to induce pexophagy (Feng, He et al. 2014, Sargent, van Zutphen et al. 2016). As a result of pexophagy not modulated by PEX5, neuronal PEX5 levels would be lower than if PEX5-induced pexophagy were occurring; PEX5 levels are known to increase in situations of high oxidative stress or starvation (Walton, Brees et al. 2017, Eun, Lee et al. 2018). This bulk autophagy mechanism could be a way for cortical neurons to conserve energy and resources, and also underscores the secondary role that peroxisomes play to mitochondria, which possess many of the same antioxidant enzymes that peroxisomes do. By reducing peroxisomal activity, overall metabolism and energy use is also reduced. At the same time, with the major role that peroxisomes play in synthesizing plasmalogens and other cellular lipids, if peroxisomes are being degraded at a faster rate than normal, neuronal lipid homeostasis may be inhibited (Baes and Aubourg 2009, Berger, Dorninger et al. 2015).

Experiments exploring this potential mechanism will be discussed in Chapter 5, the future directions chapter.

A second mechanism that explains these lower PEX5 levels is that PEX5 may be aberrantly degraded by polyubiquitination, which marks PEX5 for degradation in the proteasome (Katarzyna and Suresh 2016, Sargent, van Zutphen et al. 2016, Uzor, McCullough et al. 2020). Cortical neurons may be degrading PEX5 to recycle resources, since bulk autophagy can target the same peroxisomes that PEX5 can. One would expect that lower PEX5 levels (but a higher bulk autophagy rate of peroxisomes) would eventually lead to inefficient peroxisomal lipid metabolism and antioxidant activity, as PEX5 also plays an important role in transporting peroxisomal proteins into peroxisomal membranes and matrices (Baker, Hogg et al. 2016).

A limitation of this study is that we did not investigate peroxisomal and PEX5 levels in cortical neurons of symptomatic BACHD mice. This is an immediate future direction, and is discussed in length in Chapter 5. At the same time, this unpublished data is the first to suggest that peroxisomal alterations occur in a model of HD as the disease progresses, as shown by our cellular culture model. Due to the limitations of modeling HD *in vitro* before symptoms manifest, we analyzed brain slices from the BACHD mouse model to answer whether a peroxisomal protein involved in pexophagy (PEX5), and the number of peroxisomes are indeed affected. We found a reduction in PEX5 and the number of peroxisomes (by ACAA1 puncta count) in premanifest BACHD cortical slices. Taking all our evidence together, we conclude that, in HD, the neuronal pexophagy process increases aberrantly, leading to fewer peroxisomes and lower PEX5 levels in neurons.



After observing that PEX5 levels were reduced in premanifest BACHD mouse cortical neurons, we wondered if PEX5 levels were negatively affected in the aging mouse brain. After all, HD is an age-related disease, and in normal brain aging, changes to the cortex also occur (Gray, Shirasaki et al. 2008, Nana, Kim et al. 2014, Vorisek, Syka et al. 2017). While some studies indicated a link between peroxisomes and Alzheimer's disease, it was also unclear if PEX5 levels were affected at all in the normal, aging brain (Kou, Kovacs et al. 2011, Dorninger, Forss-Petter et al. 2017, Islinger, Voelkl et al. 2018). That question brought us to the second part of this dissertation: investigating PEX5 (and other peroxisomal protein) levels in the aged brains of male and female mice.

## **Chapter 4: Aging lowers PEX5 levels in cortical neurons in male and female mouse brains**

This chapter is based upon “Aging lowers PEX5 levels in cortical neurons in male and female mouse brains,” a first-author publication accepted by *Molecular Cellular Neuroscience*, August 2020.

### **Introduction**

Peroxisomes are organelles present in most cells, excluding mature red blood cells (Gronowicz, Swift et al. 1984, Amaral, Castillo 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, Grunau et al. 2009). In the central nervous system, peroxisomes generate myelin sheath lipids, an important part of axonal communication and conduction (Baes and Aubourg 2009, Trompier, Vejux et al. 2014). 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, Van Veldhoven et al. 2009). Peroxisomes are degraded via a selective recycling process known as pexophagy, the autophagy of peroxisomes (Iwata, Ezaki et al. 2006, Nordgren, Wang et al. 2013, Abdrakhmanov, Gogvadze et al. 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 et al. 2020).

Peroxisomes accumulate in fibroblast models of senescence, indicating a possible defect in clearance with age (Legakis, Koepke 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, Ly 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, Yang et al. 2012). Interestingly, PEX5 plays a major role in modulating peroxisomal biogenesis by importing peroxisomal matrix proteins into the peroxisomal membrane (Stanley, Filipp 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, Dalen et al. 2005), it is unknown how peroxisomal proteins in general are affected as the brain ages, particularly in neurons (Uzor, McCullough et al. 2020).

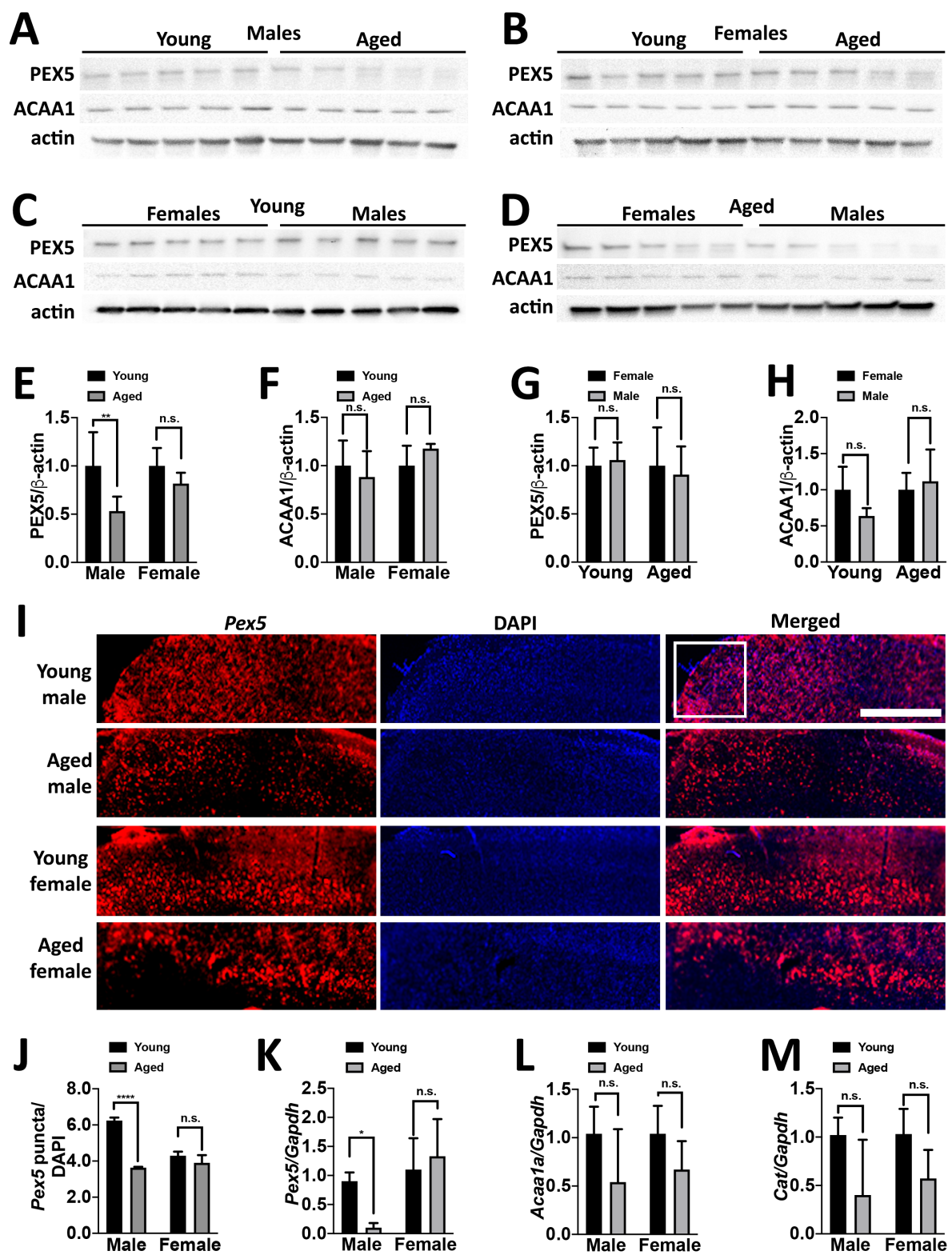
Here, we hypothesized that aging lowers the amount of PEX5 protein and *Pex5* expression in the mouse brain. To test this, we ran a Western blot probing for PEX5 protein levels using young (3-month-old) and aged (32-months+) mouse brains of both sexes. Next, to determine if *Pex5* expression was affected 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.

## **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. 4.1A**) and females (**Fig. 4.1B**).



**Figure 4.1: PEX5 levels differ in young and aged male mouse brains**

4.1A, B: PEX5 and ACAA1 immunoblots of young and aged male (A) and female brain lysates (B) are shown. 4.1 C, D: PEX5 and ACAA1 immunoblots comparing aged male and female (C) and young male and female brain lysates (D) are shown. 4.1 E: The quantification of the PEX5 Western blots in 1A and 1B. Bars are mean  $\pm$  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. 4.1 F: The quantification of the ACAA1 Western blots in 1A and 1B. Bars are mean  $\pm$  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. 4.1 G The quantification of the PEX5 Western blots in 1C and 1D. Bars are mean  $\pm$  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. 4.1 H: The quantification of the ACAA1 Western blots in 1C and 1D. Bars are mean  $\pm$  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. 4.1 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. 4.1 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. 4.1 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. 4.1 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.

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. 4.1C, 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. 4.1E**). On the other hand, there was no significant difference in ACAA1 levels between young and aged male and female samples (**Fig. 4.1F**). 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. 4.1G**). There was also no significant difference between ACAA1 levels in young and aged male and female samples (**Fig. 4.1H**).

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, Ly 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. 4.1I**). 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. 4.1J**). 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. 4.1K**). 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-

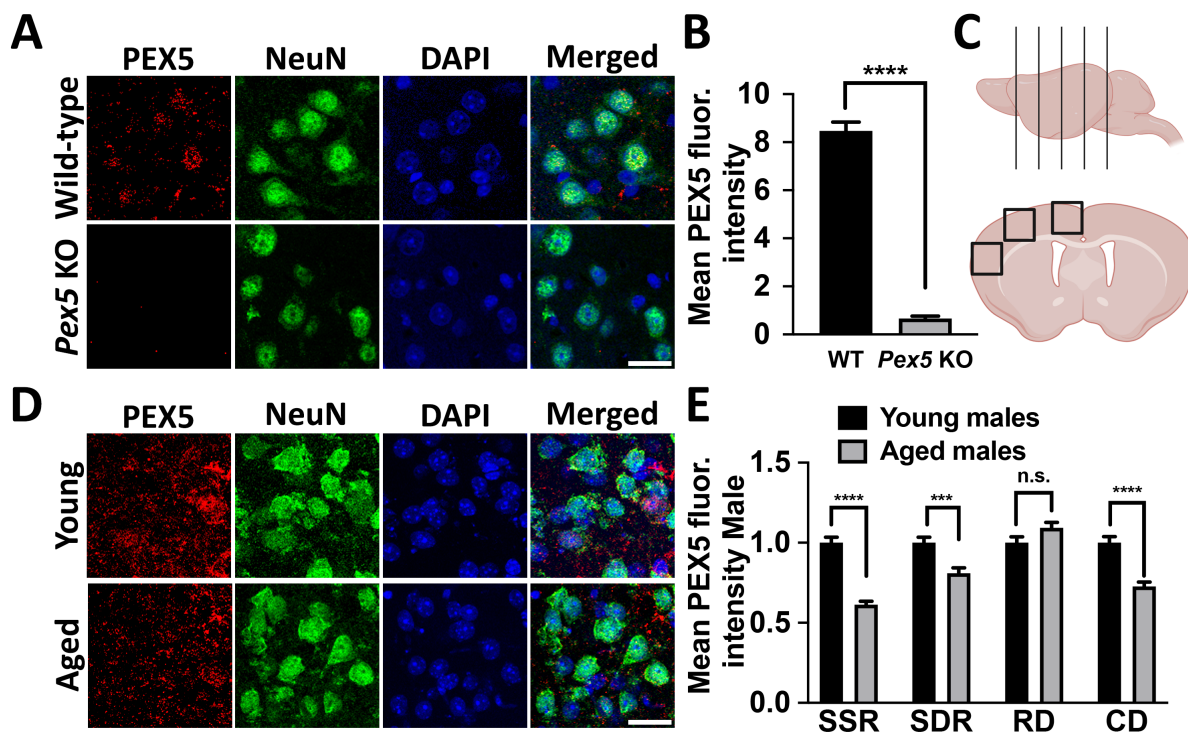


qPCR analysis of genes for two peroxisomal proteins transported by PEX5, *Acaala* (ACAA1) and *Cat* (catalase) showed no significant change in expression levels with age in both male and female cortices (**Fig 4.1L, M**).

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

While the brain samples we examined via Western blot 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, McEvoy 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<sup>-/-</sup>* (*Pex5* KO) knockout mouse (Bottelbergs, Verheijden et al. 2010). Only the wild-type brain exhibited positive PEX5 fluorescence, indicating that our antibody was specific (**Figure 4.2A, B**).



**Figure 4.2: PEX5 levels are lower in cortical neurons of aged male mouse brains, in comparison to young male brains**

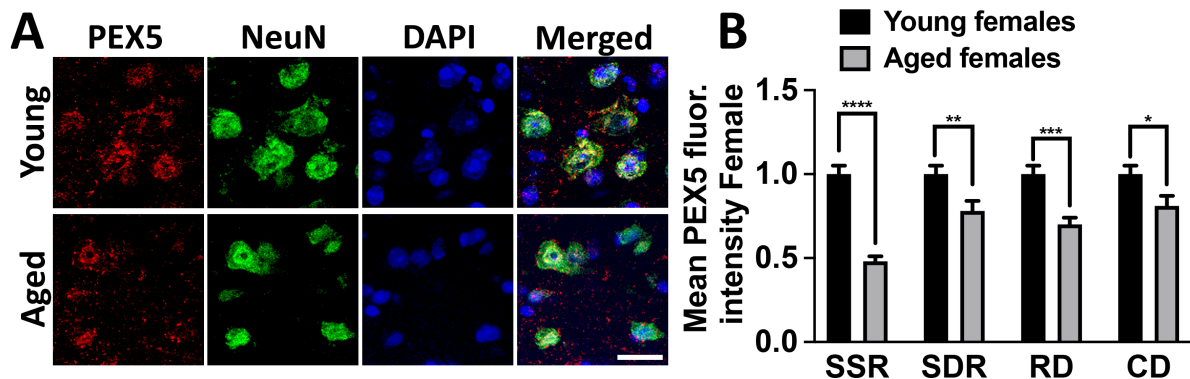
4.2A,B: Representative images of wild-type and *Pex5* KO mouse 12 week-old sagittal brain sections stained for PEX5 (red), neuronal marker NeuN (green), and DAPI (blue), and the quantification of PEX5 fluorescence intensity. Bars are mean  $\pm$  SEM,  $n=150$ . \*\*\*\* $P<0.0001$ . Scale bar=15  $\mu$ m. Unpaired t-test was run to determine statistical significance. 4.2C: Representative scheme of brain sections (top) analyzed for PEX5 immunohistochemistry in young and aged groups; sections of cortex (bottom) analyzed in PEX5 immunohistochemistry experiments. 4.2D,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  $\pm$  SEM,  $n = 4$ ; about 400 cells per section were analyzed. \*\*\*\* $P<0.0001$ , n.s. = not significant. Scale bar = 15  $\mu$ m. Two-way ANOVA was

run for each region, adjusting for multiple comparisons with Sidak's multiple comparisons test.

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 retrosplenial, somatosensory, and motor cortices (**Figure 4.2C**). 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 (**Figure 4.2D, 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 (**Figure 4.3A, B**).



**Figure 4.3: PEX5 levels decrease in aged female neurons in the mouse cortex compared to young female mice**

4.3A,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  $\pm$  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  $\mu\text{m}$ . Two-way ANOVA was run for each region, adjusting for multiple comparisons with Sidak's multiple comparisons test.

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 (Carvalho, Pinto et al. 2007, Wang and Subramani 2017, Wang, Xia et al. 2017, El Magraoui, Brinkmeier et al. 2019). It also plays a major role in peroxisomal degradation via pexophagy, a peroxisome-specific form of autophagic clearance (Deosaran, Larsen et al. 2013, Subramani 2015, Sargent, van Zutphen et al. 2016, Tripathi, Zhang et al. 2016, Nazarko 2017, Pascual-Ahuir, Manzanares-Estreder et al. 2017, Sedlackova, Kelly et al. 2020, Uzor, McCullough et al. 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, Grou et al. 2014, Walton, Brees 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, Ly 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, Dammer 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, Arias et al. 2012, Nordgren, Francisco et al. 2015, Zhang, Tripathi et al. 2015, Yu, Feng et al. 2017, Moruno-Manchon, Lejault et al. 2020).

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, Hickey et al. 2009, Campesi, Straface et al. 2013, Chen, Hu et al. 2013, Koenig, Sateriale et al. 2014, Olivan, Calvo et al. 2014, Weis, Toniazzo et al. 2014, Camuzard, Santucci-Darmanin et al. 2016, Demarest, Waite 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, Cheng 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, Xia 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, Toyama 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, Payne 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, Koepke 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 (Homma, Tsunoda et al. 1994, Wang, Van Veldhoven et al. 2013, Mecocci, Boccardi 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, Kovacs 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 (Carvalho, Pinto et al. 2007, Zhang, Tripathi et al. 2015, Sargent, van Zutphen et al. 2016, Wang and Subramani 2017, Costello, Zalckvar et al. 2019, El Magraoui, Brinkmeier 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 (Yamamoto, Völkl et al. 1988, Osumi, Tsukamoto et al. 1991, van Roermund, Ijlst et al. 2014, Sargent, van Zutphen et al. 2016, Barros-Barbosa, Ferreira et al. 2019). 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 (Marcassa, Kallinos et al. 2019, Riccio, Demers et al. 2019, Jo, Park et al. 2020). 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.

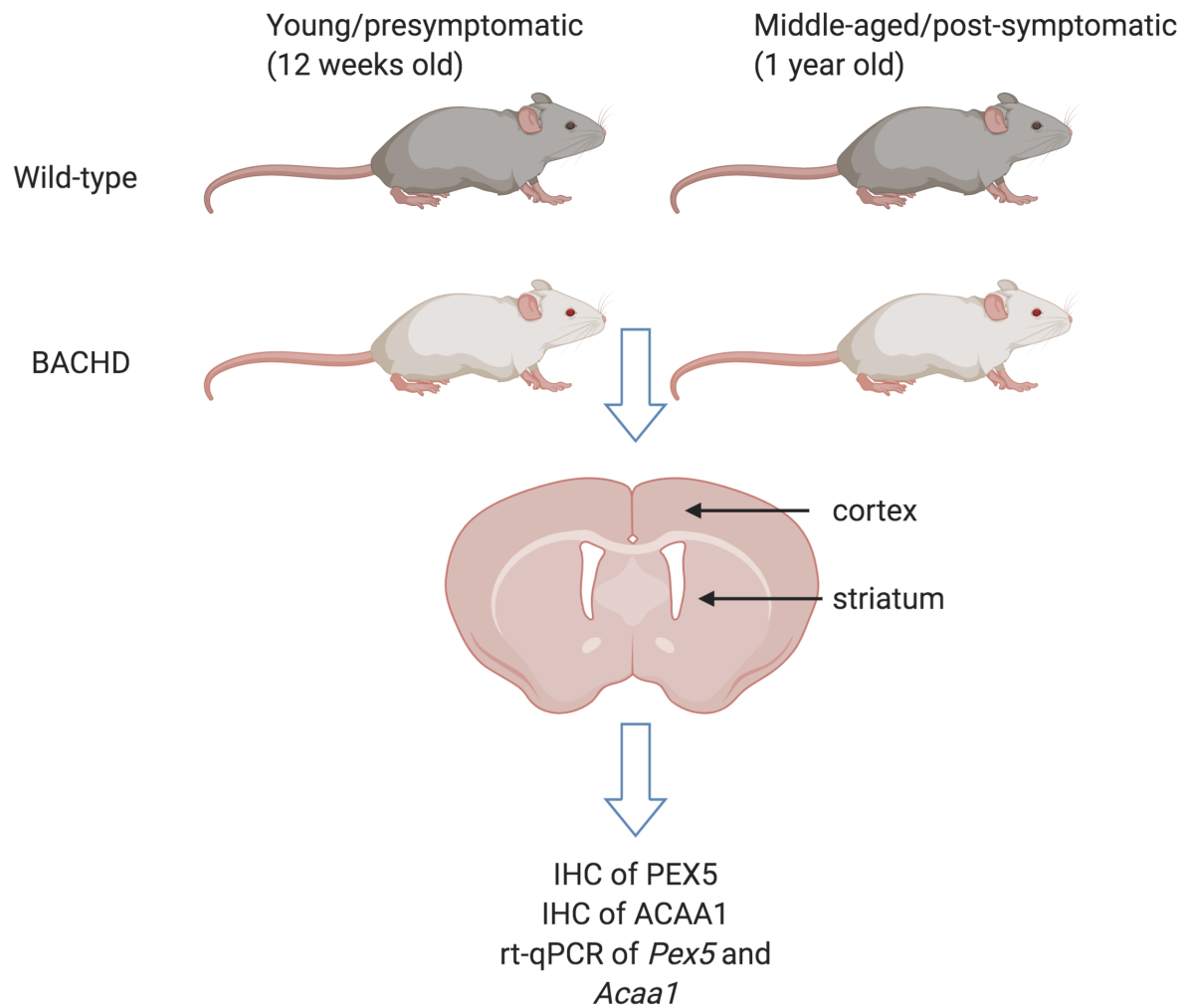
## **Chapter 5: Future directions**

### **I. Markers of peroxisomes and pexophagy are affected in two models of Huntington disease**

#### **Determining a mechanism of PEX5 repression in Huntington disease mouse models**

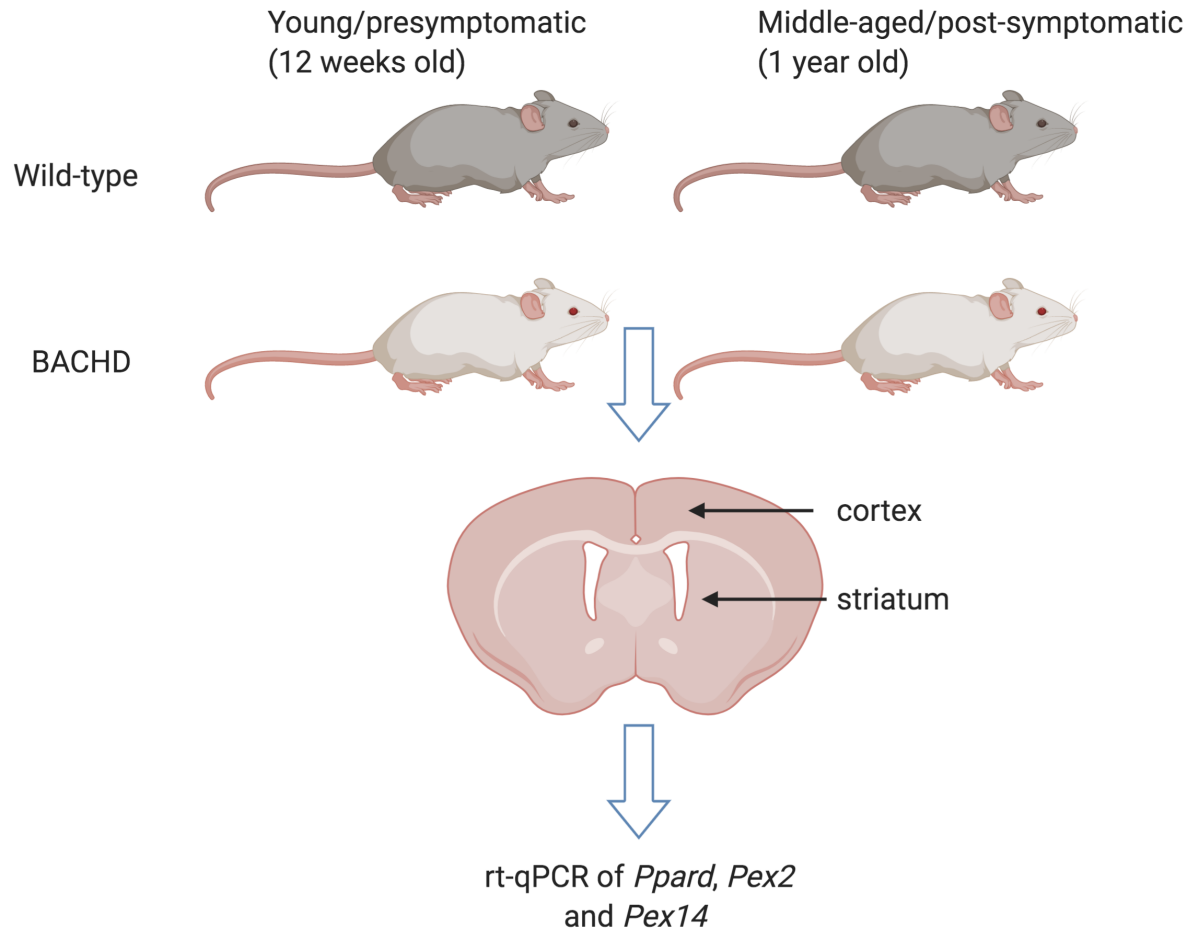
As previously mentioned in chapter 2, the largest limitation in our work on peroxisomal markers in HD is that we did not investigate those markers in symptomatic BACHD mice. Also, the striatum, which is the first region affected by and very sensitive to HD pathogenesis, was not analyzed for changes in pexophagy or peroxisomal number (Sturrock and Leavitt 2010, Ehrlich 2012, Golas 2018). Therefore, a relatively simple next step would be to repeat the experiment with four groups: 12-week-old wildtype mice, 12-week-old BACHD mice, year-old-wildtype mice, and year-old-BACHD mice. They will all be sacrificed at the same time, and their brains will undergo fixation and treatment in 20% sucrose to prepare for slicing. Their brains will be sliced at 20  $\mu$ m, and the slices that are equivalent to 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) will be mounted on slides. The slides will undergo immunohistochemistry protocols staining for either PEX5 (peroxisomal protein/pexophagy marker) or ACAA1 (peroxisomal marker); each slice will be imaged at three locations of the cortex/striatum to determine fluorescent intensity and puncta number. An immediate next step is to determine if these protein levels are affected by gene expression. To do this, dissected cortices of young (12-week-old) and middle-aged (year-old) wildtype and BACHD mice will undergo RT-qPCR for *Pex5* and *Acaal*, to determine if their expression is changed by mHtt expression before and after symptoms occur. With the

resulting data, one can conclude the changes to peroxisomes and a key peroxisomal protein in a system where mHtt is expressed (**Figure 5.1**).



**Figure 5.1: Scheme of future direction investigating (1) PEX5 and ACAA1 levels and (2) *Pex5* and *Acaa1* gene expression in cortices and striata of wildtype and BACHD mice, at pre- and post-symptomatic ages. Created in BioRender.**

After determining protein levels and gene expression, the next step will be to determine a mechanism of aberrant pexophagy. We know from previous studies that autophagy machinery is impaired in models of Huntington's disease; this previous data may be pointing us to a change to possible regulators of peroxisome homeostasis, the peroxisome proliferator-activated receptors, or PPARs. PPARs come in different forms:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ; it has been previously reported that PPAR- $\delta$  is impaired in mouse models of HD (Dickey, Pineda et al. 2016). Knocking out PPAR- $\delta$  affects myelination in the corpus callosum of mouse mutants, indicating a possibly negative effect on peroxisomal lipogenesis (Peters, Lee et al. 2000). Therefore, an interesting experiment (to run in tandem with the one mentioned previously) would potentially build a mechanism linking PPAR- $\delta$  activity to PEX5 activity in our HD mouse models. To do so, we could generate a PPAR- $\delta$  knockout mouse, and determine if PEX5 levels and gene expression have changed, in comparison to age-matched wild-type mice. If PEX5 levels have gone down (in comparison to control) in the absence of PPAR- $\delta$ , then PPAR- $\delta$  could be a potential master regulator of PEX5. To confirm this in our HD mouse models, PPAR- $\delta$  gene expression could be analyzed in WT and BACHD cortices via RT-qPCR, with the proposed result being that pre- and post-symptomatic BACHD mice may have lower expression of *Ppard* in comparison to their wild-type counterparts, due to the expression of mHtt. Therefore, one could conclude that mHtt represses PPAR- $\delta$  activity, leading to a reduction in PEX5 levels. Other peroxisomal genes such as *Pex2* and *Pex14* (both involved in peroxisomal protein import) could also be explored, to fully elucidate the role that mHtt-repressed PPAR- $\delta$  may have on their expression (Islinger, Voelkl et al. 2018, Barros-Barbosa, Ferreira et al. 2019) (**Figure 5.2**).



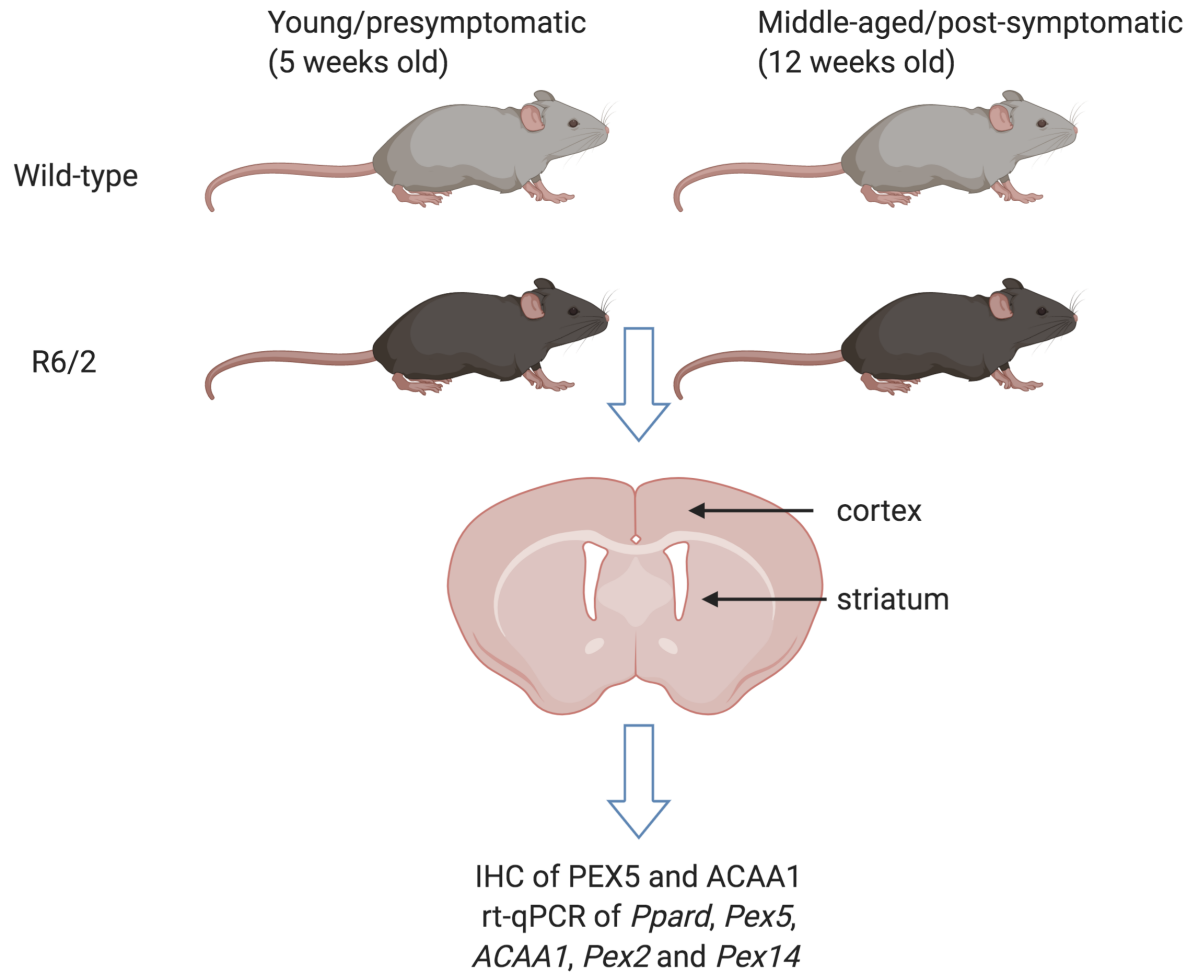
**Figure 5.2:** Scheme of second future direction investigating *Ppard* expression levels in cortices and striata of wildtype and BACHD mice, at pre- and post-symptomatic ages.

Created in BioRender.

### **The effect of HD severity on peroxisomal markers**

Another question remains, such as: does peroxisomal health change in more severe forms of HD? To answer this question, the proposed experiment will investigate peroxisomal health in the R6/2 mouse model. This mouse model expresses exon 1 of the human huntingtin gene that contains 150 CAG repeats, and dies around the age of 3 months, with animals being presymptomatic up until 5 weeks of age (Li, Popovic et al. 2005, Cepeda-Prado, Popp et al. 2012). Therefore, the experiments proposed for BACHD cohorts can be repeated in R6/2 models to determine if severe HD pathogenesis has a more severe effect on peroxisomal health in cortical and striatal neurons (**Figure 5.3**).





**Figure 5.3: Scheme of third future direction investigating (1) PEX5 and ACAA-1 levels, (2) *Pex5* and *Acaa1* expression and (3) *Ppard* expression levels in cortices and striata of wildtype and R6/2 mice, at pre- and post-symptomatic ages. Created in BioRender.**

## **Conclusion**

With these future directions, we propose to flesh out a full mechanism of peroxisomal aberration in the brains of mouse models of HD.

## **II. Aging lowers PEX5 levels in cortical neurons in male and female mouse brains**

In the peroxisomal biology sub-field, there are still unanswered questions about how peroxisomal function and pathways are affected in the aging brain. We know about peroxisome dysfunction in liver and kidney disease, but the scientific community is still trying to understand peroxisomes in the brain (see Chapter 1). In peroxisomal biogenesis disorders pathology is expected, but what about an aged brain without dementia? What peroxisomal changes occur in such a brain?

To start answering the latter question, we looked at neuronal PEX5 levels in aged male and female mice, where we observed a reduction in the cortex, compared to younger counterparts; *Pex5* expression was lower in aged male cortices in comparison to younger male cortices, while there was no difference in female samples (see Chapter 4). So, in conclusion, PEX5 levels are reduced in the aged mouse cortex, regardless of sex. While this data may show a potential age-related reduction, it still leaves the author with more questions: Why is there no significant difference in cortical *Pex5* expression between females of different ages? While we have discussed the potential mechanisms previously, we will now turn to potential, future experiments to tease out this potential sex difference.

## **Screening for sex-associated master regulators of PEX5**

The first experiment to run would be to determine the master regulators of PEX5 that may be sex-associated. To do that efficiently, we will switch to cell culture, using cell lines that are derived from male and female sources as separate cohorts (prostate vs. ovarian, for instance), and perform a CRISPR-Cas9 loss-of-function screening (Vesikansa 2018, Ouyang, Liu et al. 2019).

### **Knocking in candidate genes**

After determining candidate genes (write more on this), we will then express them in male mice using a CRISPR-Cas9 system, and age them until 32 months of age (as in Chapter 4) (Scott and Gruzdev 2019). We will then compare cortical *Pex5* expression levels in young (3-month-old) and aged (32-month-old) wild-type and KO male mice, using the RNAScope assay. If the candidate genes are indeed master regulators of *Pex5*, then overexpressing them will increase *Pex5* expression levels in the brains of aged KO male mice.

### **Conclusion**

By planning for, and running the experiments laid out in this future directions section, we will be able to evaluate suitable candidate genes that may modulate *Pex5* expression levels based on sex.

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