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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Exploring transmitophagy and striatal vulnerability in Huntington's disease: the role of astrocytic dysfunction

Imme Aerts

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
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Exploring transmitophagy and striatal vulnerability in Huntington's disease: the role of astrocytic dysfunction.

Imme Aerts[1], Alba Pereda[2, 3, 4] and Prof. Silvia Gines[2, 3, 4] and Prof. dr. David Wilson[1]

[1] University Hasselt, Campus Diepenbeek, Agoralaan building D. research group, Biomedical Research Institute, Universiteit Hasselt, Campus Diepenbeek,[2] Departament de Biomedicina, Facultat de Medicina. Institut de Neurociències. Universitat de Barcelona, Barcelona, Spain. [3] Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain. [4] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain.

Running title: Transmitophagy in Huntington's disease*

To whom correspondence should be addressed: Silvia Gines, Tel: +34 628 71 71 13; Email: silviagines@ub.edu

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ABSTRACT

Huntington's disease (HD) is a hereditary neurodegenerative disorder caused by a mutation in the huntingtin gene. The striatum is the most affected brain region, with D2-type medium spiny neurons (MSNs) showing greater vulnerability, although the mechanism behind this is still unclear. Transmitophagy, a recently described form of intercellular mitochondrial exchange between neurons and astrocytes, supports neuronal health, but its role in HD is not yet well understood. This study investigates how astrocyte-mediated mitochondrial transfer is affected in HD using primary striatal cultures from wild-type (WT) and R6/1 mice, a mouse model of HD. First, mitochondrial membrane potential and oxidative stress in astrocytes were measured via live-cell imaging to assess mitochondrial function. Second, mitochondrial mass in the astrocyte-conditioned medium (ACM) was quantified as an indirect measure of mitochondrial release. Third, the impact of ACM on A2A receptor-expressing (D2-type) MSNs was evaluated using Sholl analysis. The results showed no significant differences between WT and R6/1 astrocytes in oxidative stress or mitochondrial membrane potential, indicating preserved mitochondrial function. Western blot analysis of ACM also showed no significant differences in mitochondrial mass. Neurons treated with R6/1 ACM did show significantly increased branching compared to WT-treated neurons. These findings suggest that astrocyte-derived mitochondria from R6/1 mice can positively influence the neuronal morphology. This may reflect early compensatory mechanisms before clear astrocytic pathology is present. Our results underscore the importance of studying astrocyte-neuron interactions in early stages of HD, where subtle but meaningful changes may contribute to disease progression.

INTRODUCTION

Epidemiology and clinical features of Huntington's disease

Huntington's disease (HD) is a rare autosomal dominant neurodegenerative disorder with an estimated prevalence of 5-10 cases per 100,000 individuals in most European countries, North and South America, and Australia (1-4). HD is characterized by motor, psychiatric, and cognitive symptoms, with a clinical diagnosis typically happening around 45 years of age after a prodromal period of approximately 15 years (3-5). This phase follows a presymptomatic period that is characterized with subtle motor, cognitive, and behavior changes that are seen before the more obvious motor symptoms manifestation. After motor signs start, the disease progressively worsens, with symptoms like involuntary movements (such as chorea), coordination and speech impairments, mood changes, depression and difficulty swallowing.

HD patients have a premature mortality within the next 15 years after diagnosis (1, 3, 4, 6). Currently, no treatments are available to stop or slow the progression of the disease. The available treatments are mostly focused on managing the symptoms instead of addressing the underlying cause of the disease (2, 5-8).

Genetic Basis and Neuronal Vulnerability in Huntington's disease

HD is caused by an abnormal expansion of CAG repeats in the huntingtin (*HTT*) gene, which is located on chromosome 4 (1-4). In non-affected individuals, the *HTT* gene usually contains between 6 and 35 CAG repeats. The risk of developing HD increases as the number of repeats increases (2, 4, 5). Patients with more than 40 repeats are observed to have full penetrance of the disease, while those with 36-39 repeats may experience later disease onset (5, 7). The higher the number of repeats, the earlier symptoms usually appear. Adult-onset HD is typically seen in individuals carrying 40–50 repeats, while 50–120 repeats are associated with the juvenile form of the disease (4).

The *HTT* gene encodes huntingtin, a protein found throughout the body that plays a critical role in important cell functions such as transcriptional regulation, intracellular trafficking, and maintaining synapse formation (4, 6, 9). HTT expression is highest in the basal ganglia in the brain, which is a group of subcortical nuclei critical for motor and cognitive functions (1, 6, 9, 10). Within this network, the striatum is the most affected region in HD, showing selective degeneration of GABAergic medium spiny neurons (MSNs) (1, 4, 6, 10). MSNs constitute-over the 90% of the striatal neurons, and up to 95% degenerate during the progression of HD (6, 11, 12).

MSNs are divided into two main subtypes based on their expression of dopamine receptors and projection targets. D1 receptor-expressing MSNs form the direct pathway, which facilitates voluntary movements. The D2 receptor-expressing MSNs are part of the indirect pathway, which suppresses involuntary movements (10, 12-14). In the case of HD, MSNs of the indirect pathway (D2-expressing MSNs) are more vulnerable and begin to degenerate earlier, which contributes to the hyperkinetic motor symptoms that are observed in the early stages of HD. As the MSNs in the direct pathway (D1R-expressing) also begin to degenerate, patients often develop hypokinetic symptoms, such as bradykinesia and rigidity (12, 13, 15, 16). In addition to the D2 receptor, D2-MSNs have also been shown to express the adenosine A2A receptor (12, 13). This receptor belongs to the G protein-coupled receptor (GPCR) family and is mainly coupled with Gs proteins, which stimulate adenylyl cyclase (17). Several studies have shown that A2A receptors are co-expressed with D2 dopamine receptors in D2-MSNs within the striatum (18, 19). Importantly, this co-expression appears to be specific to D2-MSNs, as A2A receptors are not found in neurons that express D1 receptors and belong to the direct pathway.

Astrocytes in Huntington's disease

Glial cells are found throughout the central nervous system (CNS) (18, 20-22). Although estimates vary, it is generally accepted that glial cells are at least as or more numerous than neurons (18, 20, 21). Approximately 20-40% of all cells in the mammalian brain are specialized glial cells known as astrocytes, however their proportion can vary by species and brain regions (21, 23). Astrocytes play an essential role in maintaining neuronal function and survival by regulating ion homeostasis, clearing excess neurotransmitters, providing metabolic support, and maintaining blood-brain barrier integrity (22-28).

In HD, astrocytes play a direct role in disease pathogenesis through a loss of these supportive functions. Although astrocytes are not the main affected cells in HD, they express the mutated protein, which alters their normal physiological roles (29-32). One of the earliest observed problems in HD astrocytes is their reduced glutamate uptake, which can lead to excitotoxicity and contributes to the degeneration of striatal neurons through non-cell-autonomous mechanisms (29-31). In addition, mutant huntingtin (mHtt)-expressing astrocytes show reduced expression of glutamate transporters, such as GLT-1. This makes it more difficult to regulate extracellular glutamate levels and synaptic transmission (30-32). This dysfunctional astrocytic environment reduces metabolic and synaptic support under stress conditions, thereby increasing neuronal vulnerability.

Mitochondrial Dysfunction in Huntington's Disease

Mitochondria are essential organelles responsible for producing adenosine triphosphate (ATP) through oxidative phosphorylation, supporting critical cellular processes such as metabolism, calcium regulation, apoptosis, and aging (33-35). They can occupy up to 25% of the cytoplasmic volume in many eukaryotic cells (33). Despite having relatively lower mitochondrial density in neurons compared to other cells such as muscle cells, the brain requires a large and continuous energy supply and consumes significantly more oxygen and glucose than other organs (36). This highlights the brain's high dependence on optimal mitochondrial function, as mitochondrial dysfunction can lead to energy deficits, oxidative stress, and ultimately, neuronal damage (35-37).

Previous studies of mitochondrial dysfunction in HD showed ultrastructural abnormalities in the mitochondria of postmortem neuronal brain tissue from human patients (35, 38). This was further supported by animal studies showing that the systemic administration of 3-nitropropionic acid (3-NP), a mitochondrial toxin and irreversible inhibitor of mitochondrial complex II, induced the selective degeneration of neurons in the caudate-putamen in rodents and non-human primates. This mimics both the anatomical and behavioral features of HD (39-42). Clinical signs of progressive weight loss in HD patients despite caloric intake, suggest an underlying systemic metabolic disorder. This is supported by a reduced cerebral glucose metabolism, particularly in the basal ganglia, even before clinical symptoms appear (43-46). However, these metabolic deficits have primarily been examined in the context of neuronal energy demand and function. Additionally, several studies have reported significant reductions in the enzymatic activity of mitochondrial complexes I, II-III, and IV in neuronal populations of the striatum, while other brain regions appear relatively unaffected (38, 47-49).

To meet these metabolic and energetic cellular demands, mitochondria undergo continuous fission and fusion events (35). Under physiological conditions, a balance between these processes maintains mitochondrial morphology, integrity, and cellular distribution of the organelles (35, 50). However, this balance seems to be disrupted in HD, especially in neurons expressing mHtt, where abnormal fission and impaired fusion lead to mitochondrial fragmentation and dysfunction (51). Mitochondrial fission is mostly regulated by the cytosolic GTPase dynamin-related protein 1 (Drp1) and the adaptor protein, mitochondrial fission 1 protein (Fis1), while fusion is regulated by the outer membrane proteins mitofusin 1 and 2 (Mfn1 and Mfn2) as well as optic atrophy 1 (OPA1) (52-56). Altered expression levels of these proteins have been observed in neuronal cell lines, animal models and postmortem brain tissue (57-59).

These findings emphasize that most studies on mitochondrial dysfunction have been focussed on neuronal cells. However, much less is known about mitochondrial function in astrocytes in HD. This is an important gap, as astrocytes also rely on mitochondria to support their roles in neurotransmitter clearance, metabolic support, and antioxidant defense. Emerging evidence suggests that HD astrocytes also show mitochondrial dysfunction, impaired energy metabolism and increased reactive oxygen species (ROS) production (60, 61). These issues include less efficient lactate transfer to neurons and increased production of ROS, which can further damage nearby cells (61). Structural damage in astrocytic mitochondria has been observed in both HD models and human postmortem tissue (61). Additionally, astrocytic glycolytic metabolism was selectively impaired in the striatum of HD patients, suggesting astrocyte dysfunction (62). Using fluorescence lifetime imaging microscopy, it has been observed that the mitochondria in the striatum of a HD mouse model were not functioning correctly. The glucose levels were low, while fatty acid breakdown products were high, which caused astrocytes to switch from using glucose to fatty acids (fatty acid oxidation or FAO). Typically, this limits ROS levels, but in these mice, damaged fat accumulates in the striatum, resulting in elevated ROS levels and neuronal damage (63). A more recent study observed altered $NADH/NAD^+$ ratios, reduced complex II levels, increased fatty acid oxidation, and increased ROS levels in an HD mouse model (64). Together, these findings indicate that astrocyte mitochondrial impairment may

contribute to striatal vulnerability and should be investigated further to fully understand their role in HD progression.

Transmitophagy in Huntington's disease

Recent evidence has proposed another layer on the mitochondrial quality control processes. Thus in animal models and human studies it has been suggested that mitochondria can be released into extracellular fluids such as blood and cerebrospinal fluid, for the exchange between cells (65-69). This process, known as intercellular mitochondrial transfer, has two different functions. Mitochondria can act as danger signals that cause inflammation in stress conditions, however, the transfer of healthy mitochondria can support damaged cells and promote regeneration (65, 66, 69). Davis et al. were the first to describe transmitophagy: a special process where damaged mitochondria, usually from neurons, are transferred to nearby astrocytes so that they can break them down, and vice versa (see Figure 1) (70). In their study, they observed that mitochondria from retinal ganglion cell axons were internalized and degraded by nearby astrocytes. This revealed a poorly understood mechanism of mitochondrial quality control in the CNS (70). Transmitophagy has also been observed in Parkinson's disease models, in which damaged mitochondria from dopaminergic neurons are transferred to astrocytes and thereby reducing neuroinflammation (71)

Here, Morales et al. described a form of transmitophagy mediated by spheroids from dopaminergic neurons to nearby astrocytes. They suggested that aging astrocytes could impair this pathway, which could contribute to the pathogenesis of Parkinson's disease (71). In addition, in Alzheimer's disease (AD), human induced pluripotent stem cell (iPSC)-derived astrocytes have been shown to take up and degrade neuron-derived mitochondria in both *in vitro* and *in vivo* models (72). The study showed that this transmitophagy process is altered with aging. Their findings suggest that both AD pathology and aging selectively disrupt crucial transmitophagy components (72). While transmitophagy was first discovered in retinal ganglion cells, there has been limited research on its function in other parts of the nervous system. Despite the growing interest in how the mitochondrial exchange can influence the neuronal health, there is still no published work investigating if transmitophagy occurs or is altered in the context of Huntington's disease. This represents a clear gap in our understanding of HD pathogenesis.

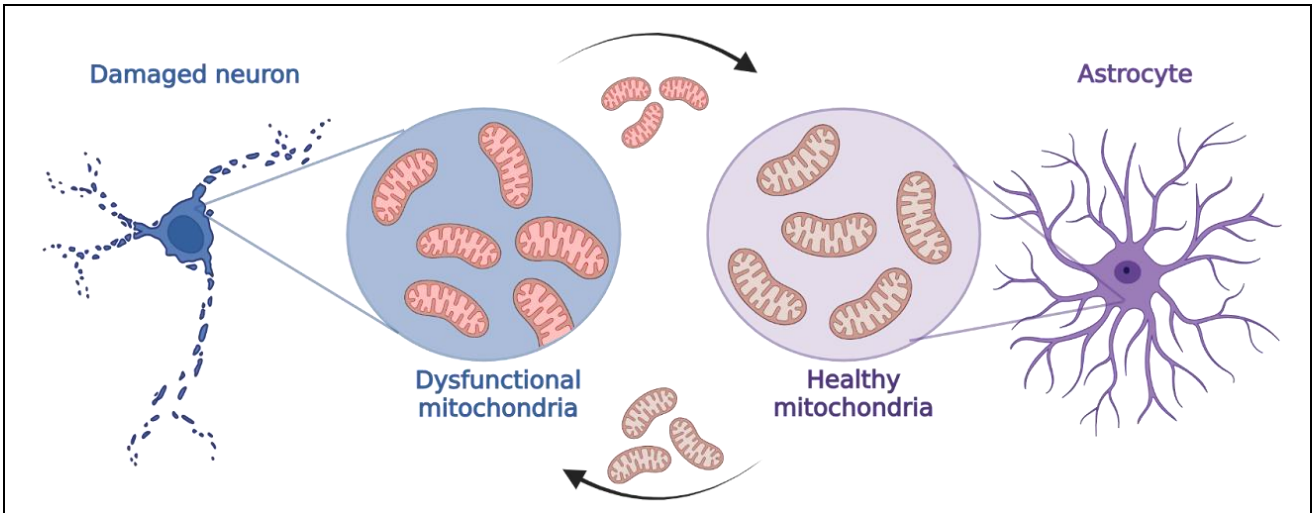

Fig. 1 – **Schematic representation of transmitophagy.** Damaged neuronal mitochondria are transferred to neighboring astrocytes where they are degraded. At the same time, healthy astrocytic mitochondria are transferred back into the damaged neuron, thereby restoring its bioenergetic capacity and promoting neuronal recovery.

This study aims to investigate whether mitochondrial function is altered in HD astrocytes and whether the astrocyte-mediated mitochondrial transfer to neurons is affected. Three objectives related to astrocyte-mediated transmitophagy are investigated. The mitochondrial function by analyzing the mitochondrial membrane potential and the levels of oxidative stress WT and R6/1 striatal astrocytes through live cell-imaging. These measurements can be used to characterize the mitochondrial metabolism in HD astrocytes to gain insight into whether their mitochondria are functioning correctly. Second, the mitochondrial mass of astrocyte-conditioned medium (ACM) derived from WT and R6/1 astrocytes is examined as an indirect measure of intercellular mitochondrial transfer. The ACM refers to the culture medium of astrocytes. This process allows the accumulation of secreted factors and cellular components, including transferred mitochondria. Alterations in mitochondrial mass can be interpreted as potential changes in the release of mitochondria by astrocytes. Lastly, the impact of the internalization of astrocytic mitochondria on neuronal morphology is evaluated using Sholl analysis to determine if altered astrocytic-mediated mitochondrial function negatively affects neuronal branching. This approach is expected to provide new insights into how astrocyte-mediated mitochondrial dysfunction in HD may compromise neuronal support and contribute to disease progression.

EXPERIMENTAL PROCEDURES

Animal model

R6/1 transgenic mice expressing a fragment of the human *huntingtin* gene with 115 or 145 glutamine repeats (73) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Female wild-type (WT) mice were crossed with male R6/1 mice to generate age-matched heterozygous WT and R6/1 littermates on a B6CBA background. Genotyping was performed by PCR of tail biopsies. Mice were housed in mixed genotype groups at 19-22°C under a 12:12 h light/dark cycle with ad libitum access to food and water. All procedures complied with national and European regulations and were approved by the Animal Care and Ethics Committee of the Generalitat de Catalunya.

Cultures

Primary neuronal cultures

Striata from E17.5 WT mouse embryos were dissected and mechanically dissociated using a fire-polished glass Pasteur pipette. The resultant cells were seeded (80 000 cells/well) onto 12mm coverslips pre-coated with 0,1mg/mL poly-D-lysine in Neurobasal medium (Gibco, #21103-049), with the addition of 2% B27 (Gibco, Cat. #17504-044) and 1% GlutaMAX (Gibco, Cat. #35050-038), in order to sustain serum-free conditions. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. Neurons were used for further experimentation at day *in vitro* 14 (DIV14).

Primary astrocyte cultures

Striata from WT and R6/1 mouse pups at post-natal day 0-3 (P0–3) were dissected and mechanically dissociated using a fire-polished Pasteur pipette. Cells were seeded into 0,1mg/mL poly-D-lysine-coated 6-well plates in DMEM (Gibco, Cat. #11574486) supplemented with 10% FBS (Sigma Aldrich, Cat. #F7524), 90mMglucose, and 1% penicillin/streptomycin (Diagnovum, Cat. #D910) and maintained at 37ºC and 5% CO_2). At DIV5, cells were shaken overnight to remove non-astrocytic cell types. At DIV7, the cells were separated using a two-minute typsinization process. Then, they were centrifuged at 250 g for ten minutes at room temperature and seeded into new plates. The seeding densities were 350,000 cells/well for the analysis of mitochondrial mass and assessment of neuronal branching, and 40,000 cells/well for the live cell experiment. Astrocytes were used for further experimentation at DIV14 or DIV16.

Astrocyte-conditioned medium treatment

To study mitochondrial transfer from astrocytes to neurons, WT and R6/1 astrocytes (DIV18) were labeled with 200 nM MitoTracker™ Red CMXRos (Invitrogen, Cat. #M7512) in 1% GlutaMAX supplemented Neurobasal for 30 minutes at 37ºC. After treatment, cells were washed with phosphate-buffered saline (PBS), and incubated with fresh GlutaMAX supplemented Neurobasal. After 24 hours, the astrocyte-conditioned medium (ACM) was collected, centrifuged at 1,000g for 10 minutes

at RT, and used to replace half of the medium in WT neuronal cultures. Neurons were treated with either WT or R6/1 ACM supernatant for 24 h at 37 °C. After 24h, the neurons were fixed with 4% paraformaldehyde-PBS for 10 minutes, followed by three PBS washes. To avoid paraformaldehyde crosslinking, coverslips were treated with 0,1 M glycine-PBS for 15 minutes. After three final PBS washes, coverslips were stored in 0,0025% azide-PBS at 4 °C.

Neuronal branching assessment

ACM-treated neuronal cultures were permeabilized with PBS-1% BSA-0,1% saponin for 10 minutes, followed by blocking with 15% PBS-Normal Horse Serum (NHS) in PBS for 30 minutes at RT. Coverslips were incubated overnight at 4 °C in the dark with anti-adenosine receptor A2A antibody (1:500, Santa Cruz Biotechnology, Cat. #sc-32261). The following day, coverslips were incubated with Alexa Fluor® 488 AffiniPure® Donkey Anti-Rabbit IgG (1:100, Jackson ImmunoResearch, Cat. #711-545-152) for 1 hour at RT in the dark. All antibodies were diluted in PBS with 5% NHS-PBS. Finally, the coverslips were washed and mounted with DAPI-Fluoromount-G® (Southern Biotech, Cat. #0100-20). Neuronal branching was assessed by confocal imaging using a Zeiss LSM880 microscope with a 63× 1.40 aperture number (NA) oil objective. Images were taken with a 0,6× digital zoom and 0,3 µm z-stacks. Sholl analysis was performed in ImageJ using the Concentric Circles and Cell Counter plugins with eight concentric circles (50-400 µm from soma) to quantify neurite branching by counting the intersections with each circle.

ACM mitochondrial mass analysis

Astrocytic mitochondria from striatal ACMs were precipitated at 2,000g for 10 minutes. The resulting pellet was resuspended in 1mL of PBS, transferred to Eppendorf tubes, and centrifuged again under the same conditions. The final pellet was resuspended in 10 µL lysis buffer (50mM Tris base [pH 7,5], 150mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM sodium-orthovanadate and protease inhibitor cocktail). Samples were denatured in sample buffer (62,5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0,1% (w/v) bromophenol blue) and boiled at 100°C for 5 minutes.

Mitochondrial mass from ACMs was assessed by Western Blot. Samples were resolved in a denaturing 12% SDS-polyacrylamide gel electrophoresis at 30 mA and transferred to a nitrocellulose membrane (Amersham, Cat. #10600002) for 1h at 100V at 4ºC. Unspecific unions were blocked with 10% non-fat powdered milk in Tris-buffered saline 0,1% Tween-20 (TBS-T) for 1 hour at RT. Membranes were incubated overnight at 4°C with primary antibodies (Supp. Table 1) followed by the appropriate secondary antibodies (Supp. Table 2) for 1 hour at RT. Bands detection was performed using Luminol Reagent (Santa Cruz, Cat. #sc-2048). Bands densitometry was quantified using Image Lab software (Bio-Rad) and normalized to astrocytes cell count from each sample.

Analysis of mitochondrial membrane potential and oxidative stress in live astrocyte cultures

Mitochondrial membrane potential and oxidative stress were studied in live Wt and R6/1 striatal astrocytes in primary cultures. Cells were incubated at 37°C for 30 minutes with 20 nM TMRM (Invitrogen, Cat. #T668) and 200 nM MitoTracker Green (Invitrogen, Cat. #M7514) in Krebs buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose) to assess mitochondrial membrane potential and mass. For oxidative stress assessment, cells were incubated with 5 µM MitoSOX Red (Invitrogen, Cat. #M360058) under the same conditions. After incubation, astrocytes were washed twice with PBS and maintained in Krebs buffer. Live-cell imaging was performed at 37ºC and 5% CO_2 using the Opera Phenix High-Content Screening System (63x 1.4 NA oil objective). Mitochondrial membrane potential was imaged without stacks and oxidative stress with 0,3 µm Z-stacks. Data from 25 frames per sample were analyzed. Mitochondrial membrane potential was quantified using the TMRM-to-MitoTracker Green intensity and area ratios. Oxidative stress was measured as MitoSOX Red intensity normalized to the analyzed area.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0. Results are expressed as mean ± SEM. Outliers were identified via the ROUT method, and data normality was assessed using the Shapiro-Wilk test. Two-group comparisons used either Student's *t*-test or the Mann-Whitney test based on distribution. Two-way ANOVA with Sidak's multiple comparison test was used for multi-group comparisons. A 95% confidence interval was applied, with $p < 0,05$ considered statistically significant.

RESULTS

Analysis of mitochondrial membrane potential and oxidative stress in live astrocyte cultures.

To better understand whether mitochondrial function is altered in astrocytes derived from the R6/1 HD model, we first assessed the intrinsic properties of their mitochondria. Specifically, we examined markers of oxidative stress and mitochondrial membrane potential to determine whether R6/1 astrocytic mitochondria display functional impairments compared to WT controls (Figure 2A). The astrocytes were stained with MitoSOX Red to detect mitochondrial superoxide production or with MitoTracker Green to label all mitochondria and with TMRM (red) to selectively label mitochondria with an intact membrane potential. To evaluate the mitochondrial function relative to mitochondrial content, the area ratio of TMRM to MitoTracker Green staining was calculated. The quantification of MitoSOX Red intensity showed no significant difference in oxidative stress levels between WT and R6/1 astrocytes. Similarly, the TMRM/MitoTracker Green area ratio revealed no significant difference in mitochondrial membrane potential between the two genotypes (Figure 2B).

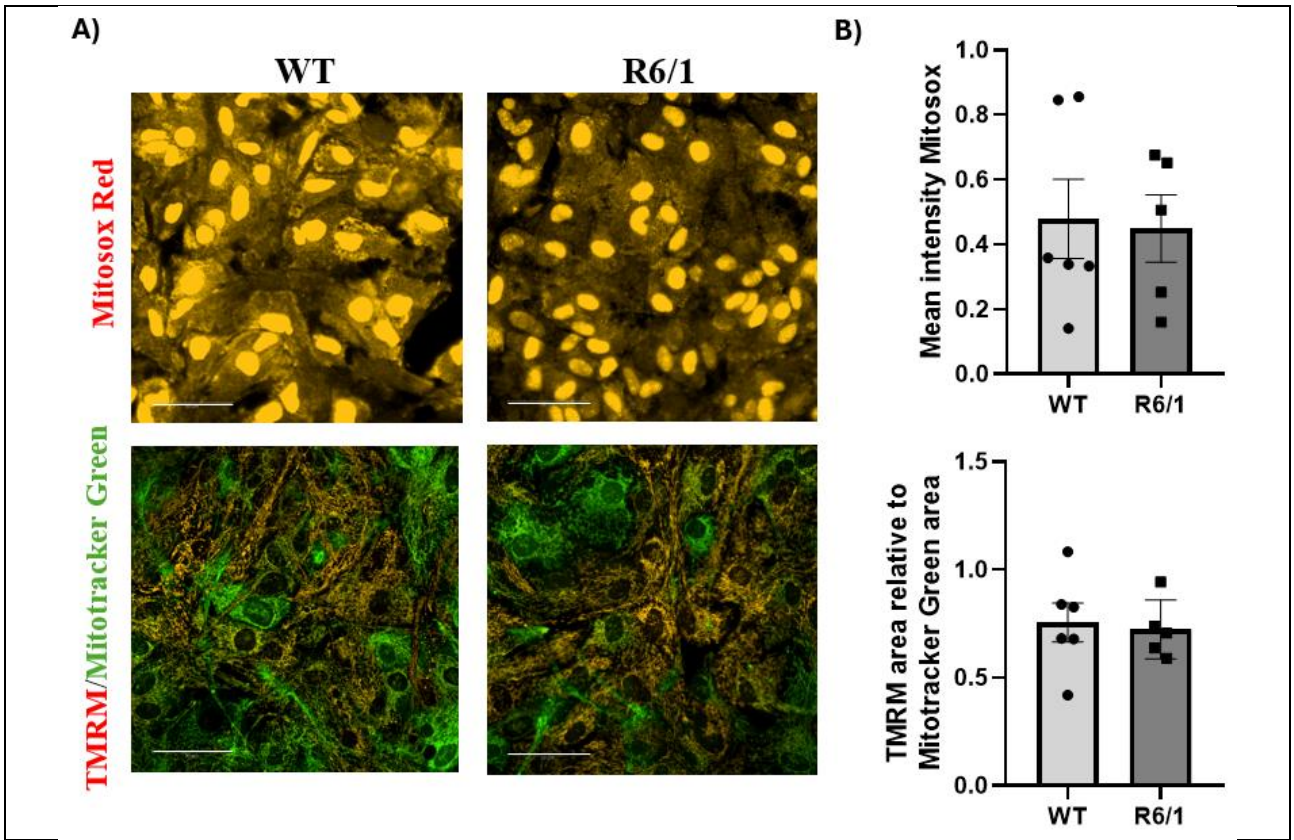

Fig. 2 – **Assessment of mitochondrial oxidative stress levels and mitochondrial membrane potential in living astrocytes.** To assess the mitochondrial oxidative stress levels and mitochondrial membrane potential, astrocytes were stained with either 5 µM Mitosox Red or 20 nM TMRM (red) and 200 nM Mitotracker Green respectively at DIV15. **A)** Opera images of Mitosox Red (upper panel) and TMRM (red) and Mitotracker Green (green) fluorescence for WT and R6/1 astrocytes. Scale bar 50 µm. **B)** Quantification of the intensity of Mitosox Red (upper graph) and the intensity of TMRM and the area ratio of TMRM and Mitotracker Green (lower graph). Data from two different cultures was analyzed with unpaired t-test (5-6). Data are presented as mean ± SEM.

ACM mitochondrial mass analysis

The general aim of this project is to evaluate whether extracellular mitochondria released by WT and R6/1 astrocytes viability differentially affects the neuronal viability. Since the mitochondria can be secreted into the extracellular space and potentially internalized by neurons, we examined whether the astrocytic genotype influences the amount of released mitochondria. To address this point, we quantified by Western Blot the mitochondrial markers translocase of outer mitochondrial membrane 20 (TOMM20) and the voltage-dependent anion channel (VDAC) in the ACM derived from WT and R6/1 striatal astrocytic cultures (Figure 3A). Both proteins are markers of the mitochondrial outer membrane. The analysis revealed no significant difference in the levels of these mitochondrial proteins between the ACM of WT and R6/1 (TOMM20 p-value = 0,3867; VDAC p-value = 0,3823) (Figure 3B).

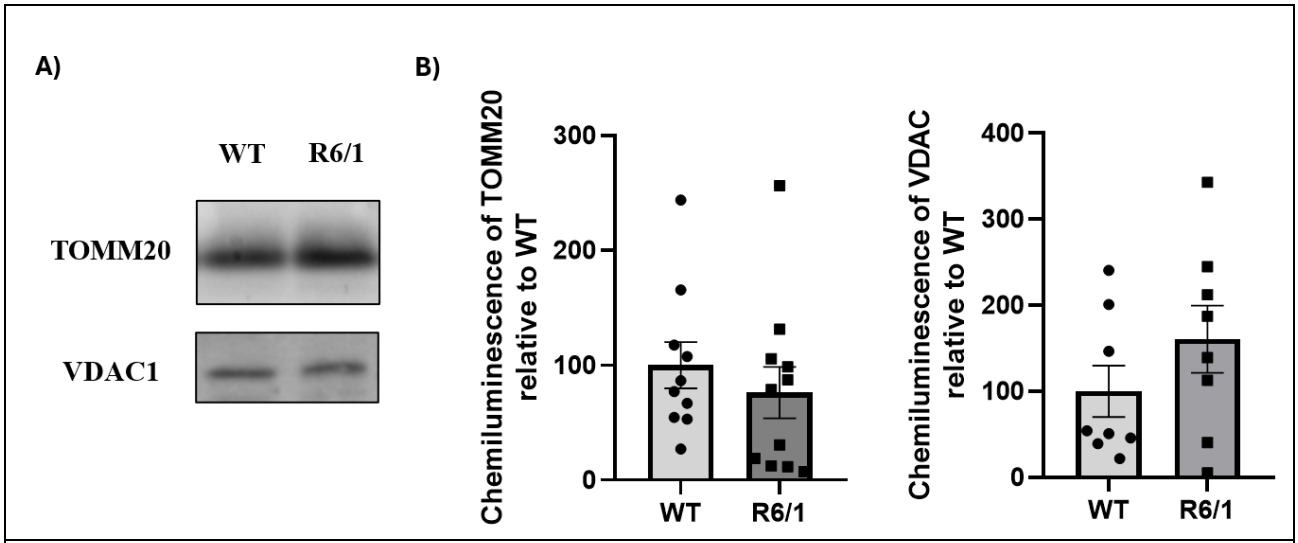

Fig. 3 – **R6/1 and WT astrocytes release a similar amount of mitochondria into the extracellular medium. A)** Immunoblotting bands of TOMM20 and VDAC from WT and R6/1 ACM. **B)** Quantification of the TOMM20 chemiluminescence relative to the cell count. Data was analyzed using the Mann-Whitney test (p-value = 0,3867). **C)** Quantification of the VDAC chemiluminescence relative to the WT chemiluminescence. Data was analyzed using the Mann-Whitney test (p-value = 0, 3823). Data from three different cultures are presented as mean ± SEM (n = 10-11). TOMM20: translocase of outer mitochondrial membrane 20; VDAC: voltage-dependent anion channel

Neuronal branching assessment

Astrocytes play a crucial role in maintaining neuronal health, and the exchange of mitochondria between astrocytes and neurons may contribute to structural stability and serve as a mechanism of neuroprotection and metabolic support. However, it remains unclear whether mitochondria derived from diseased astrocytes (R6/1HD model) can alter neuronal structure. As previously mentioned, D2-type MSNs are particularly vulnerable to the effects of mHtt. To distinguish between the D1 and D2 MSNs, we used an antibody targeting the A2A adenosine receptor, which is selectively expressed in D2 MSNs. To determine the effect of internalizing astrocyte-derived mitochondria on the morphology of A2A striatal MSNs, we assessed dendritic branching with Sholl after treatment with WT or R6/1 ACM (Figure 4A). The Sholl analysis measures the number of dendritic intersections at increasing distances from the soma. No changes in branching were detected; the evaluation of total intersections showed no major differences between the two treatment conditions (p = 0,9758) However, a significant difference was observed in total branching levels between genotypes (p-value = 0,0002) (Figure 4B.

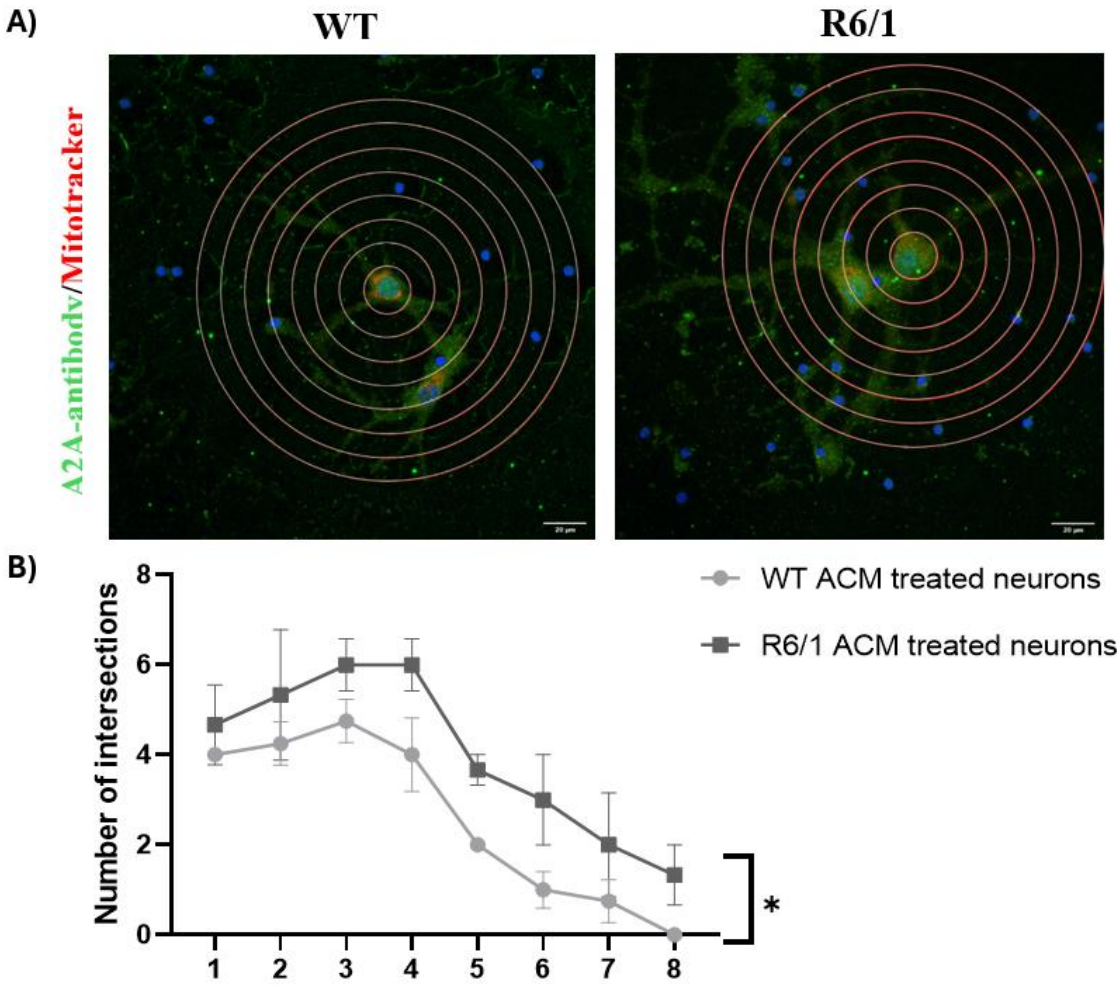

Fig. 4 – **Effect of WT or R6/1 ACM treatment on morphology of A2A striatal neurons.** WT striatal neurons were treated at DIV15 with WT or R6/1 ACM that contained mitochondria stained with Mitotracker Red CMXRos. **A)** WT striatal neurons were stained with an A2A-receptor antibody in green and their nuclei in blue (DAPI). Scale bar 20 µm. **B)** The Sholl analysis was conducted for both groups. Data was analyzed with Two-way Anova and Sidak's multiple comparison test (p-value = 0,9758). A significant difference between total branching levels and genotypes was observed (p-value = 0,0002). n =3-4 neurons for both genotypes from one culture. Data are presented as mean ± SEM.

DISCUSSION

The mHtt gene is expressed throughout the body, but Htt expression is highest in the basal ganglia (6, 9). In HD, the striatum is known to be the most affected region, specifically the D1 and D2 MSNs (6, 10, 11). Consequently, numerous studies have focused on these neuronal population (12, 13, 15, 16). However, recently, there has also been interest in investigating the effects of mHtt on striatal astrocytes. It has been demonstrated that mHtt aggregates accumulate in these supporting cells, which may influence the progression of HD (29-32). Both cell types play a crucial role in transmitophagy, a process by which mitochondria are exchanged between these cells to support damaged cells and promote regeneration (70). Mitochondrial dysfunction is also a hallmark of HD. This has mainly been studied in neurons, but little is known about the effects of mitochondrial function in astrocytes (35, 38). To address this knowledge gap, this thesis investigates how astrocyte-mediated mitochondrial transfer to neurons (transmitophagy) is affected in HD.

A notable finding was that there was no significant difference in the oxidative stress levels and mitochondrial membrane potential in living astrocytes of both genotypes. This means that R6/1 and WT astrocytes both show normal levels of oxidative stress and do not show less polarized mitochondria. This was not expected, as the aggregation of mHtt promotes the generation and accumulation of ROS (74). Therefore, oxidative stress, due to the accumulation of ROS, is being considered as one of the key players in HD pathogenesis (74, 75). Several studies observed increased ROS levels in the striatum of R6/1 and R6/2 HD mouse models (76, 77). Lopes et al. found that increased oxygen consumption combined with elevated ROS levels in early stages of HD reinforces the hypothesis that mitochondrial and redox dysregulation may contribute to the pathogenesis of HD. This has been observed in human and mouse model carriers of mHtt (78). In addition, O2- is generated during respiration and primarily by complexes I and III (79, 80). These complexes, along with others, form the electron transport linked to proton translocation through the mitochondrial intermembrane space, forming an electrochemical proton gradient (electromotive force) and a mitochondrial transmembrane potential ($\Delta\psi m$) (74). Both are required for the generation of ATP (74, 79). However, in HD, the activity of complexes II, III and IV are reduced and the $\Delta\psi m$ is disrupted, which affects the mitochondrial bioenergetics (35, 47, 49). In another study, brain mitochondria were isolated from two lines of YAC72 mice expressing “low” and “high” levels of full length-mHTT. These showed depolarized membranes, with brain mitochondria from the YAC72 high expressor depolarizing more rapidly after Ca2+ stimulation (81). In mitochondria from chimeric human-mouse mHTT-expressing cells (striatal progenitor cell lines), a similar defect in $\Delta\psi m$ was found in response to increasing Ca2+ concentrations (82). Although these studies indicate that oxidative stress levels and mitochondrial membrane potential should be different between the two genotypes, it should also be mentioned that these studies were not performed in striatal astrocytes. Most studies have used fibroblasts or striatal cells with no further indication. There are several reasons we can think of as explanations for these results, namely that the effects of mHtt cannot yet be seen in astrocytes because they are proliferative cells. The R6/1 mouse line is a widely used HD model that shows progressive and rapid decline in motor skills and cognitive and social behavior starting at 12 to 14 weeks of age (83). In this experiment, astrocytes are about 2-3 weeks old, and astrocytes are more efficient than neurons at clearing aggregates, making them more resistant to mHtt aggregation (84, 85). This may lead to the explanation that the toxic effects of mHtt aggregates are not yet measurable in these astrocytes. Another reason is that the size of the groups analyzed, is too small to obtain statistical significance. Higher number or replicates would be necessary.

Because mitochondria can be secreted into the extracellular space by astrocytes and possibly also be taken up by neurons, we investigated whether the astrocytic genotype (WT vs. R6/1) could influence the amount of released mitochondria (65-69). If there were alterations in this, it could indicate genotype-dependent disruptions, which could possibly contribute to neuronal vulnerability in HD. To assess this, the mitochondrial mass in the ACM was analyzed. No significant changes in mitochondrial mass were observed after Western

blot analysis of the ACM of either genotypes. This suggests that astrocytes of both genotypes may secrete comparable amounts of mitochondria in the extracellular medium. However, it is important to remember that TOMM20 and VDAC only indicate the presence of mitochondria, not their functional state. Therefore, these results provide no information about mitochondrial quality or activity.

One possible explanation for the lack of differences is that R6/1 astrocytes are still resistant to the toxic effects of mutant huntingtin (mHtt) (83-85). A relevant comparison can be made to a study by Lampinen et al. who examined transmitophagy in Alzheimer's disease (AD), focusing on the degradation of neuronal mitochondria by astrocytes (72). They found no changes in transmitophagy in young astrocytes (2-3 months old), but significant increases in mitochondrial degradation were observed in AD astrocytes derived from older mice (6-11 months old) (72). A similar pattern was seen in iPSC-derived astrocytes from a symptomatic AD patient with the PSEN1 ΔE9 mutation, compared with isogenic corrected controls (72). These findings suggest that age and disease progression may be critical factors in modulating transmitophagy, which could explain the absence of detectable changes of the HD model. Another possible explanation is that changes in mitochondrial release may represent a cellular stress response. Because our data showed no significant increase in oxidative stress or disruption of mitochondrial membrane potential in R6/1 astrocytes, it is possible that these cells have not yet reached a certain stress threshold that would lead to altered mitochondrial release. It is also important to emphasize that the ACM used in this study was isolated from pure astrocyte cultures. This condition lacks the *in vivo* environment and complexity of the brain, where multiple cell types such as neurons, microglia and oligodendrocytes, interact and influence the astrocytic behavior. The absence of these signals in the culture may limit astrocyte activation and stress, which makes the reflection of observed mitochondrial mass in the ACM less accurate for the *in vivo* situation.

Dendritic arborization is essential for synaptic integration and neuronal connectivity. Disruptions to this process are often seen in neurodegenerative disorders (86-88). Since mitochondria play an important role in supporting the neuronal function and development, we investigated whether the astrocytic genotype influences the neuronal morphology. Via Sholl analysis, we examined the impact of ACM derived from WT or R6/1 astrocytes on the dendritic branching of A2A-positive striatal neurons, as these neurons are the D2 MSNs that degenerate most rapidly in HD. After counting all intersections, no significant difference was observed in the distance from the cell body. However, a significant difference was observed in total branching levels between genotypes. In this case, neurons treated with R6/1 ACM displayed more branching than those treated with WT ACM. These results contradict expectations and findings from other studies. Lerner et al. studied Golgi-stained MSNs in the striatum of 20–26 month old WT and HD knock-in mice. After analyzing the MSNs using Sholl analysis, they found reduced dendritic arborization in the HD knock-in MSNs (89). Subsequently, the same results were observed in MSNs of the striatum in late-stage HD and in transgenic R6/2 mice (90, 91). However, similar reductions in dendritic branching have been observed in other disorders as well. For instance, Wang et al. found fewer dendritic branches in striatal neurons in patients with FOXP1 syndrome, which is a neurodevelopmental disorder (92). Klein Gunnewiek et al. demonstrated that neurons with a high percentage of mutated mtDNA exhibit reduced arborization in mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (93). These findings underscore the fact that reductions in neuronal branching occur in other neurological disorders as well.

Although there is much evidence expecting a reduced branching in WT neurons treated with R6/1 ACM, Lebouc et al. found that dendritic maturation in D2-MSNs of R6/1 mice is accelerated, with an early increase in branching that normalizes from the third day after birth (P3) (94). This better represents the *in vivo* situation, but the main focus is on developmental observations within brain tissue (94). In contrast, our research focuses more on the functional effect of mitochondria on neurons and less on the general morphology of brain structures. By specifically looking at mitochondrial influence on neuronal development and function, we take a fundamentally different approach. Therefore, it

should be noted that the methods used and the research objectives in the study by Lebouc et al. and in our research are completely different, which also influences the interpretation of the results.

There may also be other reasons why R6/1 astrocytic mitochondria may have an effect on the branching of WT neurons. Mitochondria consist of mitochondrial DNA (mtDNA), proteins, small molecules, peptides, lipids, and nucleic acids (95, 96). If there is a difference in these components between WT and R6/1 mitochondria due to the toxic effects of mHtt, it could influence neuronal branching. Alternatively, R6/1 mitochondria may be smaller, which enables them to move more easily to areas that require high energy, such as synapses. This could explain the alteration in branching (97, 98). Internalized mitochondria may also cause changes in the neuron itself. Since mitochondria influence calcium homeostasis, R6/1 mitochondria may influence this process and alter the control of dendritic responses (33-35, 99). Further investigation into these possible causes may provide us with more insight into why this phenomenon occurs in primary striatal A2A neurons, and may help clarify whether this increased branching is a temporary effect or reflects a compensatory mechanism trying to rescue the A2A neurons that are more susceptible for the toxic effects of mHtt.

Strengths and weaknesses

The results in this study are preliminary data and should therefore carefully be interpreted. The sample sizes in all experiments were relatively small, which limits the statistical power. In addition, some methodological choices also bring limitations. The experiments were performed in monocultures, which means that there was no interaction with other cell types such as microglia or neurons/astrocytes, while *in vivo* they play an important role in cell-cell communication and stress responses. Moreover, since all experiments were performed *in vitro*, it makes it very difficult to directly translate the findings to the complex environment of the living brain. Furthermore, primary astrocytes were used for the experiments (2–3 weeks old), which may explain why there are no clear signs of pathology yet. To enhance the reliability and interpretation of the results, follow-up experiments with larger numbers, longer culture times and possibly co-cultures with other cell types are recommended.

Future perspectives

The findings in this study provide new insights into the mitochondrial behavior of astrocytes in HD, particularly with regard to their role in transmitophagy. Although no significant differences were found in oxidative stress, mitochondrial membrane potential, or mitochondrial mass between WT and R6/1 astrocytes, this outcome is highly informative in itself and provides a basis for future research.

An important consideration is the possibility that the pathological mitochondrial effects of mutant huntingtin (mHtt) have not yet manifested in the primary astrocytes. Future research should therefore include longer culture periods to mimic aging, astrocyte-neuron co-culture systems and possibly the use of D1-MSNs as an additional comparison, given their vulnerability in HD. These steps would bring the research closer to the complex *in vivo* brain environment and give more translationally relevant data. From a broader perspective, these findings can also be socially relevant, as mitochondrial dysfunction is increasingly recognized in many neurodegenerative diseases beyond HD. Understanding and in the end targeting the mitochondrial transfer between astrocytes and neurons may open new insights for therapeutic intervention, by contributing to early diagnosis or disease-modifying treatments. This makes the research potentially interesting for pharmaceutical companies, biotech companies, and healthcare providers, particularly those that are focused on mitochondrial therapies. Studying the quality and function of mitochondria transferred from astrocytes to neurons represents a new angle that has not yet been widely explored. Future experiments could combine live cell imaging, mitochondrial labeling, and co-culture systems to assess both the functional outcomes in neurons and the fate of the transferred mitochondria. Such methodologies would provide unique insights into intercellular mitochondrial dynamics and offer a potential advantage over more traditional approaches that focus exclusively on neuronal pathology.

CONCLUSION

This study investigated how astrocyte-mediated mitochondrial transfer to neurons is affected in a cellular model of HD. We specifically investigated whether R6/1 astrocytes show changes in oxidative stress, mitochondrial membrane potential, or mitochondrial release capacity. The experiments showed that there are no significant alterations in oxidative stress, mitochondrial membrane potential in R6/1 astrocytes. In addition, it was found that the R6/1 astrocytes released normal amounts of mitochondrial mass into the extracellular medium. However, when A2A neurons were treated with R6/1 ACM, they unexpectedly showed increased branching.

Based on these results, we conclude that the mitochondrial effects of mHtt in primary cultured astrocytes may not yet be apparent at the levels of oxidative stress. It is possible that mitochondrial dysfunction has not yet reached a detectable level in the astrocytes. This unexpected finding of increased branching of A2A neurons after R6/1 ACM treatment suggests a temporary effect or the presence of compensatory or indirect mechanisms. This underscores the need for follow-up research using longer culture periods and possibly involving other cell types via co-cultures, which could also provide valuable insights into the complex interaction between cells in the brain.

Further research into the quality, function, and impact of mitochondria transferred from astrocytes to neurons could not only contribute to a better understanding of the role of astrocytes in HD, but also provide starting points for new treatment strategies for neurodegenerative diseases in general.

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GenAI and Grammarly were used for spelling and grammar control.

SUPPLEMENTARY INFORMATION

Supplementary Table 1 – **Primary antibodies used for Western Blot.** List with molecular weight, host species, used dilution, source and ID of each primary antibody.

Antigen	Molecular weight (KDa)	Host species	Dilution	Source	ID
TOMM20	16.3	Rabbit	1: 1 000	ProteinTech	11802-1-AP
VDAC	39	Mouse	1 : 1 000	Abcam	ab14734

Legend: TOMM20: translocase of outer mitochondrial membrane 20; VDAC: ATP synthase F1 subunit alpha

Supplementary Table 2 – **Secondary antibodies used for Western Blot.** List of secondary antibodies with the used dilution, source and ID.

Secondary antibody	**Dilution**	**Source**	**ID**
Anti-rabbit IgG	3,3 : 10 000	Promega	WB401B
Anti-mouse IgG	3,3 : 10 000	Promega	WB402B